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Habitat characteristics influence the breeding of Rose's dwarf mountain toadlet *Capensibufo rosei* (Anura: Bufonidae)

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Direct anthropogenic factors (e.g., habitat loss, fragmentation, and degradation) threaten many amphibian populations, however some declines have occurred in supposedly pristine environments with no obvious causes. These enigmatic declines may be due to shifts in environmental factors influencing development and ultimately adult survival. Rose's mountain toadlet *Capensibufo rosei* has undergone such an enigmatic decline, with several populations presumed to be locally extinct at historic breeding sites. The two remaining breeding sites (Silvermine (SILV) and Cape of Good Hope (CGH)) on the Cape Peninsula of South Africa were monitored for three years (2012-2014) for life history traits and ecological requirements. Males congregate at ephemeral pools during the middle of the austral winter, with females arriving to lay eggs and then immediately leaving. Breeding only occurs in a few of the available pools. We hypothesised that larval development in colder, deeper pools would result in smaller-bodied tadpoles, and ultimately in relatively smaller adults. Pools at SILV were significantly deeper and colder compared to CGH, with breeding occurring in pools that were 27.05±10.21 mm and 21.55±6.95 mm deep at SILV and CGH, respectively. Contrary to expectations, breeding adults and developing tadpoles at SILV were larger than CGH individuals. The percentage of non-developing eggs at CGH was high compared to SILV and other anuran species. Development within this threatened species may be influenced by pool characteristics, which could provide clues as to the factors that influenced local extinctions in historical populations.

Key words: amphibian; aquatic habitat; bufonid; development; montane; tadpole

INTRODUCTION

Amphibians are a speciose vertebrate group; however, the rates at which constituent populations are declining are rapid compared to the estimated background rate of extinction (Beebee & Griffiths, 2005; McCallum, 2007; Hussain, 2012). Declines have been attributed to a number of factors including climate change, direct anthropogenic influences (e.g., habitat degradation), and invading pathogens (e.g., Stuart et al., 2004; Wake & Vredenburg, 2008), and are often due to combinations of multiple factors (Blaustein et al., 2011; Hussain, 2012). Some species are distributed in supposedly pristine habitats, but are still undergoing population declines or local extinctions of populations (Stuart et al., 2004). These are termed 'enigmatic declines', as the cause for the decline is not immediately apparent. To understand enigmatic declines, it is beneficial to understand the life history of a species and the conditions under which the species thrives.

One species undergoing an enigmatic decline (Cressey et al., 2015) is Rose's mountain toadlet *Capensibufo rosei* (Hewitt, 1926). The species was assessed as Vulnerable (SA-FRoG & IUCN 2010), but phylogenetic analyses showed that *C. rosei sensu lato* is divided into multiple cryptic species inhabiting separate mountain ranges (Tolley et al., 2010), and the taxonomy of the genus is currently under revision (Channing et al., 2017). *C. rosei sensu stricto* is found only on the Cape Peninsula of South Africa (Fig. 1; Tolley et al., 2010; Cressey et al., 2015) on montane plateaux within fynbos, a Mediterranean-type heathland. Only two breeding populations are known, both within Table Mountain National Park (TMNP; Cressey et al., 2015), prompting this species to be prioritised nationally for conservation work (including surveying, monitoring and taxonomy; Measey 2011). Broad scale surveys across the distribution revealed that these two populations (Cape of Good Hope (CGH) and Silvermine (SILV)) may be the only populations remaining, as previously known breeding sites no longer support breeding populations (Cressey et al., 2015). This species has tested negative for the chytrid fungus (*Batrachochytrium dendrobatidis*; see Tarrant et al., 2013), leaving the cause and/or causes for these apparent local extinctions unclear, as many of the historical breeding sites are within protected areas. Furthermore, the Cape of Good Hope population is genetically depauperate (Cressey et al., 2015; Da Silva et al., 2016) suggesting that it may have undergone a recent bottleneck and therefore may be at higher risk to environmental changes. An understanding of the

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breeding phenology and habitats at the two breeding sites would provide a baseline for understanding the potential factors influencing the enigmatic decline of the species.

C. rosei breeds in ephemeral pools fed by rainfall during the austral winter (June to August; Rose, 1962; Grandison, 1980; de Villiers, 2004). Interestingly, this species is the only southern African amphibian that does not have an advertisement call, and the tympanum and middle ear elements are absent (Grandison, 1980). Breeding in *C. rosei* follows an explosive breeding strategy similar to other bufonids. Ad hoc observations (GJM & KAT Pers. Obs., 2008) suggest that at the beginning of the breeding season (July-August), a large cohort of males forms at the breeding pools and remains there throughout the breeding season. Females seem to arrive individually, spending enough time at the breeding site to lay their eggs, and then departing. Thus, at any point in time, it is likely that the sex ratio at a breeding puddle is highly skewed toward males. *C. rosei* females lay relatively small clutches of strings of eggs (approximately 100 eggs, 2–3 mm diameter of egg jelly once hydrated; Grandison 1980; Liedtke et al., 2014), and tadpole development to metamorphosis follows the typical anuran metamorphosis cycle (de Villiers, 2004).

The aims of the current study were to investigate breeding phenology and tadpole development for *C. rosei* at the two known breeding sites on the Cape Peninsula and to characterise environmental features of the breeding pools. Data were collected over three consecutive years (2012-2014), including timing of the breeding, tadpole and adult sizes, and pool characteristics (depth and temperature). We expected that adults would converge on the pools close to one of the full moon periods in the middle of the austral winter season, given that the lunar cycle has been linked with amphibian breeding (Grant et al., 2009), and that they would only select pools within a specific depth range. Breeding pools begin to dry after winter rainfall has ceased, so it is possible that the choice of pool is made based on specific characteristics (i.e. particular depth, which is related to temperature). In other amphibians, variation in growth rates of tadpoles and resultant size of adults have been found, due to either densities of tadpoles (e.g., Denton & Beebee, 1993; McDiarmid & Altig, 1999; Green & Middleton, 2013) differences in the pool characteristics (e.g., Darrow et al., 2004) and other environmental factors (such as predators; Relyea, 2001). Therefore, we hypothesised that deeper, colder pools would lead to the development of smaller-bodied tadpoles.

METHODS

Field work and sampling

Data were collated during the austral winter and spring (July-November) over three consecutive years: 2012-2014. Breeding sites were visited approximately once a week in July until breeding was observed and thereafter twice a week from late August to September, whilst the adult males aggregated at the breeding pools, and then approximately every five days from October to November, when eggs were observed in the pools, and as the tadpoles grew and metamorphosed. Once metamorphs started to disperse from the pools into the surrounding vegetation, sampling efforts were continued (~5 days) until it was determined that all metamorphs had left the breeding area. GPS points for each pool investigated were collected using a Garmin GPS.

Breeding phenology

Dates were recorded for the convergence of adults at the pools, appearance of eggs, hatching of tadpoles, appearance of metamorphs, and dispersal of adults. The dates that pools were observed to have dried up after the breeding season, as well as the dates of the full moon, were recorded.

Pool characteristics

During winter, many small pools form at the two sites, but only some are used for breeding (hereafter referred to as breeding pools). Across the three sample years, some pools utilised in one year were not utilised in the following year. Prior to breeding, we identified a number of potential pools (five or six at each site for each sample year) of varying depths to monitor for environmental characteristics and breeding activity. Although a number of pools were formed during the rainy season in the SILV and CGH areas, we chose pools in areas where male *C. rosei* were observed to aggregate in previous years. During breeding, some of these pools were not utilised (hereafter referred to as non-breeding pools), but we continued to monitor them for comparison with breeding pools. In addition, the surrounding areas were surveyed to potentially find other breeding pools, however none were found (over and above the ones included in this study).

Pool depths of breeding and non-breeding pools were measured using a graduated ruler (10 random placements within the puddle where depth was measured to the nearest millimetre) at each pool, on every sample day. All statistical analyses were performed in R Studio v.0.98.1103, using R version 3.2.0 (R Core Team 2015). All data were tested for normality using the Shapiro-Wilk test (package: 'stats', function: 'shapiro.test'; R Core Team 2015). The mean values of the ten measurements were compared using the Wilcoxon two-sample rank sum test (package: 'stats', function: 'wilcox.test'; R Core Team 2015). Dates when pools dried up were recorded if this occurred before metamorphosis was complete, and metamorphs had moved off into the surrounding vegetation.

Pool temperature was measured every hour using Thermochron® iButtons® (model: DS1921G-F5#; Maxim Integrated Products, Inc.) placed underwater, encased in silicone capsules, in multiple pools before breeding commenced and were removed once the metamorphs had all left the breeding site. In 2012 and 2013, temperatures of breeding pools were measured, whilst in 2014, temperatures of both breeding and non-breeding pools were measured. Maximum and minimum temperatures recorded for each day (recorded from 18/09/2012 - 26/10/2012, 26/07/2013 - 17/10/2013

and 10/07/2014 - 05/11/2014) were extracted. The daily ranges of water temperatures (difference between the maximum and minimum recorded) were calculated. Differences between the temperature ranges in breeding and non-breeding pools within and between CGH and SILV were compared using a Wilcoxon two-sample rank sum test. Linear model regressions were conducted for the maximum and minimum daily temperatures of breeding pools, to investigate the change pool temperatures over the breeding time period (package: 'stats', function: 'lm'; R Core Team, 2015). Linear model regressions and a Pearson's product-Moment correlation test was conducted between pool depths and pool mean temperatures, for both sites combined, to investigate the relationship between depth and temperature (package: 'stats'; functions: 'lm' and 'cor.test'; method = 'Pearson'; R Core Team, 2015).

Morphological analyses

Adults were captured by hand and temporarily retained in a plastic container during which time they were measured and then photographed on grid paper. Although sub-adult males (ranging in size from 10 mm to 13 mm) were observed at the breeding sites, adults were considered to be individuals with SUL > 15 mm. Body length (snout to urostyle length: SUL) was measured using a graduated ruler in the field (years 2012-2014). Adult *C. rosei* were sexed on site by visual inspection of the angle of the posterior body region (between hindlimbs): males have angular, hooked posteriors (Fig. 1).

Measurements of eggs (jelly diameter), embryos (either diameter or lengths), tadpoles (lengths) and metamorphs (lengths) were made on the photographs using ImageJ v.1.50i (http://imagej.nih.gov/ij/; Abramoff et al., 2004). The proportion of non-developing eggs were counted on photographs of in situ eggs and tadpoles, obtained after sliding a laminated (waterproof) 1-cm^2 grid paper underneath them. The usual stages of embryonic development in tadpoles (i.e. Gosner staging system) were not easily distinguishable from the photographs, and so the various phases of tadpole development and metamorphosis were divided into eight distinct groups (Fig. 3): [A] Within egg, embryonic development, aquatic stages: (1) Fertilised embryo (ball-shaped; blastula, gastrula and neurula stages of development); (2) Tailbud stage (anterior and posterior part of embryo developed, but head and tail are not distinct from body); (3) Late tailbud stage (head and tail distinct); [B] Hatched from egg, aquatic stages: (4) Tadpole with tail; (5) Metamorph (tail and hindlimbs); (6) Metamorph (tail and both hindlimbs and forelimbs); [C] Terrestrial stages: (7) Metamorph (miniature toad; tail resorbed); (8) Fully developed, mature adult. Body lengths at each phase were compared between sites and across the years (see Fig. 3). For phases 3-6, lengths were measured separately in two ways: (1) the sum of the body and tail (total length), and (2) the body lengths only. Sexes of the metamorphs could not be determined,

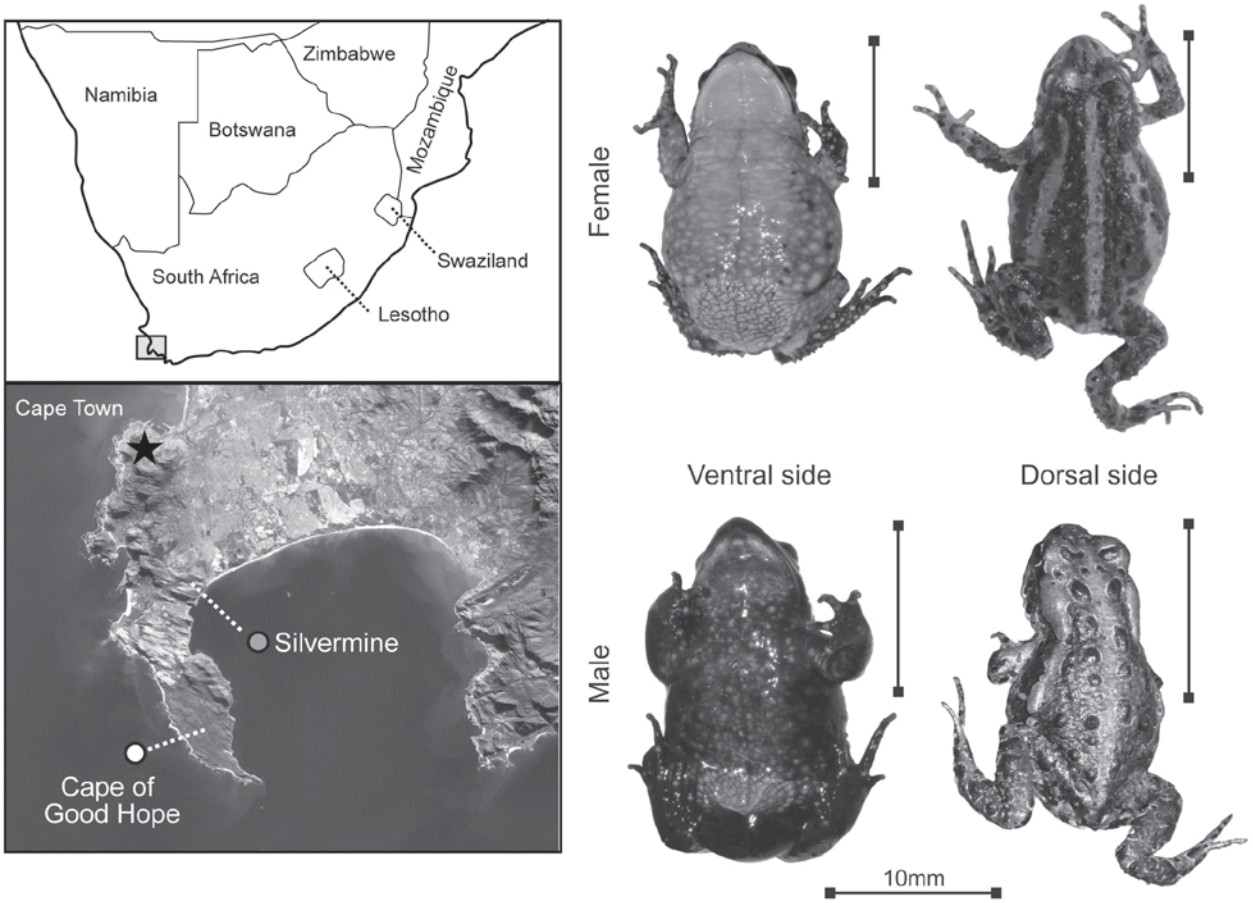

Figure 1. Map showing the general location of the two breeding sites (left) and photographs of the ventral and dorsal views of female and male *C. rosei* (right; photographs by SE). Topographic map of the Cape Peninsula obtained from http://en-gb.topographic-map.com/places/Cape-Town-1310815/ (AfriGIS (Pty) Ltd).

so only the adult measurements were divided by sex for analysis purposes.

Adult body lengths for both females and males, as well as the overall lengths and body lengths of tadpoles, were compared between the sites. Egg jelly diameters were compared between the two sites using a Welch two-sample t-test (package: 'stats', function: 't.test'; R Core Team 2015). Wilcoxon rank sum tests and Welch two-sample t-tests of the body lengths, and separately of the total lengths, at each phase of development, were conducted between sites. For the adults, Wilcoxon rank sum tests were conducted between sexes within the sites, and of the sexes separately, between sites. Generalised linear models were used to investigate the effects of environmental variables (pool depth, pool temperature) on tadpole total length, and body length only, at phases 4 to 6, separately (package: 'stats', function: 'glm', family = 'gaussian'; R Core Team, 2015).

RESULTS

Breeding phenology

Breeding and the development of the young (from convergence of adult males on pools to departure of metamorphs) over the three year study period varied from 73-86 days (ca. 10-12 weeks), with time from male aggregation to juvenile dispersal (breeding time) being shorter at CGH (mean ± s.d.: 73.7 ± 18.2 days) compared to SILV (mean ± s.d.: 85.7 ± 3.1 days). Adult males arrived once pools had formed after the heaviest rainfall month (June) in the region (South African Weather Service Statistics; http://www.weathersa.co.za), during which pools of water formed at the two sites. The adult males began to arrive at the pools ranging from the beginning of July to the end of August (Table 1), and remained at the breeding pool for approximately three weeks to a month (SILV: 12-21 days, mean = 18.0 ± 4.3 days; CGH: 10-30 days, mean = 20.0 ± 10.0 days) with the convergence of adult males being on average earlier at CGH (beginning to middle of July) than at SILV (beginning of August) (Table 1). The arrival of the adult males may have coincided with one of the full moons in either July or August within the respective years (Table 1), though the dates recorded depended on the timing of sampling efforts, as sampling in 2012 commenced only once adults were observed at the pools. Females arrived individually close to the observed date of the convergence of males and remained at the pool long enough to lay their eggs before departing. Thus, the sex ratio was highly biased towards males at the pools (Table S1). At both sites, tadpoles hatched from eggs after approximately 18.2 ± 6.5 days (range: 12-30 days at SILV and 15-20 days at CGH) with back legs developing about 23.5 ± 6.8 days after hatching (range: 28-31 days at SILV and 14-23 days at CGH) and front legs 10.0 ± 5.7 days thereafter (range: 9-12 days at SILV and 3-10 days at CGH). The time from the appearance of the front legs to when metamorphs (tail resorbed) had all left the breeding pools, moving into the surrounding vegetation, was on average 26.7 ± 8.6 days (range: 24-33 days at SILV and 15-36 days at

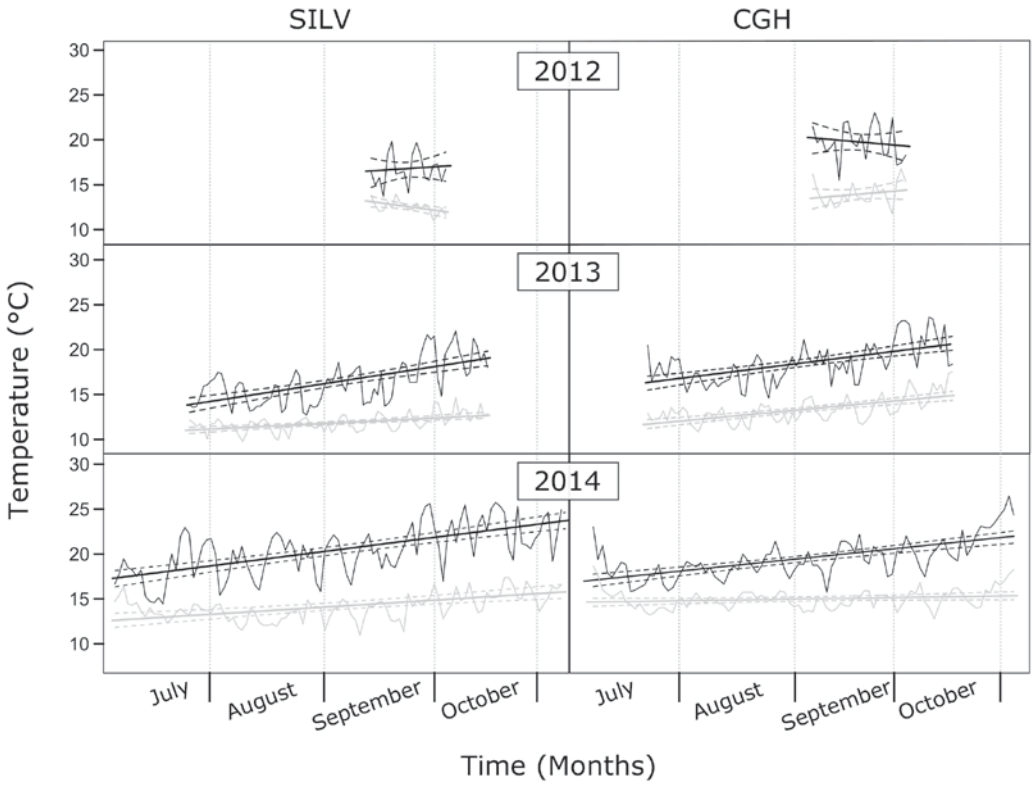

Figure 2. Pool water temperatures for daily values of breeding pools (averaged across all breeding pools) at each site during three consecutive years (2012-2014), measured over the sampling period of July to October (x-axis). The values of maximum (black) and minimum (grey) pool temperatures, as well as the mean (straight solid black and grey lines) and 95% confidence intervals (straight dotted black and grey lines) are shown.

Table 1. Breeding times for the five years at the two study sites, including the dates of the full moons at the beginning of the breeding season. Key to site abbreviations: SILV = Silvermine, CGH = Cape of Good Hope.

Site	Year	2nd full moon	First observation of females or eggs deposited in pools	convergence of adult males	Bulk of eggs lais	All adults left	First development of back legs	First development of front legs	First appearance of metamorphs (tails resorbed)	All metamorphs left	Pools all dried	Approx. days of breeding	Number of days adults remained
SILV	2012	Aug 31	Aug 01	Aug 01	Aug 10	Aug 20	Sept 23	Sept 25	Oct 05	Oct 28	Oct 28	89	21
	2013	Aug 21	Jul 27	Aug 01	Aug 03	Aug 18	Sept 23	Oct 12	Oct 18	Oct 24	Nov 05	85	18
	2014	Aug 10	Jul 30	Aug 10	Aug 10	Aug 21	Sept 10	Sept 19	Oct 15	Oct 22	Nov 06	83	12
CGH	2012	Aug 31	Aug 10	Aug 10	Aug 14	Aug 20	Sept 14	Sept 17	Sept 25	Oct 02	Oct 10	54	10
	2013	Aug 21	Aug 10	Jul 28	Aug 07	Aug 18	Sept 18	Sept 23	Oct 03	Oct 12	Oct 18	77	21
	2014	Aug 10	Jul 28	Jul 07	Jul 15	Aug 06	Aug 21	Aug 31	Sept 19	Oct 05	Oct 15	90	30

Table 2. Depths of breeding and non-breeding pools (means ± standard deviation) for the sampling years separately at each site, measured in millimetres during the breeding period. Results of the Wilcoxon two-sample rank sum tests, testing differences between breeding and non-breeding pool depths in each year. Key to site abbreviations as in Fig. 1. Key to headings: W = Wilcox rank sum test index. Significant P-values ($P < 0.05$) are indicated in bold font.

	Breeding (n)	Non-Breeding (n)	W-value	*P*-value	Which pools were deeper?
SILV:					
2012	25.28 ± 9.22 (6)	28.77 ± 19.88 (4)	4694	0.99	Statistically equal
2013	31.41 ± 10.67 (5)	35.46 ± 11.50 (3)	841	0.05	Non-breeding
2014	23.32 ± 9.62 (6)	28.04 ± 11.75 (6)	340.5	**0.005**	Non-breeding
CGH:					
2012	21.47 ± 6.86 (4)	16.63 ± 3.80 (2)	2062	**0.004**	Breeding
2013	20.56 ± 5.43 (3)	17.47 ± 6.12 (2)	424.5	0.21	Statistically equal
2014	23.13 ± 8.78 (8)	18.90 ± 6.81 (3)	229.5	0.17	Statistically equal

Table 3. Pool depths comparisons between sites (CGH and SILV) for each sampling year and compared using a Wilcox rank sum test (W-value).

Year	Breeding or non-breeding?	W-value	P-value	Which is deeper (CGH or SILV)?
2012	Breeding	5353.5	0.02	SILV
	Non-breeding	2455	<0.0001	SILV
2013	Breeding	2097.5	<0.0001	SILV
	Non-breeding	540.5	<0.0001	SILV
2014	Breeding	722.5	0.66	SILV
	Non-breeding	187	0.02	SILV

Table 4. Wilcoxon two-sample rank sum tests between pool temperature characteristics. Key to headings: mean maximum temperatures (MaxT), mean minimum temperatures (MinT), and range of temperatures (RangeT). Significant p-values (P < 0.05) are indicated in bold font.

comparison data A	Comparison data B	W-value	P-value	Which group is larger?
Range T				
All years – SILV – breeding	All years – CGH – breeding	235430	**0.0001**	B
2012 – SILV – breeding	2012 – CGH – breeding	1691.5	**< 0.001**	B
2013 – SILV – breeding	2013 – CGH – breeding	15747	**0.002**	B
2014 – SILV – breeding	2014 – CGH – breeding	96780	0.71	A=B
2014 – SILV – non-breeding	2014 – CGH – non-breeding	19155	**< 0.001**	B
2014 – SILV – breeding	2014 – SILV – non-breeding	109010	**< 0.001**	A
2014 – CGH – breeding	2014 – CGH – non-breeding	42602	0.09	A=B
MaxT				
2014 – SILV – breeding	2014 – SILV – non-breeding	95606	**< 0.001**	A
2014 – CGH – breeding	2014 – CGH – non-breeding	43482	0.20	A=B
MinT				
2014 – SILV – breeding	2014 – SILV – non-breeding	73040	0.18	A=B
2014 – CGH – breeding	2014 – CGH – non-breeding	46646	0.97	A=B

CGH). These ranges were measured for the breeding season as a whole, and not for individuals. The timing of the metamorphosis for an individual would thus fall within these ranges, however, the developmental timing of the tadpoles may be staggered (i.e. not all individuals developing at the same time, due to the differential deposition of eggs in the breeding pools).

Pool characteristics

Breeding and egg deposition was confined to two or three pools at each site, despite a number of other pools present in the area. In all years, metamorphosis had been completed before pools had dried up (both non-breeding and breeding pools dried up only after metamorphs had dispersed into the surrounding vegetation). At Silvermine, breeding pools were consistently shallower compared to non-breeding pools across all years,

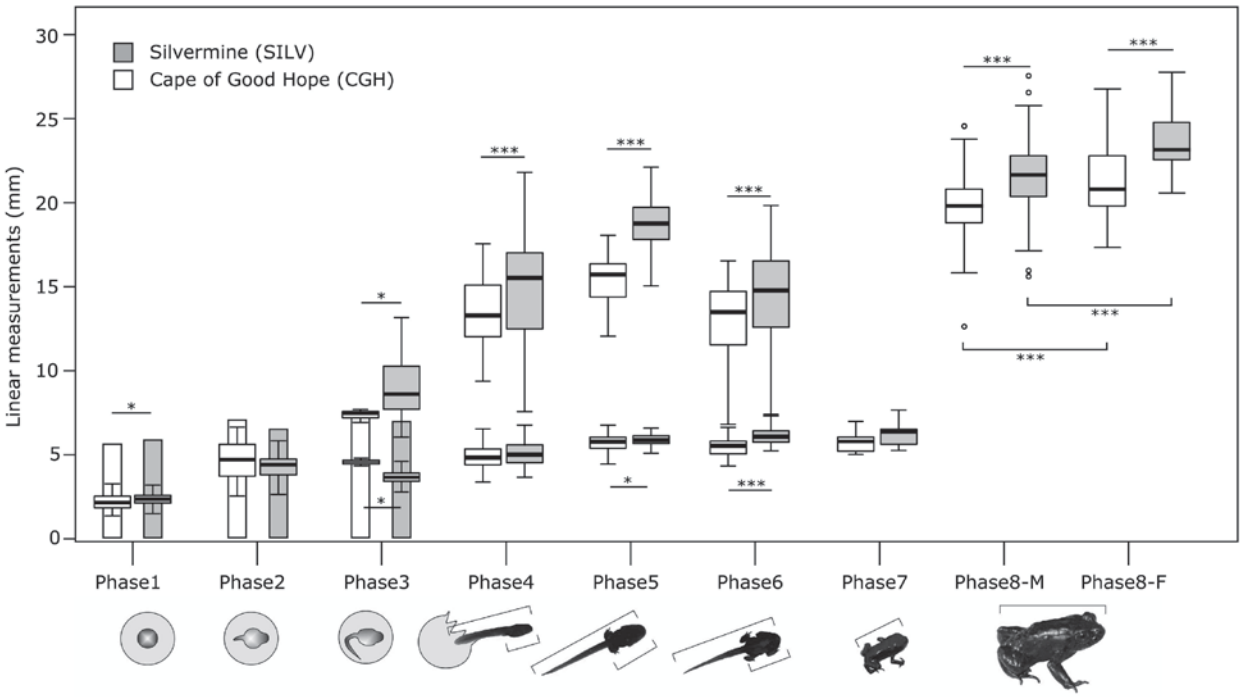

Figure 3. Boxplots (median and quartiles) of the sizes of *C. rosei* at various phases in the life-cycle of the species (figures below the graph), from the two breeding sites at SILV (grey) and CGH (white). The adult phase (phase 8) is divided by sex (F = female, M = male). At phases 3 to 6, the upper boxplots indicate total lengths (body and tail) and the lower boxplots indicate body length only. Barplots are shown in the first three phases indicating mean egg jelly diameter. Significant differences in lengths are indicated using lines with stars above or below boxplots (*: 0.05 < *P*-value < 0.01; ***: *P*-value < 0.001) between sites (above boxplots), and between sexes within the sites (adult phase; below boxplots).

though only significantly different in years 2013 and 2014 (Table 2). In contrast, breeding pools at CGH were consistently deeper than non-breeding pools, though only significantly so in the year 2012 (Table 2). Breeding pools at SILV were significantly deeper than those at CGH overall (W = 21422, $P < 0.0001$), due to the differences in the years 2012 and 2013 (Table 3), with depths of breeding pools at SILV ranging between 3.3 and 51.8 mm, and at CGH ranging between 5.9 and 43.9 mm (Table 2). Pools not utilised for breeding (non-breeding pools) at SILV were significantly deeper than those at CGH overall (W = 7441, $P < 0.0001$), due to differences found in all three sampling years (Table 3), with non-breeding pools at SILV ranging between 7.8 and 88.6 mm, and those at CGH ranging between 5.9 and 43.9 mm (Table 2). During the first larval development stages (phase 1), the depths of the breeding pools at SILV ranged from 16.8 – 48.4 mm (mean ± s.d. = 29.25 ± 7.54 mm), whilst at CGH the depths ranged from 11.0 – 19.2 mm (mean ± s.d. = 15.84 ± 3.85 mm). This indicated that pools chosen for breeding were on average twice as deep at SILV than those at CGH.

Pool temperatures ranged on average over the three sample years from 7.0 to 31.5 °C (mean ± s.d. = 14.16 ± 4.31 °C) at SILV, which was significantly colder than at CGH (8.5 – 36.5 °C; mean ± s.d. = 16.11 ± 4.40 °C) (Table 4). Daily range of temperatures ("RangeT"; Table 4) of breeding pools was significantly larger at CGH compared to those at SILV during 2012, 2013, as well for non-breeding pools in 2014 (Table 5). The RangeT in breeding pools between sites in 2014 were similar (Table 4). Breeding pools at SILV in 2014 had higher mean MaxT values, resulting in the significantly larger RangeT experienced in breeding pools, relative to non-breeding pools (Table 5). There were no significant differences in either of the three value sets between breeding and non-breeding pools at CGH during 2014 (Table 5).

The pool temperatures increased as the seasons progressed from winter to spring (Fig. 2). Regression analyses of the maximum and minimum daily temperatures of the breeding pools throughout the austral winter exhibited positive slopes in the years 2013 and 2014 (though the negative slopes in the year 2012 may be due to the smaller sample size; Table 6). The lower intercept values and larger slopes at SILV in the maximum and minimum temperatures indicate that SILV breeding pools were colder during the middle of winter (June and July), but were progressively warmer than CGH breeding pools by the end of the breeding season in November (Table 6; Fig. 2). The linear regression and correlation test indicated a significantly negative relationship between pool depth and pool temperature; thus deeper pools were cooler (r = -0.56; R2 = 0.32; slope = -0.07; intercept = 15.64; t = -23.24; $P < 0.0001$).

Morphological analyses

C. rosei exhibits female-biased sexual size dimorphism (SSD) (Table 7; Fig. 3), with significant differences in body sizes found at both sites between the sexes (Mann-Whitney U test: SILV: W = 20700, $P < 0.0001$; CGH: W = 51859, $P < 0.0001$). Body sizes differed between SILV and CGH individuals. At SILV, adult breeding males ranged in SUL size from 16.00 - 30.00 mm (mean ± s.d. = 21.80 ± 1.87 mm), whilst those at CGH ranged from 16.00 - 27.00 mm (mean ± s.d. = 20.28 ± 1.63 mm) (Table 7; Fig. 3). A small number of metamorph males (10-15 mm SUL) were caught at CGH during 2013 and 2014, indicating perhaps that maturation to a breeding age takes longer than a year. Females ranged in size from 20.77 - 28.00 mm (mean ± s.d. = 23.94 ± 1.73 mm) at SILV, whilst at CGH females ranged in size from 17.52 - 27.00 mm (mean ± s.d. = 21.32 ± 2.29 mm). Both sexes from SILV were significantly larger than CGH individuals (Table 7; Fig. 3).

Egg jelly diameters remained relatively consistent throughout the embryonic stage, before being ruptured during the hatching of the tadpoles (Table 7). The proportion of non-developing eggs (averaged over the three sample years) were significantly higher at CGH (0.60 ± 0.13) compared to those at SILV (0.23 ± 0.05) (t_{53} = -8.15, P < 0.0001). Egg density and tadpole recruitment was not estimated as variables routinely used in these estimations were not measured for this study (only pool depth measurements were taken for the three study years, and not pool width or length).

The two sites differed in their embryo and tadpole sizes (Table 7, Fig. 3). When the total body length of the embryos and tadpoles, as well as only the body lengths, were compared between the sites, SILV individuals were consistently larger than those at CGH, except during Phases 2, 4 and 7 (Table 7, Fig. 3). Generalised linear model analyses showed that the depths of the pools, and

Table 5. Pool temperatures for pools in which breeding occurred for both sites for the years 2012, 2013 and 2014. Key to site abbreviations in Table 1. Key to heading abbreviations as in Table 4.

Sites and years	Breeding or non-breeding	MaxT (˚C)	MinT (˚C)	RangeT (˚C)
SILV:				
2012	Breeding	16.87 ± 2.73	11.99 ± 1.32	4.88 ± 2.86
2013	Breeding	15.85 ± 3.18	10.39 ± 1.27	5.50 ± 3.41
2014	Breeding	18.89 ± 4.34	11.51 ± 2.00	7.39 ± 4.37
	Non-breeding	17.17 ± 4.41	12.62 ± 3.71	4.55 ± 3.63
CGH:				
2012	Breeding	19.97 ± 2.72	12.81 ± 1.45	7.16 ± 3.26
2013	Breeding	18.37 ± 2.68	12.11 ± 1.69	6.26 ± 2.39
2014	Breeding	20.87 ± 4.12	13.05 ± 2.54	7.19 ± 4.10
	Non-breeding	20.34 ± 3.93	13.14 ± 2.80	7.82 ± 4.15

Table 6. Regression analyses of the pool temperatures in the breeding pools at the two sample sites. Key to headings: R^2 = Variance estimate, slope = slope of the regression, intercept = intercept of the regression, t-value = index for the t-test to investigate level of relationship between the variables, p-value = significance value. Significant p-values ($P < 0.05$) are indicated in bold font.

Sampling year and site	R^2	slope	intercept	t-value	*P*-value
MaxT:					
SILV					
2012	0.28	0.05	12.51	0.52	0.61
2013	0.40	0.07	11.49	7.41	**<0.0001**
2014	0.39	0.08	14.17	8.23	**<0.0001**
CGH					
2012	0.02	-0.05	24.06	-0.63	0.53
2013	0.36	0.06	14.37	6.57	**<0.0001**
2014	0.42	0.06	15.23	8.04	**<0.0001**
MinT:					
SILV					
2012	0.28	-0.08	18.06	-2.57	**0.02**
2013	0.28	0.03	9.02	5.66	**<0.0001**
2014	0.18	0.04	9.31	4.75	**<0.0001**
CGH					
2012	0.06	0.06	8.22	1.13	0.26
2013	0.41	0.05	9.34	7.25	**<0.0001**
2014	0.04	0.01	12.36	1.86	0.07

Table 7. Morphological measurements (in millimeters) and comparisons between the sites. Egg jelly diameters, embryo and tadpole lengths (both overall lengths and body lengths only), and adult lengths per sex, at each breeding site. Results of comparison statistical analyses, investigating differences in morphological measurements between sites, are shown. Indices indicate type of comparative test done: t = Welch two-sample t-test, W = Wilcox rank sum test. Key to site and heading abbreviations as in Table 1. Significant p-values ($P < 0.05$) are shown in bold font.

	SILV		CGH		Comparison tests		
	n	Mean ± s.d. (mm)	n	Mean ± s.d. (mm)	df	Index	*P*-value
Egg diameter							
Phase 1	304	5.70 ± 0.68	67	5.81 ± 0.83	82		0.36
Phase 2	87	6.35 ± 0.66	15	6.85 ± 0.62	17.6		0.05
Phase 3	61	6.79 ± 0.73	3	7.20 ± 0.73	2.13		0.42
Overall length							
Phase 1	304	2.35 ± 0.45	67	2.37 ± 0.87	15.7		**0.01**
Phase 2	87	4.36 ± 0.73	15	4.75 ± 1.21	9		0.24
Phase 3	61	8.78 ± 1.75	3	7.42 ± 0.39			**0.01**
Phase 4	348	14.86 ± 2.85	79	13.33 ± 2.04			**<0.0001**
Phase 5	87	18.73 ± 1.48	54	15.37 ± 1.45			**<0.0001**
Phase 6	174	14.26 ± 3.03	81	12.69 ± 2.58	16.8		**<0.0001**
Phase 7	9	6.27 ± 0.73	17	5.81 ± 0.53	3		0.53
Body length							
Phase 3	61	3.87 ± 0.46	3	4.75 ± 0.23	2.82		**0.01**
Phase 4	348	5.02 ± 0.68	79	4.80 ± 0.72	94.5		0.06
Phase 5	87	5.87 ± 0.43	54	5.67 ± 0.53	2		**0.02**
Phase 6	174	6.11 ± 0.48	81	5.45 ± 0.57			**<0.0001**
Adult length							
Females	57	23.94 ± 1.73	455	21.32 ± 2.29		W = 4148.5	**<0.0001**
Males	88	21.81 ± 1.87	901	20.28 ± 1.63		W = 299980	**<0.0001**

Table 8. Generalised linear model analysis investigating the effect of variables on tadpole overall lengths and body lengths only, at three stages of larval development (phases 4-6; sample size indicated). Key to parameter abbreviations: Depth = Pool depth; Temp.mean = Mean daily pool temperatures; Temp.range = Range of daily temperatures. Significant p-values ($P < 0.05$) are shown in bold font. Coefficients not defined due to singularities are indicated with a dash.

	Phase 4	**n=79**		**Phase 5**	**n=54**		**Phase 6**	**n=81**	
	Estimate	**t-value**	**P-value**	**Estimate**	**t-value**	**P-value**	**Estimate**	**t-value**	**P-value**
Overall tadpole length									
(intercept)	-136.13	-3.80	**0.001**	23.38	16.3	**<0.001**	-424.96	-1.252	0.221
Depth	6.49	4.09	**<0.001**	-0.35	-5.01	**0.0001**	20.84	1.314	0.200
Temp.mean	4.55	3.50	**0.001**	-0.06	-0.41	0.686	27.56	1.331	0.194
Temp.range	12.29	3.07	**0.004**	-0.11	-0.58	0.571	1.05	1.364	0.183
Depth: Temp.mean	-0.06	-0.67	0.510		-	-	-1.33	-1.361	0.184
Depth: Temp.range	-0.80	-2.64	0.012		-	-		-	-
Temp.mean: Temp.range									
Depth:Temp.mean:Temp. range									
Tadpole body length only									
(Intercept)	-55.46	-4.60	**<0.001**	9.12	7.911	**<0.001**	-88.29	-0.527	0.603
Depth	2.62	4.90	**<0.001**	-0.11	-1.997	0.062	4.32	0.552	0.586
Temp.mean	1.79	4.09	**<0.001**	-0.06	-0.495	0.627	5.78	0.566	0.576
Temp.range	5.03	3.74	**0.001**	-0.05	-0.348	0.732	0.36	0.945	0.353
Depth:Temp.mean	-0.01	-0.61	0.548		-	-		-0.57	0.576
Depth:Temp.range	-0.33	-3.29	**0.002**		-	-		-	-
Temp.mean:Temp.range		-	-		-	-		-	-
Depth:Temp.mean:Temp. range		-	-		-	-		-	-

the mean and range of the pool temperatures of the sites have significant effects on the body lengths (body length only and overall body lengths), at various stages of larval development (Table 8). Depth had a significantly positive effect on body lengths at phase 4 (deeper pools housed larger-bodied tadpoles), and significantly negative effects at phase 5 (deeper pools housed smaller bodied tadpoles). Mean daily pool temperatures and the range of daily temperatures both had significantly positive effects during phase 4 of larval development, but not at the other phases. There was a significantly negative interaction effect between pool depth and temperature range during phase 4. This indicates that environmental variables (depth and temperature) had the most significant effects during phase 4 of larval development.

DISCUSSION

C. rosei appears to have specific habitat requirements for breeding, only selecting pools with certain characteristics in terms of depth and temperature. Breeding pools at both sites were approximately 20-30mm deep, compared to the variable deeper and shallower depths of non-breeding pools, although pools at SILV were statistically significantly deeper and cooler than at CGH. Furthermore, we found that the tadpoles developing in the colder, deeper breeding pools at SILV had larger body lengths, and adults at SILV were also larger-bodied.

The breeding dynamics at the two sites differed in respect to timing of development: individuals at SILV took longer to develop than those at CGH. Breeding generally commenced close to a full moon in the middle of the winter season, similar to other anuran phenology (Grant et al., 2009), though the exact recorded dates of breeding were dependent on sampling efforts. Amphibian embryonic development has been shown to occur at a faster rate in warmer waters (Moore, 1939; Volpe, 1957; Berven et al., 1979; Morrison & Hero, 2003), possibly accounting for the faster development times at CGH. Egg deposition and development time from tadpole to metamorph stage (Phases 5-7) occurred earlier at CGH compared to SILV. Accelerated metamorphosis occurring in response to pool desiccation has also been shown in other anurans (Crump, 1989; Loman, 1999; Richter-Boix et al., 2006; Székaly et al., 2010). During the period studied, however, metamorphosis was near completion for *C. rosei*, and metamorphs (some with tail that were not fully resorbed) had moved off into the surrounding vegetation, often, but not always, before pools had dried up. In previous dry years, pools dried, leaving pre-metamorphs stranded (GJM & KAT Pers. Obs., 2009), though this was not the case during the sampling years for the present study. So the earlier, and shorter, breeding times of the CGH toadlets may be linked with the warmer waters of the shallower breeding pools, as well as the potential of the pools drying up faster (due to their shallower depths and possible faster rates of evaporation).

The adult body size ranges measured are smaller than values reported in the literature (20-40mm; de Villiers 2004), but the previously reported values are likely to have been confounded by the taxonomic confusion mentioned in the introduction. The differences in body size between the two breeding sites showed SILV adults

to be larger than their cohorts at CGH, and contrary to our hypothesis, embryos and tadpoles at SILV were larger-bodied than at CGH. In treefrogs *Hyla versicolor* (Relyea & Hoverman, 2003) and wood frogs *Rana sylvatica* (Relyea, 2002), shorter tail lengths and longer bodies in tadpoles were induced by high-competition larval environments. In other anurans, greater density, and therefore increased competition for resources of tadpoles (e.g., Denton & Beebee, 1993; Green & Middleton, 2013) was linked to variation in growth rates of tadpoles, and resultant size of adults. Anuran tadpoles have been shown to adapt either physiologically to low dissolved oxygen levels in the water (e.g., by changing the P50 level of haemoglobin in the blood) or anatomically (e.g., by developing longer tails with larger surface area for oxygen uptake into the bloodstream) (Burggren & Mwalukomo, 1983; McDiarmid & Altig, 1999). Lower dissolved oxygen levels are found in deeper waters (Wetzel, 2001), but oxygen saturation is lessened in warmer waters. The pools at the two breeding sites in this study, although significantly different in mean depths, are still considered to be shallow in comparison to other water bodies used by anurans for breeding purposes (e.g., lakes, ponds etc.). The characteristics of the pools at each site may be influencing the development of the larvae, resulting in differing body size, though further research into other site-specific characteristics (pool volumetric size, surrounding vegetation coverage, shading percentage of the pools, water characteristics such as pH and oxygen levels) would be beneficial for conclusive estimation of the factors that provide a good breeding environment for this threatened species. Another potential explanation for the significantly smaller adults at CGH could be due to the age structure at each site; SILV may have a larger percentage of older adults, and therefore are on average larger-bodied. As such, further study is needed at each site, investigating whether dissolved oxygen levels in the pools is affecting the development of the tadpoles, including the algal density in the breeding pools that may be providing dissolved oxygen. For the moment we can conclude that the colder, deeper pools at SILV may be one of the factors influencing the development of their larger bodies, as well as the longer development times observed, however further investigations of other water characteristics are needed for conclusive determination of factors influencing tadpole development in these breeding sites.

One interesting result from our study is the high proportion of non-developing eggs (aggregated across the years 2013 and 2014) found at CGH (ca. 60% non-developing), possibly attributable to the embryonic development being interrupted in some way. Many potential mechanisms could explain these observations; for example, a response to temperature, pH or salinity levels (e.g., Gosner & Black, 1957; Schlichter, 1981; Freda, 1986), background levels of herbicide or pesticide (e.g., Hayes et al., 2006), or increased risk of pathogen infection through UV-B exposure, (e.g., Kiesecker et al., 2001). Such variations in the ecosystem characteristics acting on fertilisation and/or embryonic development may partly explain enigmatic declines within the historical populations. In the two sites studied, we do not expect that pesticide or herbicide contaminations are influencing the development, as both sites are in protected areas that are not close to agricultural lands. If, however, the CGH population consisted of a larger proportion of younger adults compared to SILV, the large proportion of non-developing eggs at CGH could be as a result of lower fertilisation success of younger males.

With the lack of breeding populations at historically known sites, which are not impacted directly by anthropogenic land transformation or the presence of the chytrid fungus, an understanding of the reproduction at the two remaining breeding sites of *C. rosei*, and the factors influencing the development of the embryos, is crucial. In this study, we show that development in this threatened species is linked to pool characteristics, and that toads will only breed in pools with specific thermal and depth profiles, despite other nearby pools being available. Alteration to habitats, whether intentional or inadvertent, could lead to breeding sites becoming unsuitable resulting in local extinctions of breeding populations. Thus, further study on additional pool characteristics, such as water conductivity, macrophyte cover, and shade, are needed to fully understand the influences of environment on *Capensibufo* development and morphology.

An understanding of the life history in a species that is undergoing an enigmatic decline, such as *C. rosei*, enables us to understand which level conservation efforts are needed to target in order to prevent further population declines. Investigating the fertilisation success and survival estimates of a species is a first step to ensuring that recruitment during each breeding season is successful, which has been conducted for *C. rosei* (Becker, 2014). Secondly, the effect that environment has on the development, and in turn the morphology, of individuals is needed, as changes in the environment could have detrimental effects on development and in turn breeding success. These data could then be compared to sites where populations have declined or become locally extinct, and, if deemed necessary, conservation strategies can be implemented to mitigate further declines. Lastly, assessments of factors influencing adult survival (such as predation, disease and toxicity in the environment) and reproductive potential are needed, which we suggest are the next steps to understanding the enigmatic decline of *C. rosei*.

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