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Extensive geographic variation in testes size and ejaculate traits in a terrestrial-breeding frog

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ABSTRACT

Ejaculate traits vary extensively among individuals and species, but little is known about their variation among populations of the same species. Here, we investigated patterns of intraspecific variation in male reproductive investment in the terrestrial-breeding frog *Pseudophryne guentheri*. Like most anurans, breeding activity in *P. guentheri* is cued by precipitation, and therefore the timing and duration of breeding seasons differ among geographically separated populations, potentially leading to differences in the level of sperm competition. We therefore anticipated local adaptation in sperm traits that reflect these phenological differences among populations. Our analysis of six natural populations across a rainfall gradient revealed significant divergence in testes and ejaculate traits that correspond with annual rainfall and rainfall seasonality; males from the northern and drier edge of the species range had significantly smaller testes containing fewer, smaller and less motile sperm compared to those from mesic central populations. These findings may reflect spatial variation in the strength of postcopulatory sexual selection, likely driven by local patterns of precipitation.

1. Introduction

Much of the striking interspecific diversity in ejaculate traits has been attributed to differences in the levels of sperm competition [i.e. the competition between the ejaculates of two or more males for fertilization; 1]. For example, the broad theoretical prediction that species experiencing high levels of sperm competition should invest proportionately more in spermatogenesis [2, 3] is well supported from comparative studies that report variation in relative testes size [4] and sperm traits (e.g. size, speed and viability) [5-10].

Despite the success of sperm competition theory in explaining interspecific diversity in testes and ejaculates, we know less about the factors driving intraspecific variation in these traits. In theory, differences in environmental or ecological conditions among populations of the same species should influence mating dynamics and levels of sperm competition, and thus promote local adaptation in associated reproductive traits. For example, abiotic variables such as temperature and precipitation can influence the seasonality and length of the breeding season [11], operational sex ratios (OSR – the ratio of males to females ready to mate) [12, 13], resource availability [14], and ultimately the level of sperm competition and patterns of reproductive investment [15, 16]. However, intraspecific studies investigating male gamete traits along ecological gradients thought to correlate with the level of sperm competition have yielded mixed results [17-23] and we mostly lack an understanding of the drivers of intraspecific variation in male reproductive investment.

In most anuran amphibians, breeding phenology is linked to precipitation [24, 25]. For example, anurans occupying arid or semi-arid habitats tend to be opportunistic breeders, initiating short bouts of breeding activity following rainfall events [26, 27]. Under such conditions, the simultaneous arrival of many females during ‘explosive’ breeding events is expected to result in a more balanced OSR and more relaxed male-male (including sperm) competition [15]. By contrast, terrestrially breeding amphibians rely on consistent moisture for the successful development of their eggs, and may show prolonged breeding activity that coincides with seasonally occurring rainfall (thus exhibiting less balanced OSRs and heightened intrasexual selection). Therefore, local precipitation may influence breeding systems and sperm competition in amphibians [28], driving clinal divergence in male reproductive investment.

Here, we investigated intraspecific variation in ejaculate traits and testes investment along a precipitation cline in the terrestrial-breeding and externally fertilizing frog *Pseudophryne guentheri* (figure 1a; for further detail of study species see Electronic Supplementary Material [ESM]). *Pseudophryne guentheri* is distributed across regions of southwestern Australia that experience between ~300 and 1250 mm of rainfall per year (ESM figure S1). The arrival of males and females at a breeding site coincides with moist conditions following rainfall, and therefore differences in precipitation between populations may have implications for sperm competition. Our aim was to test whether male reproductive investment differs predictably among six populations across a rainfall gradient. We focused on a range of traits putatively tied to sperm competition, including testis size, sperm density, sperm motility and sperm length. Although we lack specific evidence for *P. guentheri*, previous work on other frog species indicates that sperm competition selects for increased testis size and greater sperm length (both sperm head and tail length) [29-33], while a single study of sperm competition in a related myobatrachid frog *Crinia georgiana* reported a competitive advantage in favour of males with slower-swimming sperm [34]. We expect that the window of mating opportunity is smaller for populations where rainfall is infrequent, resulting in ‘explosive’ breeding patterns in these dryer sites and consequently a relaxation in the level of sperm competition (and associated male reproductive investment) compared to more mesic habitats.

2. Material and methods

(a) Ethics statement

All animal work was conducted in accordance with the University of Western Australia’s (UWA) Animal Ethics Committee (permit number RA/3/100/1466). Fieldwork was conducted under permit SF010807 issued by the Western Australian Department of Biodiversity, Conservation and Attractions.

(b) Animal collection and study sites

Male *P. guentheri* were collected from six breeding choruses (Table 1) located along west-east transects at approximately two latitudes: four central sites and two sites near the northern limit of the species’ range (ESM figure S1). Breeding aggregations occur along drainage lines, but environmental conditions, including the climate, differ considerably among sites [Table 1; see also 35].

Ten to 19 (mean = 15.8 ± 1.6 SE; Table 1) calling males were collected by hand from their breeding burrows in May and June 2016. Males were then transported to the University of Western Australia in Perth and individually housed in terraria containing damp sphagnum moss and fed a diet of small insects. Terraria were kept in a controlled-temperature room at 16°C with an 11/13 h light/dark photoperiod to mimic winter conditions. In order to control for differences in body size or condition affecting sperm traits, snout-to-vent length (SVL; ± 1 mm) and standard mass (mass of a fully hydrated frog with bladder water drained; ± 0.001 g) were measured for each male upon arrival at the laboratory.

(c) Testes weight and sperm density

Sperm and testes traits were measured three to eight days after animals were collected using standard procedures (see ESM Supplementary Methods). Animal holding time did not significantly influence ejaculate traits (MANCOVA; Pillai's trace = 0.119, F = 1.42, P = 0.201). Residual testes tissue was removed from the macerate, blotted dry and weighed. The sperm suspension volume (µl) was then calculated as the mass of both testes (mg) – (testes residual tissue mass (mg) + volume of SAR added in µL). The sperm density in testes macerates was measured in an improved Neubauer haemocytometer (Hirschmann Laborgeräte, Eberstadt, Germany). The total number of sperm in testes was estimated by multiplying the sperm density (spermatozoa/µl) by the sperm suspension volume (µl).

(d) Sperm motility

A subsample of sperm suspension from each male was used to analyse sperm motility. The sperm density of the subsample was adjusted to a standard of 4×10^6 sperm per ml (using 1:1 SAR) to minimise any effects of sperm concentration on motility. Sperm motility was assessed using computer-assisted sperm analysis (CASA, CEROS 2 sperm tracker, Hamilton-Thorne Research) immediately after sperm activation. From these analyses, we selected curvilinear velocity (VCL) as a measure of sperm swimming speed and beat-cross frequency (BCF) as an estimate of the frequency with which sperm cells cross their smoothed path (see ESM Supplementary Methods). We also obtained a measure of the proportion of sperm exhibiting progressive motility in the sample.

(e) Sperm length

Within 24 h of sperm collection, samples containing inactive sperm were placed under a phase-contrast microscope (Olympus BX41) and photographed at X 800 magnification. Tail length, head length and head width of 10 intact sperm per male were later measured using ImageJ (see ESM Figure S2) [36].

(f) Statistical analysis

All analyses were performed using R version 3.4.3 [37]; some data were transformed to ensure they complied with the assumptions of parametric tests (see ESM Supplementary Methods).

To test whether sperm quantity, motility or length traits differed significantly among populations, we performed a single multivariate analysis of covariance (MANCOVA) in which population was entered as the fixed factor and ejaculate traits as the dependent variables. We tested for multicollinearity of the dependent variables using the “olsrr” package in R [38] (see ESM Table S2 for final VIF values). Models including the number of sperm in testes had high levels of multicollinearity (VIF ≥ 10), and we consequently removed this trait from the MANCOVA. Male standard mass was added as a covariate to control for potential allometric relationships between body size and sperm traits.

To examine whether abiotic variables thought to influence the seasonality and length of the breeding season of *P. guentheri* explained some of the differences in ejaculate traits among populations, a single MANCOVA was performed with ejaculate traits as the dependent variables and annual rainfall, rainfall seasonality (see ESM) and male standard mass as covariates. As above, to avoid multicollinearity, the number of sperm in testes was removed from the list of dependent variables in the model. ANCOVAs were performed for each ejaculate trait when the MANCOVA was significant, and male standard mass was added as a covariate in all analyses.

3. Results

Ejaculate traits differed significantly among populations (MANCOVA; Pillai's trace = 0.673, F = 21.602, P < 0.001), and male standard mass was a significant covariate (Pillai's trace = 0.401, F = 7.043, P < 0.001). Furthermore, annual rainfall (Pillai's trace = 0.588, F = 14.822, P < 0.001) and rainfall seasonality (Pillai's trace = 0.669, F = 20.967, P < 0.001) significantly influenced ejaculate traits, with male standard mass being significant (Pillai's trace = 0.244, F = 3.348, P = 0.002). Our univariate analyses confirmed that individual sperm quantity, motility and morphology traits were significantly affected by annual rainfall and rainfall seasonality (Table 2). Males from mesic populations with lower values for rainfall seasonality had proportionally larger testes than those from xeric sites (figure 1b, Table 2) and their sperm density was greater (figure 1c, Table 2). Annual rainfall and rainfall seasonality also significantly affected sperm motility parameters. Sperm from males from more mesic sites swam more quickly (VCL; figure 2a), exhibited greater motility (% motile; figure 2b) and had significantly higher flagellar beat frequencies (BCF; figure 2c) than the spermatozoa of more xeric males (see Table 2). Furthermore, sperm taken from males originating from mesic populations had significantly longer tail (2d) and head lengths (2e) on average, resulting in greater total sperm lengths (Table 2). Sperm from more mesic males was also narrower (figure 2f). Representative images of sperm from mesic and xeric populations are shown in figure 2g and 2h, respectively.

4. Discussion

Our findings demonstrate striking patterns of intraspecific variation in testes size and sperm traits in *Pseudophryne guentheri* sampled across a rainfall gradient. In general, males from xeric populations had smaller testes containing sperm at a lower density compared to males from mesic populations. These patterns were also apparent in our analyses of sperm size and motility, where we found that spermatozoa were smaller and swam more slowly in the xeric populations.

One critical factor likely to influence the level of sperm competition is the length of the breeding season, which can influence breeding systems and promote local adaptation in traits tied to sperm competition [28]. In natural populations of *P. guentheri*, seasonal breeding choruses may last six weeks at central sites (NM, personal observations for populations 1-3), while we speculate that populations inhabiting northern sites might breed year round, in response to less predictable rainfall [e.g. 39], and in a more ‘explosive’ fashion [40], where many females simultaneously arrive at a breeding site. This is likely to lead to a more balanced OSR in drier sites and therefore relaxed male-male competition [12]. Therefore, inter-population variation in the level of sperm competition, may account for the differences in reproductive investment reported here, although other factors (e.g. differences in resource availability across a rainfall gradient and/or genetic factors) may also play a role. Our findings therefore support our initial prediction that males from populations of *P. guentheri* experiencing a drier climate would exhibit reduced investment in testes and ejaculate traits. Indeed, an undescribed *Pseudophryne* species [44] sampled from an even drier region in the north (see ESM figure S1) showed ejaculate traits consistent with low reproductive investment (ESM figure S3).

There is widespread support from interspecific comparative studies of anurans [29-33], and more broadly across animal taxa, to show that sperm competition selects for increased relative testis size [4], while a single comparative study of 67 Chinese anurans also suggested that sperm competition selects for longer sperm [33]. Less is known about how selection imposed through sperm competition influences sperm velocity, although comparative [e.g. 7] and experimental [reviewed in 41] studies generally support the view that greater levels of sperm competition favour faster-swimming sperm [but see 34]. Our findings at the intraspecific level broadly complement these macro-evolutionary patterns by revealing possible adaptations to locally variable levels of sperm competition, although we acknowledge that experimental work is needed to determine how the traits considered here function during sperm competition.

There is tentative evidence that postcopulatory sexual selection can drive diversification of gamete traits and thus lead to reproductive isolation and speciation [42-45]. For example, variation in sperm length, driven by sperm competition and cryptic female choice, may promote reproductive isolation in insects [46]. Although competitive fertilisation experiments for *P. guentheri* are required, we predict that sperm from northern males will compete poorly with sperm from central males, given the smaller quantity of sperm produced, the slower swimming speed of spermatozoa, and altered sperm morphology. Thus, the marked intraspecific variation in sperm traits and reproductive investment in *P. guentheri* may indicate that xeric populations near the range edge and mesic populations are, at least partially, reproductively isolated.

In summary, we found a striking divergence in testes size, sperm quantity, motility and length among *P. guentheri* populations that corresponded with annual rainfall and rainfall seasonality. Together, these findings that males from xeric sites exhibit lower reproductive investment in testes and sperm compared to males from the more mesic centre of the species range are consistent with emerging evidence that patterns of sexual selection can diverge among populations of the same species [47], most likely driven by local climatic factors. Irrespective of the drivers of these disparate patterns, differences in gamete traits between populations may lead to reproductive isolation [43, 44]. The divergence between xeric and mesic populations provides a mechanism for cryptic speciation in this widespread Australian genus [48, 49], and crossing populations will conclusively answer whether successful interbreeding is possible.

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Author contributions. T.S.R-B, N.J.M. & J.P.E. conceived the study. T.S.R-B conducted the field and laboratory work, conducted the statistical analyses and wrote the first draft of the paper. All authors contributed towards subsequent versions of the manuscript. All authors agree to be held accountable for the content therein and approve the final version of the manuscript.

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Table 1. Site locations, rainfall characteristics and the number of males sampled (*N*) for each *P. guentheri* population.

Collection site	Pop. No	Longitude	Latitude	*N*	Number of days between first rainfall* (≥ 5mm/day) and sperm collection date (± SD)	Annual mean temperature (°C)	Annual mean precipitation (mm)	Days of rain (> 1mm) in May-July	Days of rain (> 5mm) in May-July
Chidlow	1	31°53'05.5"S	116°18'48.0"E	17	27 ± 11	17.2	788	35	20
Flint Plot	2	32°17'01.4"S	116°31'24.1"E	12	11 ± 6	6.7	654	34	18
Pingelly	3	32°28'25.9"S	116°58'27.9"E	19	7 ± 5	16.6	428	28	13
Dudenin	4	32°49'17.4"S	117°53'01.0"E	18	12 ± 4	16.5	358	23	9
Binnu	5	28°02'30.8"S	114°39'36.0"E	19	6 ± 5	19.9	352	23	10
Mullewa	6	28°31'07.3"S	115°38'11.4"E	10	6 ± 4	20.5	329	22	9

* of the breeding season (May-June 2016)

Note: Populations are numbered by increasing aridity. Climate data were obtained from the Bureau of Meteorology and are interpolated values for the specific coordinates of each population, averaged from 1980 to 2017.

Table 2. Analysis of covariance (ANCOVA) test results for sperm trait comparisons among males from six populations across a rainfall gradient. *P* values are corrected for multiple comparisons following Bonferroni.

	Dependent variables	Effect	*Mean SS*	*F*	*df*	*P*	Sig.
Sperm quantity	Mass of both testes (mg)	Annual rainfall	2.92	11.01	1	0.010	*
		Rainfall seasonality	16.45	62.03	1	< 0.001	***
		Male standard mass	2.18	8.20	1	0.042	*
	Sperm density (sperm/µl)	Annual rainfall	41.16	23.53	1	< 0.001	***
		Rainfall seasonality	97.12	55.53	1	< 0.001	***
		Male standard mass	10.62	6.07	1	0.125	ns
Sperm motility	VCL (µm/s)	Annual rainfall	324.00	37.38	1	< 0.001	***
		Rainfall seasonality	187.40	21.62	1	< 0.001	***
		Male standard mass	11.00	1.27	1	0.263	ns
	Proportion of motile sperm (%)	Annual rainfall	0.000045	0.04	1	0.836	ns
		Rainfall seasonality	0.013934	13.47	1	0.003	**
		Male standard mass	0.000788	0.76	1	0.385	ns
	BCF (Hz)	Annual rainfall	5.13	9.07	1	0.027	*
		Rainfall seasonality	1.86	32.87	1	< 0.001	***
		Male standard mass	3.56	0.06	1	0.802	ns
Sperm morphology	Sperm tail length (µm)	Annual rainfall	32.46	5.19	1	0.200	ns
		Rainfall seasonality	300.63	48.02	1	< 0.001	***
		Male standard mass	1.70	0.27	1	0.603	ns
	Sperm head length (µm)	Annual rainfall	0.25	63.48	1	< 0.001	***
		Rainfall seasonality	0.05	12.62	1	0.005	**
		Male standard mass	0.03	7.67	1	0.054	ns
	Sperm head width (µm)	Annual rainfall	0.16	46.11	1	< 0.001	***
		Rainfall seasonality	0.11	32.22	1	< 0.001	***
		Male standard mass	0.01	2.789	1	0.787	ns

FIGURES

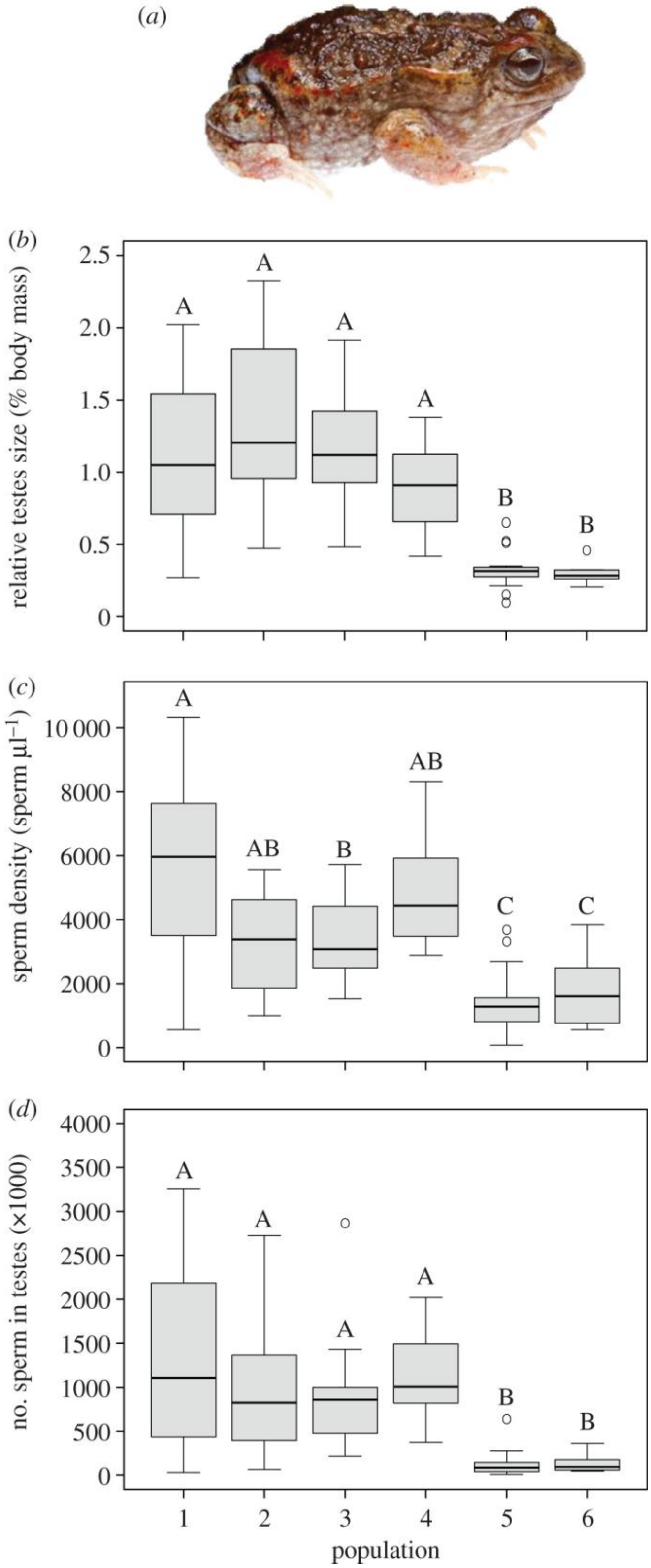

Figure 1. Image of (a) a male *P. guentheri*, followed by box plots showing median (thick lines) and lower and upper quartile values (box width) for (b) relative testes size, (c) sperm density and (d) total number of sperm within the testes in six *P. guentheri* populations. Populations that do not share the same letters are significantly different (Bonferroni post-hoc tests, $P < 0.05$).

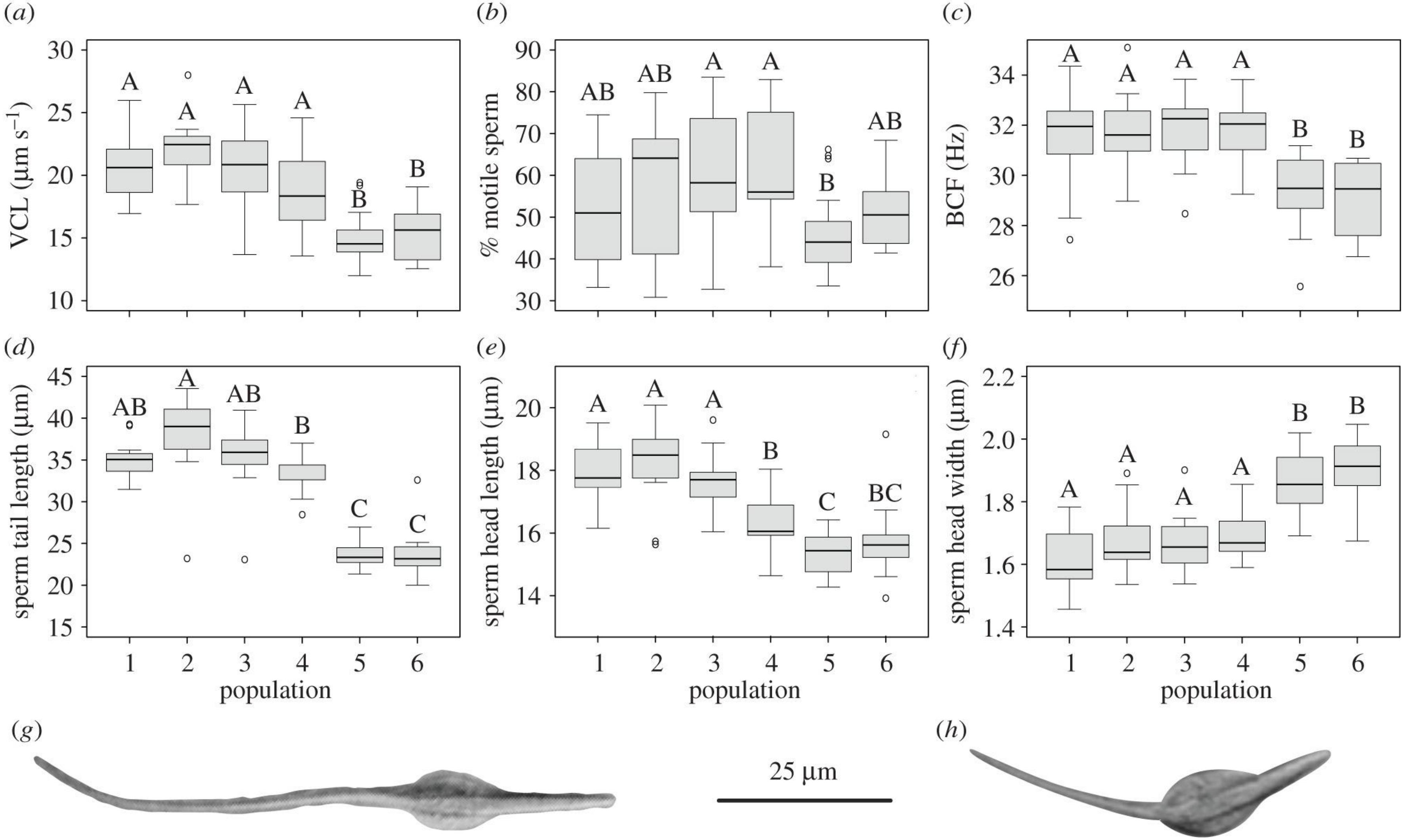

Figure 2. Box plots of sperm motility (a-c) and sperm dimensions (d-f) in six P. guentheri populations. Each box shows the lower and upper quartile values and the thick line indicates the median value. Populations that do not share the same letters are significantly different (Bonferroni post-hoc tests, $P < 0.05$). Micrographs of representative spermatozoon for central males (populations 1 – 4) and northern males (populations 5 and 6) are shown at g) and h) respectively.