ELECTRONIC SUPPLEMENTARY MATERIAL

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

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Supplementary methods

Study species

Crawling frogs, *Pseudophryne guentheri*, are small (26-33 mm snout-to-vent length, SVL) myobatrachid frogs endemic to the southwest of Western Australia [1]. Breeding takes place in autumn and winter months following seasonal rainfall. Males excavate burrows in areas that are likely to be flooded and call at the burrow's entrance to attract females [2]. After a female has selected and approached a male, mating occurs inside the burrow. The male grasps the female and, after a pre-ovipositional clasping period which may last several hours in related species [3], externally fertilises egg clutches of 60 to 300 eggs as they are released. After mating, the male usually remains with the eggs and resumes calling. Woodruff [3] observed that in some *Pseudophryne sp*., the female either leaves the breeding area after mating if all eggs were deposited, or otherwise remains in the chorus to subsequently mate again. Within the genus *Pseudophryne*, Woodruff [3] estimated that fewer than half the females lay all their eggs at once and seasonal monogamy or successive polygamy are common. Encapsulated embryos develop terrestrially and hatching occurs when burrows flood in late winter [4]. Tadpoles complete their development in ephemeral water bodies in about three months [2].

While multiple paternity in *P. guentheri* has not been investigated directly, our field observations lead us to speculate that sperm competition occurs in this species. For example, on several instances multiple males have been found in one burrow at central breeding sites, including occasions when more than one female was present. We have also observed a burrow containing a male at the bottom, with a side channel located in close proximity and occupied by another, smaller male. Byrne *et al.* [5] estimated that the risk of sperm competition (i.e. the probability that a female has mated with another male) is comparatively high in this species (sperm competition index 3 out of 4; ranks were allocated with regards to density of breeding aggregations, proximity of males and male-male interactions) due to close proximity of calling males at the breeding sites and evidence of male-male interactions, such as territorial calling [6].

Sperm collection

Three to eight days after collection, males were sacrificed via ventral immersion in <0.03% benzocaine solution for 10 min, followed by double pithing. Both testes were removed, blotted dry and weighed to the nearest 0.1 mg (precision balance XS204, Mettler Toledo, Melbourne, Australia) and placed on ice. Testes were then macerated in 20 to 615 µL (adjusted according to the weight of the testes) of chilled standard amphibian ringer (SAR; 113mM NaCl, 2mM KCl, 1.35 mM $CaCl_2$, and 1.2 mM $NaHCO_3$), which mimics the osmolality within the male reproductive tract and keeps the spermatozoa in an inactive state [7]. Anuran sperm can be stored in the buffer for extended periods (days - weeks) without experiencing substantial declines in sperm motility [8, 9].

Sperm motility

For each male, 1 μL of sperm suspension was mixed with 10 μL of 25% SAR to activate the spermatozoa. This sperm solution was immediately pipetted into two uncoated wells of a 12-well multitest slide (MP Biomedicals, Aurora, OH, USA), 4 μL in each well, and covered gently with a coverslip. An average of 202 ± 11 SE motile sperm tracks were measured per sample, with cell movements below 2.5 μm/s considered to be static. The CASA assays generated seven motility parameters. Three of these parameters described components of sperm velocity, including average path velocity (VAP) - the mean velocity of the sperm head along its average trajectory, curvilinear velocity (VCL) - the mean path velocity of the sperm head along its actual trajectory, and linear velocity (VSL) - the mean path velocity of the sperm head along a straight line from its first to its last position. The remaining four motility parameters included sperm linearity ((VSL/VCL) × 100), the beat frequency of the sperm's flagellum (BCF), the amplitude of the lateral sperm head displacement (ALH), and the wobble coefficient ((VAP/VCL) × 100). Both VCL and BCF were used in our final analyses of sperm motility (see main text), but the broad patterns reported in this study remained largely unchanged for all parameters. Finally, the CASA assays also generated a measure of the proportion of sperm exhibiting progressive motility (> 2.5 μm/s) in the sample.

Statistical analyses

In order to ensure that data complied with assumptions of normality, Q-Q plots of residuals were inspected and data were transformed as necessary. Male standard weight, mass of both testes and sperm head length and width were subject to $\log_{10}$ transformations. Sperm density, number of sperm in testes, sperm tail length, BCF and proportion motile sperm were transformed using the Box-Cox method [10]. The sperm velocity parameters VAP and VSL were both strongly correlated with VCL (both *r*-values > 0.89, $P < 0.001$), and we therefore restricted our analysis of sperm velocity to VCL. Results did not change when conducting analyses of VAP or VSL.

Abiotic variables

Abiotic variables thought to influence the seasonality and length of the breeding season of *P. guentheri* were added as covariates to the MANCOVA to investigate whether they explained some of the differences in ejaculate traits between populations. A suite of abiotic variables were tested in the model, including annual rainfall (mm), soil moisture (%), evaporation (mm/day), average maximum, minimum and annual temperature (°C), annual rainfall variability (percentile analysis, indicating how rainfall varies from year to year), rainfall seasonality during the breeding season (% annual rainfall that falls between May and July), and average number of days/year when rainfall exceeded 5 mm. All abiotic variables were obtained from the Australian Bureau of Meteorology and are interpolated values for the specific coordinates of each population, averaged from 1880 to 2017. All abiotic variables were highly significant when tested individually. To simplify the analysis, only two abiotic variables were used in the final model, comprising annual rainfall and rainfall seasonality. Results and conclusions did not change when selecting any of the other abiotic variables.

Supplementary Tables

ESM Table S1: Coefficients of Variation (CV) for sperm tail length, head length and head width (sample size: 10 sperm cells per individual)

Population	Male ID	Mean sperm tail length	CV	Mean sperm head length	CV	Mean sperm head width	CV
1	C1	34.99	2.95	17.50	5.37	1.46	13.04
1	C2	32.86	10.02	18.21	12.29	1.55	9.34
1	C3	39.27	8.15	17.62	10.03	1.63	16.13
1	C4	36.16	3.94	17.45	8.62	1.78	6.96
1	C5	35.06	6.52	17.15	6.92	1.70	11.01
1	C6	33.65	5.46	18.25	3.62	1.62	12.05
1	C7	33.95	6.52	17.10	6.45	1.65	11.01
1	C8	33.79	5.88	18.67	7.74	1.67	12.07
1	C9	35.05	3.32	16.96	7.43	1.77	10.81
1	C10	34.39	4.43	19.06	4.36	1.51	9.30
1	C11	31.47	7.63	17.76	6.78	1.57	12.06
1	C12	35.79	5.79	17.89	9.83	1.52	11.14
1	C13	33.06	7.87	19.04	8.39	1.46	7.45
1	C14	35.32	7.64	19.52	5.42	1.58	8.46
1	C15	39.11	6.58	16.15	10.28	1.57	11.61
1	C16	35.76	7.77	17.62	3.41	1.71	7.05
1	C17	33.57	8.53	18.90	5.48	1.56	11.06
2	FL1	23.21	4.53	15.64	5.40	1.89	7.22
2	FL2	43.54	12.96	19.54	8.38	1.64	6.26
2	FL3	37.38	9.53	18.56	6.32	1.71	10.54
2	FL4	41.93	9.06	18.10	5.05	1.54	11.36
2	FL5	40.01	8.25	20.08	8.32	1.61	9.71
2	FL6	38.46	10.00	15.73	13.35	1.85	14.18
2	FL7	39.55	8.12	18.62	6.23	1.62	11.15
2	FL8	42.57	10.77	18.41	5.81	1.66	12.55
2	FL9	35.30	5.77	19.36	6.87	1.57	12.93
2	FL10	37.23	12.16	17.61	6.37	1.64	15.90
2	FL11	40.24	8.58	18.56	6.41	1.64	13.89
2	FL12	34.79	5.18	17.90	7.08	1.74	7.31
3	FA1	23.08	2.16	16.04	3.92	1.90	7.09
3	FA2	38.46	1.51	17.16	1.95	1.65	4.28
3	FA3	40.95	4.75	18.87	5.29	1.66	4.62
3	FA4	35.90	4.81	16.90	2.15	1.63	6.96
3	FA5	34.95	4.60	17.13	4.24	1.61	10.23
3	FA6	37.72	1.36	17.51	2.67	1.72	2.88
3	FA7	37.45	4.42	17.95	4.47	1.55	8.23
3	FA8	32.88	3.11	17.79	4.21	1.60	6.47
3	FA9	37.02	4.28	17.66	3.32	1.73	5.25
3	FA10	37.45	4.42	17.87	4.31	1.55	8.23

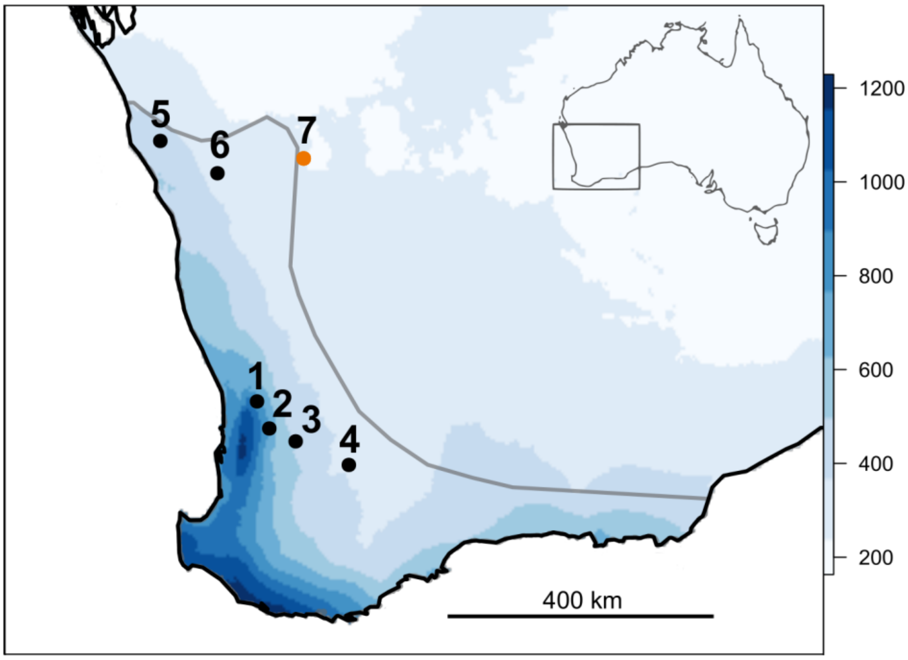
Population	Male ID	Mean sperm tail length	CV	Mean sperm head length	CV	Mean sperm head width	CV
3	FA11	37.08	4.83	17.91	8.66	1.72	6.68
3	FA12	34.70	5.45	19.61	3.67	1.59	5.61
3	FA13	37.33	3.76	18.00	3.55	1.66	6.87
3	FA14	33.75	2.32	17.70	2.32	1.66	6.82
3	FA15	34.42	2.59	17.45	3.89	1.54	9.89
3	FA16	35.47	2.07	17.09	6.78	1.75	5.20
3	FA17	34.49	3.03	16.05	7.18	1.67	5.64
3	FA18	33.54	3.68	17.93	3.85	1.62	7.83
3	FA19	36.74	3.03	18.32	4.04	1.73	7.95
4	D1	37.00	5.32	16.77	6.99	1.59	12.82
4	D2	33.05	4.78	16.78	6.40	1.71	8.36
4	D3	35.83	6.11	15.12	7.50	1.74	10.41
4	D4	36.54	8.23	16.92	11.06	1.60	7.27
4	D5	30.30	5.95	18.04	7.28	1.65	11.85
4	D6	31.12	12.88	16.31	6.65	1.67	8.30
4	D7	32.74	5.67	14.64	12.57	1.86	10.88
4	D8	28.45	5.05	16.06	8.26	1.76	8.25
4	D9	32.83	10.10	15.99	6.49	1.66	7.05
4	D10	36.16	7.87	16.05	7.83	1.60	12.49
4	D11	34.37	4.84	15.28	11.13	1.75	10.18
4	D12	32.61	5.94	15.41	9.69	1.84	10.72
4	D13	33.92	3.45	16.89	3.13	1.67	9.10
4	D14	31.19	7.20	16.05	9.02	1.64	10.29
4	D15	34.40	2.34	15.93	4.98	1.62	10.75
4	D16	34.37	6.75	17.00	6.55	1.65	10.34
4	D17	33.09	4.19	16.01	6.23	1.74	7.06
4	D18	33.89	4.64	17.38	5.62	1.68	6.70
5	B1	23.34	14.55	15.56	11.10	1.88	12.25
5	B2	23.41	5.81	15.05	4.45	1.86	10.47
5	B3	24.20	7.05	14.74	10.35	1.81	11.36
5	B4	21.75	12.34	16.42	10.18	1.69	7.94
5	B5	21.67	7.51	14.27	8.03	1.84	8.30
5	B6	22.67	8.75	15.97	6.60	1.97	7.31
5	B7	24.24	8.23	14.72	10.16	1.78	12.25
5	B8	24.85	13.38	15.83	8.30	2.02	16.33
5	B9	21.35	10.67	15.48	4.86	1.72	12.94
5	B10	23.78	11.21	15.43	12.04	1.91	15.00
5	B11	22.98	8.91	15.58	7.45	1.78	7.89
5	B12	25.69	4.38	15.94	2.61	1.84	9.78
5	B13	24.76	11.24	15.91	5.49	1.81	9.96
5	B14	21.51	10.95	14.79	11.70	1.99	11.47
5	B15	23.33	14.53	15.40	5.37	1.90	11.41
5	B16	26.96	12.42	14.54	13.11	1.96	12.43
5	B17	22.79	8.68	15.96	10.15	2.00	14.39
5	B18	23.27	7.05	14.96	6.52	1.93	9.03

Population	Male ID	Mean sperm tail length	CV	Mean sperm head length	CV	Mean sperm head width	CV
5	B19	25.39	3.15	14.41	3.77	1.78	12.28
6	M1	32.59	10.67	19.15	10.03	1.67	7.50
6	M2	20.00	8.27	14.61	8.63	1.79	8.93
6	M3	24.61	6.92	15.44	5.27	2.02	18.98
6	M4	23.18	4.88	13.92	6.44	1.85	4.65
6	M5	25.12	8.28	15.94	4.53	1.94	7.37
6	M6	22.32	8.75	15.22	12.62	1.98	8.49
6	M7	23.66	20.47	15.33	9.37	2.05	8.31
6	M8	21.04	6.50	15.84	7.52	1.97	6.31
6	M9	22.36	8.06	16.73	5.95	1.86	10.19
6	M10	23.16	8.66	15.80	5.87	1.89	9.46

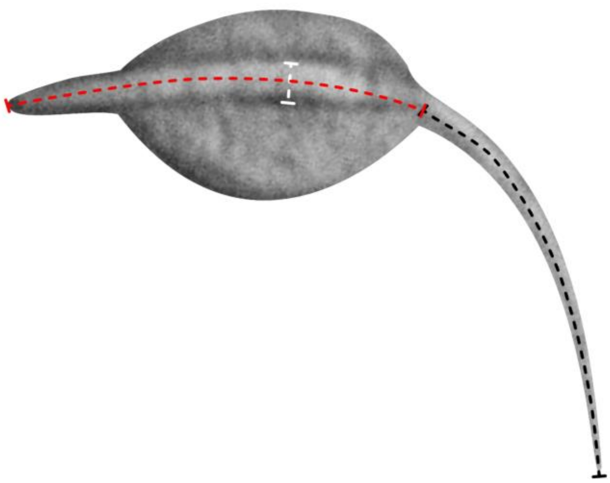
ESM Table S2: VIF values for each ejaculate trait.

Ejaculate trait	VIF
Mass of both testes	2.29
Sperm density	2.09
VCL	1.88
Proportion of motile sperm (%)	1.20
BCF	1.67
Linearity coefficient	1.54
Sperm tail length	1.51
Sperm head length	2.17
Sperm head width	2.59

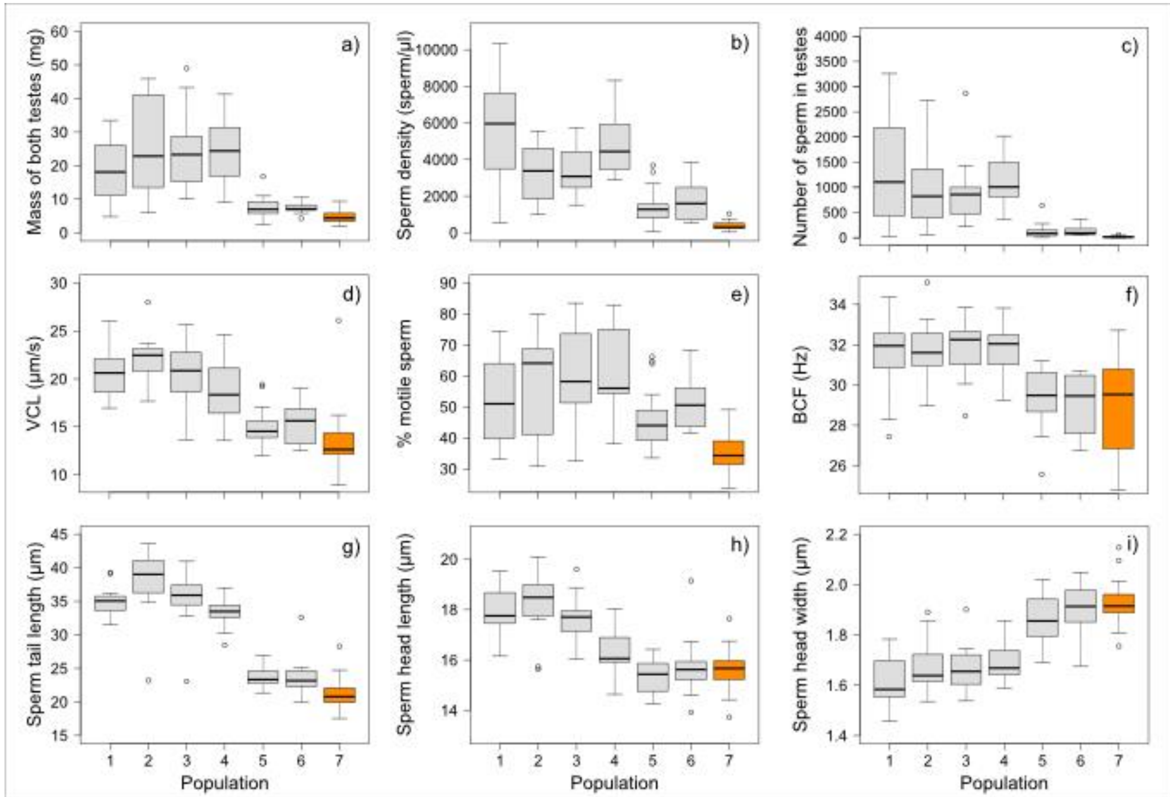
Supplementary Figures



ESM Figure S1. Map showing the distribution of *P. guentheri* in Western Australia (grey line marks the approximate range limit based on occurrence records from the Atlas of Living Australia) and six collection sites, overlaid with mean annual rainfall (mm). Collection sites were chosen with reference to a broader goal of exploring targeted gene flow in this species [11] and are numbered by increasing aridity. Northern sites are warmer and drier than the more central sites and the northern breeding habitats are more open and on sandier soils. Site 7 marked in orange shows a genetically distinct population that is likely to be a different species [12], but was thought to be *P. guentheri* at the time of collection. Male reproductive data for this population were collected in the same manner as for the *P. guentheri* populations (and on the same date as populations 5 and 6), and are shown in figure S3.



ESM Figure S2. Example of a sperm cell from a male collected from a northern population, showing how the head length (red dashed line, head width (white dashed line) and tail length (black dashed line) were measured. The structure surrounding the ventral end of the sperm head is the mitochondrial vesicle.



ESM Figure S3. Box plots showing the nine traits shown in figures 1 and 2 relative to those of an undescribed conspecific lineage (population 7) located inland of northern populations 5 and 6 (see figure S1). As for figures 1 and 2, each box shows the lower and upper quartile values and the thicker line indicates the median value. Panels are: (a) testes mass, (b) sperm density, (c) total number of sperm within the testes, (d-f) sperm motility, and (g-i) sperm dimensions. *Pseudophryne guentheri* populations are shown in light grey, and the divergent population in orange. As for Figures 1 and 2, populations are arranged in order of increasing aridity.

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