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3 **Multiple paternity in a viviparous toad with internal fertilisation**

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24

25 Abstract

26 Anurans are renowned for a high diversity of reproductive modes, but less than 1% of species
27 exhibit internal fertilisation followed by viviparity. In the live bearing West African Nimba
28 toad (*Nimbaphrynoides occidentalis*), females produce yolk-poor eggs and internally nourish
29 their young after fertilisation. Birth of fully developed juveniles takes place after nine
30 months. In the present study, we used genetic markers (eight microsatellite loci) to assign the
31 paternity of litters of 12 females comprising on average 9.7 juveniles. In nine out of twelve
32 families (75%) a single sire was sufficient; in three families (25%) more than one sire was
33 necessary to explain the observed genotypes in each family. These findings are backed up
34 with field observations of male resource defence (underground cavities in which mating takes
35 place) as well as coercive mating attempts, suggesting that the observed moderate level of
36 multiple paternity in a species without distinct sperm storage organs is governed by a balance
37 of female mate choice and male reproductive strategies.

38

39 Keywords: multiple paternity, internal fertilisation, operational sex ratio, male harassment,
40 Amphibia, *Nimbaphrynoides occidentalis*

41

42 Introduction

43 It is now well established that polyandry is more common in the animal kingdom than
44 originally assumed (Pizzari and Wedell, 2013). For females, advantages of multiple matings
45 include an increased availability of resources provided by males (Gray, 1997), enhanced
46 fertilisation success (Caspers et al., 2014; Plough et al., 2014), higher genetic heterogeneity
47 (Nichols et al., 2015; Rovelli et al., 2015) and genetic quality of offspring (Bouwman et al.,
48 2006; Byrne and Whiting, 2011; Johnson and Brockmann, 2013). In species with internal
49 fertilisation, polyandry also enables females to exert cryptic choice of fertilising males (for

50 reviews see Parker and Birkhead, 2013; Simmons, 2005). Negative effects of polyandry for
51 females can include higher predation risk (Arnqvist, 1989) and female injuries during mating
52 (Xavier, 1971), and a higher probability to suffer from sexually transmitted parasites and
53 diseases (Ashby and Gupta, 2013). Males, on the other hand, generally maximise their
54 reproductive success through rather opportunistic and sometimes coercive matings (Arnqvist,
55 1989; Arnqvist and Kirkpatrick, 2005; Szatecsny et al., 2006). However, male mating
56 strategies that involve for example territoriality can also reduce the level of polyandry
57 through female monopolisation (e.g.: Arnqvist and Kirkpatrick, 2005).

58 Apart from differential mating strategies exhibited by each sex, levels of polyandry are also
59 governed by reproductive life histories. Among the classes of vertebrates, amphibians
60 arguably demonstrate the highest diversity of reproductive modes (Duellman and Trueb,
61 1986; Wells, 2010). Caecilians and caudates are generally characterised by internal
62 fertilisation (Wake, 1993), with sperm storage organs allowing for sperm competition and
63 multiple paternity which are possibly influenced by cryptic female choice (Caspers et al.,
64 2014; Jehle et al., 2007; Kupfer et al., 2008; Rovelli et al., 2015). In anurans, on the other
65 hand, the vast majority of species fertilise their eggs externally (Wake, 1993, 2015a).

66 Polyandry in anurans is common (for a review see Byrne and Roberts, 2012) and male mating
67 tactics such as multiple amplexi (Byrne and Roberts, 1999; Byrne and Whiting, 2011), clutch
68 piracy (Vieites et al., 2004) and possibly the release of stray sperm into aquatic environments
69 (Hase and Shimada, 2014) can lead to multiple paternity within single clutches beyond the
70 control of females.

71 Internal fertilisation is a prerequisite for viviparity through uterine egg retention until an
72 advanced developmental stage (Wake, 1993, 2015a, 2015b). However, while viviparity is
73 common in caecilians, it is only exhibited by 2.7% of caudates (Buckley, 2012; Wake, 2015a,
74 2015b). In anurans, only 0.3% of species (17 out of approximately 6600, Frost, 2015) from

75 five genera are known or assumed to be viviparous, comprising three different viviparous
76 reproductive modes (Haddad and Prado, 2005; Iskandar et al., 2014). One mode is generally
77 termed ovo-viviparous, in which yolk-rich eggs are retained in the uterus and juveniles are
78 born after metamorphosis (Haddad and Prado, 2005; Lamotte and Xavier, 1972; Wake,
79 1978). This is the viviparous reproductive mode known or assumed for 15 viviparous anuran
80 species, whereas the remaining two modes are each known from only a single anuran species.
81 The second viviparous reproductive mode is generally termed viviparous, or truly viviparous,
82 in which mothers nourish their larvae during gestation and juveniles are born after
83 metamorphosis (Haddad and Prado, 2005; Xavier, 1977, 1986). Only recently the third
84 viviparous reproductive mode was discovered: *Limnonectes larvaepartus* giving birth to
85 tadpoles (Iskandar et al., 2014). In comparison to oviparous species, viviparity is often more
86 costly for females, as clutch sizes are restricted by the available intra-uterine space and
87 locomotion may be reduced (Blackburn, 1999; Shine, 1987; Wourms and Lombardi, 1992).
88 Compared to egg-laying internal fertilisers, cryptic female choice should therefore pose a
89 particular advantage to viviparous species.

90 The West African Nimba toad (*Nimbaphrynoides occidentalis*) is the only known anuran
91 representative of the truly viviparous reproductive mode (Wake, 2015b; Wells, 2010; Xavier,
92 1986). Nimba toads mate at the end of the rainy season (September-November), before
93 moving underground until the beginning of the next rainy season in March or April (Lamotte,
94 1959). After internal fertilisation of small, yolk-poor eggs (~ 500 µm in diameter, Xavier,
95 1986), females nourish their foetuses (matrotrophy) and give birth to 4-17 fully developed
96 juveniles (pueriparity) after about 9 months (Lamotte, 1959). At a longevity of 3-5 years
97 (Castanet et al., 2000), lifetime reproductive output per female is low.

98 In the present paper we focus on the Nimba toad to, for the first time, elucidate patterns of
99 paternity in a viviparous anuran with internal fertilisation. More specifically, we link our

100 findings from genetic parentage analyses of litters derived from known females with
101 behavioural field observations of male fighting and mating attempts. Together with
102 presenting data on operational sex ratios, we discuss whether internal fertilisation in the
103 Nimba toad leads to a genetic mating system which can be more controlled by females
104 compared to the external fertilisation which is exhibited by the vast majority of anurans.

105

106 Materials and Methods

107 *Field work*

108 The study was conducted in the high altitude grasslands of the Guinean Nimba Mountains,
109 West Africa. The area is characterised by a dry season from December to March and a rainy
110 season from April to November, a seasonality which has a strong influence on Nimba toad
111 abundance and sex ratios (Lamotte, 1959). A male-biased operational sex ratio (OSR) was
112 previously hypothesised to be the main driver for anuran polyandry (Byrne and Roberts,
113 2012; Hase and Shimada, 2014; Lodé et al., 2005; Sztatecsny et al., 2006). Therefore, we
114 estimated the average OSR per calendar week within the rainy season based on annual
115 monitoring data from 2007 to 2014. We included 333 areas of 5 x 5 m in size at a search
116 effort of 90 person minutes (for more details see Hillers et al., 2008; Sandberger-Loua et al.,
117 2016). Weekly OSRs were fitted to an additive generalised model using the mgcv package in
118 R (Wood, 2011).

119 We collected 12 gestating females in the field, and kept them singly in plastic terraria about 5
120 km from the capture location, feeding them with small flying insects and termites. Humidity
121 levels were kept high through moistened polyurethane foam, and the temperature was kept at
122 levels representing their natural environment (20-25°C). All females gave birth between three
123 and 10 days after capture. After birth, we clipped a tip of the second toe from the mother and
124 all offspring (following procedures described in (Grafe et al., 2011)), to store in 98% ethanol

125 for DNA analysis. All toads (mothers and juveniles) appeared in good condition after this
126 procedure and were released where the mother had been caught.

127

128 *Development of microsatellite loci for N. occidentalis*

129 We used up to eight newly developed microsatellite loci to determine the number of fathers
130 in each litter. For development of a genomic library, DNA was extracted from thigh muscles
131 of six individuals using Puregene DNA Purification Kit (Gentra Systems) according to the
132 manufacturer's recommendations. GENterprise Genomics (Mainz, Germany;
133 <http://genterprise.de>) was contracted to develop a repeat-enriched library. Twenty-seven
134 sequences containing more than eight repeats and sufficient flanking regions were chosen to
135 design PCR primers using Primer3 (Rozen and Skaletsky, 2000). Eight loci proved
136 polymorphic and unambiguously scorable based on an initial test with 4 individuals and
137 subsequent characterisation based in a further 40 individuals. Microsatellite sequences were
138 deposited in Genbank.

139

140 *Genotyping*

141 DNA for the paternity analysis was extracted using the Roche PCR template preparation kit
142 according to the manufacturer's recommendations. PCRs were performed using a 12.5 µl
143 PCR reaction volume containing 1 ×PCR-buffer, 2 mM MgCl₂, 160 µM dNTPs, 2.5 µM of
144 each primer (forward primer labelled with fluorescent IR-700 or IR-800 dye by Licor), 0.5 U
145 of Taq DNA polymerase (New England BioLabs) and 1 µl of 1:10 diluted template DNA. All
146 loci were amplified on a 2720 Thermal Cycler (Applied Biosystems, version 2.09). Loci G07
147 and D03 were amplified using a fixed annealing temperature of 57°C, and a touchdown
148 program was applied to all other loci (Nocc4, A09, C05, C10, E06 and F03). PCR conditions
149 for the two protocols were as follows: 57°C: 3 min at 94°C, 35 cycles with 30 sec. each at

150 94°C, 57°C and 72°C, followed by 20 min at 72°C; touchdown: 5 min at 94°C, 10 cycles
151 with annealing temperature decreasing 0.5°C per cycle from 63°-57°C, with 30 sec. each at
152 94°C, annealing temperature and at 72°C, followed by 25 cycles with 30 sec. each at 94°C,
153 55°C and 72°C, followed by 7 min elongation at 72°C. Allele lengths were analysed with
154 SAGA^{GT} (LICOR). Primer characteristics are given in Table 1. To minimise scoring errors,
155 every sample was amplified at least twice for each locus.

156

157 *Paternity analyses*

158 We used two complementary approaches to estimate the minimum (GERUD) and the most
159 likely (COLONY) number of sires in each litter, respectively. In a first step, we applied the
160 maximum likelihood approach implemented in COLONY2 (Jones and Wang, 2010)
161 assuming a large error rate (10%) to identify potential genotyping errors. The following
162 specifications were used: the maternal genotype was known, no candidate fathers included,
163 we gave no known population allele frequency, we used a sib-ship size prior and two runs of
164 medium length. Individuals whose alleles were classified as typing errors (0.4% of alleles or
165 one allele in 3.4% of individuals) were genotyped a third time and in all cases the allele sizes
166 were confirmed. Final results from COLONY2 runs were subsequently derived with an
167 assumed error rate of zero (all other specifications as described above), and repeated a second
168 time with the inclusion of population-wide allele frequencies. Population-wide allele
169 frequencies were derived from a dataset from 600 adult toads (Sandberger-Loua et al.,
170 unpublished). As a second approach, we applied GERUD2.0 (Jones, 2005) to estimate the
171 minimum number of fathers to explain the observed genotypes. Because GERUD2.0 does not
172 allow for missing data, we had to alternatively exclude 17% of individuals or reduce the
173 number of markers used. The statistical power was assessed by running GERUD2.0sim
174 (Jones, 2005) 10 times with 1000 iterations, assuming that the mother's genotype is known,

175 that one of 10 offspring is sired by a second male, and that either four or five loci are
176 available, based on the population wide allele frequencies. In all 10 runs, an additional father
177 was recognised in > 99% of iterations, demonstrating that only a subset of available loci is
178 sufficient for reliable paternity detection. We considered litters to contain multiple paternity
179 when GERUD and COLONY independently identified multiple fathers, and when the
180 number of most probable sires determined by COLONY was identical or larger than the
181 minimum number of fathers estimated by GERUD. The effective mating frequency (m_e), a
182 quantity which takes the actual paternity of contributing males into account, was calculated as
183 $m_e = (1/\sum pi^2)$, where pi is the proportion of offspring in a clutch sired by male i (Starr, 1984).

184

185 Results

186 The 12 Nimba toad females gave birth to an average of 9.7 young each (range: 4–16; total
187 116). We genotyped 5-7 loci for each individual and included 117 individuals in the paternity
188 analysis (12 mothers and 105 offspring, 11 young had to be discarded due to poor DNA
189 quality, Table 2). The microsatellite markers proved highly polymorphic, comprising on
190 average 14 alleles per locus (range: 7–19 alleles). All offspring could be unambiguously
191 assigned to their mothers, and in nine out of twelve families a single sire was sufficient to
192 explain the observed genotypes in each family. Three families contained multiple paternity as
193 identified by both GERUD and COLONY, with the most successful male siring between 55-
194 70% of offspring (effective mating frequency, m_e : 1.72–1.98; Table 2). The same results were
195 obtained when considering population allele frequencies (data not shown). However, whereas
196 GERUD identified two fathers in all three cases, COLONY identified two families with two
197 fathers and one family with three fathers. This discrepancy in sire number is due to GERUD
198 and COLONY identifying the minimum and the most likely number of fathers, respectively.

199 In total, 7.6% (COLONY) and 8.6% (GERUD) of juveniles were estimated to be fathered by
200 a secondary sire.

201 The operational sex ratio (OSR) significantly progressed from a female bias at the beginning
202 of the mating season, increasing to a male bias towards the end of the mating season (GAM:
203 estimate: 0.55, $t = 10.17$, $p < 0.001$, adjusted r^2 : 0.59, Fig1).

204

205 Discussion

206 This is the first study examining genetic paternity in an anuran with internal fertilisation,
207 demonstrating the occurrence of multiple sires at a moderate proportion of examined litters.

208 In line with a high diversity of reproductive modes, previous studies on frequencies of
209 multiple paternity in externally fertilising anurans revealed a range spanning from 0%
210 (Brown et al., 2010) to over 90% (Byrne and Whiting, 2011). For internally fertilising
211 caudates, multiple paternity was reported to range between 38% (Caspers et al., 2014) and
212 96% of clutches (Adams et al., 2005). Our results for the Nimba toad (25%) are lower than
213 those reported for caudates, and in the lower part of the anuran range. Polyandry allows for
214 fertility assurance, sperm competition, and possibly cryptic female choice (Birkhead, 1998).

215 In Nimba toads fertilisation rates have previously shown to be high (on average 90%,
216 Lamotte et al., 1964). It is currently unknown whether in Nimba toads mating with high
217 quality males results in more eggs being ovulated (as observed e.g. in carnivore mammals,
218 Larivière and Ferguson, 2003). In contrast to other bufonids, Nimba toads engage in a lumbal
219 amplexus through which males clasp females in the region of their ovaries (Xavier, 1971),
220 possibly inducing or facilitating ovulation. However, females kept without males ovulate and
221 develop a pseudo-gestation (Xavier, 1974), and atresia of follicles should occur if not all
222 mature eggs are ovulated due to missing induction, but is very rare (Xavier, 1971; Xavier et

223 al., 1970). Taken together, it seems likely that other factors than increased fertilisation
224 success are the main drivers for polyandry.

225 In contrast to caudates, internally fertilising anurans have no apparent sperm storage organs
226 (Wake, 2015a). In Nimba toads due to the development of a pseudo-gestation without mating
227 as well in older females (> 2 years, Xavier, 1974), inter-season sperm storage can be
228 excluded. Nevertheless, short-term sperm storage (hours to days) can still allow for sperm
229 competition and cryptic female choice (Orr and Brennan, 2015). Relative testes size is
230 generally correlated with the level of sperm competition (e.g. Dziminski et al., 2010).

231 Accounting for about 0.4% of body weight, (Gavaud, 1976; own data), Nimba toad males
232 have normal sized testes comparable to other anurans without evidence for elevated sperm
233 competition (Kusano et al., 1991). Additionally, despite a large sample size of investigated
234 females, no accumulation of sperm was found in their reproductive organs (Xavier 1971).

235 Considering that amplexus in Nimba toads can last for more than one day (Xavier, 1971),
236 cryptic female choice is only possible if spermatozoa remain viable for longer time periods
237 than mating duration. Another possibility for females to favour offspring from high quality
238 males, is to vary resource allocation. Gestating Nimba toad females secrete mucoproteins
239 from uterine epithelial cells into the uterus cavity, on which developing young are orally
240 feeding (Vilter and Lugand, 1959; Xavier, 1971, 1977). It is therefore likely difficult for
241 females to favour offspring sired by particular males. Hence, so far no indication for
242 increased sperm competition was recorded for males, nor sound indication for sperm
243 longevity or sperm storage in females, nor mechanisms enabling females to vary the provided
244 resources. Additionally, the prolonged amplexus is costly for females, as they are injured by
245 the tight grip and the nuptial spines of males (Xavier, 1971). Taken together, these
246 observations indicate that pre-mating female choice might be more important than post-
247 mating (cryptic) female choice in conjunction with sperm competition.

248 In externally fertilising anurans, male display territories or defence of resources such as
249 breeding sites can facilitate female choice (Chen et al., 2011; Hudson and Fu, 2013; Lodé and
250 Lesbarrères, 2004). Our field observations suggest similar phenomena in Nimba toads. Males
251 defend entrances to underground cavities in which Nimba toads reside during the dry season
252 and in which matings are assumed to take place (Angel and Lamotte, 1947, see Online
253 Resource 1 for a field observation). To enter these cavities at the end of the rainy season,
254 females have to pass the guarding males, providing them an opportunity for mating.

255 Individual males can be observed over several weeks around the same cavity entrances which
256 they defend against other males (Sandberger-Loua, personal observation). Males also engage
257 in antagonistic behaviour accompanied by calling and often physical combat (see Online
258 Resource 2). Unfortunately, we were not able to sample DNA in the field from males to test
259 the hypothesis that individuals winning combats at entrances to dormancy cavities sire the
260 majority of offspring in given litters. Nevertheless, for externally fertilising anurans, within-
261 clutch multiple paternity appears to be lower for territorial species (0-29%: Brown et al.,
262 2010; Chen et al., 2011; Hudson and Fu, 2013; Knopp and Merilä, 2009) compared to non-
263 territorial species (30 - > 90%: Byrne and Roberts, 1999; Byrne and Whiting, 2011; Hase and
264 Shimada, 2014; Sztatecsny et al., 2006; Vieites et al., 2004). Despite reporting on an internal
265 fertiliser, our results are in line with the proportions of polyandry reported for other anurans
266 with territorial males, suggesting that Nimba toad females may not have more control over
267 genetic sires than females of species with external fertilisation.

268 In reptiles, high levels of polyandry may mainly arise from the combination of high male
269 benefits and low female cost from frequent mating (e.g. reviewed in Uller and Olsson, 2008).
270 Similarly, for several anurans, male harassment coupled with a male-biased OSR was found
271 to be the main driver for polyandry (Byrne and Roberts, 1999; Hase and Shimada, 2014;
272 Sztatecsny et al., 2006; Zhao et al., 2016). During the breeding season, female Nimba toads

273 are most commonly found hiding in small shelters (Sandberger-Loua, personal observation),
274 probably avoiding undesired matings and harassment by males. Additionally, possibly
275 providing females the opportunity to observe males and judge on their, or their cavity's,
276 quality. Males target both single females as well as amplected pairs for mating attempts,
277 trying to dislodge the male in the latter case (Online Resource 3). Due to the viviparous
278 reproductive mode, clutch sizes are very small and a female's value hence, presumably very
279 high. This may justify the high male effort to defend territories and harass females and the
280 taken increased predation risk due to the calling and fighting activity. Mainly cavities and to a
281 lesser extent females are defended by males, possibly indicating a high value of dormancy
282 cavities for females and multiple paternity may arise if females change dormancy sites.
283 Interestingly, females also appear to physically resist mating with specific males by
284 dislodging them from their back (Online Resource 4). In externally fertilising anurans with
285 male harassment, polyandry levels similar to Nimba toads were observed (19-30%: Lodé et
286 al., 2005; Sztatecsny et al., 2006). The level of male harassment is likely related to the OSR,
287 which becomes male-biased towards the end of the mating season. We however lack the
288 information to test the hypothesis that the documented cases of multiple paternity stem from
289 matings at the end of the reproductive period. Future studies should examine the effect of a
290 male-biased OSR, female efficiency of dislodging males and the existence of mate order
291 effects on the proportions of multiply sired clutches. Due to the internal fertilisation,
292 successfully dislodging males may give Nimba toad females more control over genetic sires
293 than females of species with external fertilisation.

294

295 Conclusion:

296 Representing the first study of polyandry in an anuran with internal fertilisation, we reported
297 moderate proportions of multiple paternity in the viviparous Nimba toad. High levels of

298 sperm competition and cryptic female choice are unlikely for example due to the lack of
299 morphological adaptations, and male territoriality in combination with matings which are
300 costly for females. This suggests that pre-mating female choice is likely more important than
301 cryptic female choice to determine the observed genetic mating system in Nimba toads.
302 Taken together, we assume that the observed frequency of multiple paternity is caused by a
303 combination of female choice and male harassment. Because such effects can also be
304 observed in anurans with external fertilisation, they are not indicative for an increased female
305 control over paternity in Nimba toads compared to other anurans. Nevertheless, the ability for
306 Nimba toad females to physically resist male mating attempts in combination with internal
307 fertilisation may give females more control over the genetic mating system compared to the
308 majority of externally fertilising anurans.

309

310 Compliance with Ethical Standards

311 The study was funded by the Société des Mines de Fer de Guinée (SMFG), but the company
312 had no influence on study design, data collection, data analyses and interpretation, writing of
313 the manuscript and in the decision to submit the paper for publication, thus the authors
314 declare that they have no conflict of interests.

315 All applicable international, national, and/or institutional guidelines for the care and use of
316 animals were followed. All work complies with the guidelines for the use of live amphibians
317 and reptiles in research compiled by the American Society of Ichthyologists and
318 Herpetologists (ASIH), The Herpetologists' League (HL) and the Society for the Study of
319 Amphibians and Reptiles (SSAR), as well as to the IUCN policy statement on research
320 involving species at risk of extinction. The Ministère de l'Enseignement Supérieur et de la
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322 Technique (DNRST) granted research permits (N°085/DNPN/2007, N°103/DNRSIT/DN,

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324 N°177/MESRS/DNRST/2011, N°027/MESRS/DNRST/2012, N°061/DNRSIT/DN and
325 N°020/MESRS/DNRSIT/2014). The authorities from the Ministère de l'Environnement et du
326 Développement durable, Conakry and the Bundesamt für Naturschutz, Bonn granted CITES
327 export (N°00314,N°00492) and import permits (E-3117; E-4074), respectively.

328

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334

335 Online Resources:

336 Online Resource 1: Video1.avi, defending cavity entrance: shows a behavioural observation
337 of a male defending a cavity entrance against another male.

338 Online Resource 2: Video2.avi, antagonistic behaviours: shows behavioural observations of
339 male antagonistic behaviours as aggressive calling and fighting.

340 Online Resource 3: Video3.avi: male harassment: shows a male harassing a female, as well as
341 a male trying to dislodge an amplexed male.

342 Online Resource 4: Video4.avi: female dislodges male: gives an example of a female
343 dislodging an amplexed male from her back.

344

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513

514 Figure caption

515 Fig 1 Operational sex ratio for Nimba toads within the rainy season. Shown is the average
516 operation sex ratio (OSR) per calendar week, based on annual monitoring data in those areas
517 where females for this study were captured; data recorded between 2007 and 2014 (based on
518 333 squares (5x5m) examined for Nimba toads). The mating season is indicated in grey. The
519 line shows the predictions of the OSR of an additive generalised model. The horizontal line
520 indicates and equal OSR. The inset figure in the upper left shows a brightly coloured male,
521 the inset in the lower right a pair in amplexus. The graphic was produced in R, the inset
522 photographs added in Illustrator.