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Post-mating clutch piracy in an amphibian

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Female multiple mating and alternative mating systems can decrease the opportunity for sexual selection[1–3]. Sperm competition is often the outcome of females mating with multiple males and has been observed in many animals[1,4–7], and alternative reproductive systems are widespread among species with external fertilization and parental care[3,8–10]. Multiple paternity without associated complex behaviour related to mating or parental care is also seen in simultaneously spawning amphibians[11–15] and fishes[16] that release gametes into water. Here we report 'clutch piracy' in a montane population of the common frog *Rana temporaria*, a reproductive behaviour previously unknown in vertebrates with external fertilization. Males of this species clasp the females and the pair deposits one spherical clutch of eggs. No parental care is provided. 'Pirate' males search for freshly laid clutches, clasp them as they would do a female and fertilize the eggs that were left unfertilized by the 'parental' male. This behaviour does not seem to be size-dependent, and some males mate with a female and perform clutch piracy in the same season. Piracy affected 84% of the clutches and in some cases increased the proportion of eggs fertilized, providing direct fitness benefits both for the pirate males and the females[17]. Sexual selection—probably caused by a strong male-biased sex ratio—occurs in this population, as indicated by size-assortative mating; however, clutch piracy may reduce its impact. This provides a good model to explore how alternative mating strategies can affect the intensity of sexual selection.

Anuran amphibians have a wide diversity of reproductive modes, but external aquatic fertilization without parental care is the ancestral and most widespread strategy[18]. Only a few instances of multiple paternity have been demonstrated in frogs and those were considered to be the result of polyandrous matings, in which several males mate simultaneously with a female[11,13,14]. In the common frog *R. temporaria*, one of the most widespread Palaearctic amphibians[19], multiple paternity has been detected through allozyme analyses of tadpole kin groups, and was interpreted as being the consequence of high concentrations of spermatozoa in the water during simultaneous spawning[12].

R. temporaria is an explosive pond breeder that often reproduces immediately after the melting of the ice cover. Breeding is usually nocturnal[12,20] and males form large breeding aggregations. We monitored a high altitude population of common frogs in a medium-sized pond (540 m^2) during three consecutive breeding periods (2001–2003) in the central Pyrenean mountains, Spain (42°49′ N, 0°17′ W, about 2200 m above sea level). Breeding was exclusively diurnal due to low temperatures at night (Supplementary Information A), which permitted us to conduct detailed behavioural observations in the field and to measure and mark most individuals in this population. Males aggregated in a specific area of the pond, where clutches were also laid. Male density at the

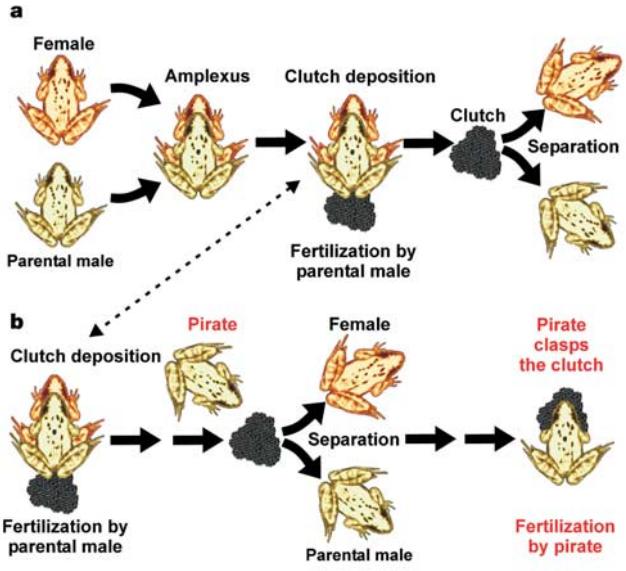

Figure 1 Schematic representation of mating systems in *R. temporaria*. **a**, Females arrive at the breeding ponds and are clasped in the axillary region ('amplexus') by a male (the 'parental' male). The female deposits a single, spherical clutch of eggs. The parental male simultaneously releases his sperm and thereby fertilizes the eggs externally. Subsequently both parents leave the clutch. **b**, 'Pirate' males search for freshly laid clutches, clasp them and release their sperm, sometimes crawling into the clutch to gain access to the internal eggs.

spawning site ranged from 0.40–4.17 males per m^2 (mean ± s.d. = 1.99 ± 1.02 males per m^2) during the reproductive period. Females arriving at the pond were approached and sometimes clasped by several males, but they only spawned while in amplexus with a single male (Fig. 1). Often hours or even days were spent before eggs were deposited at a calm site in the pond. Altogether, 119 females spawned in 2001, and the population of males was estimated at about 250 in that year. Males were slightly smaller than females (snout–vent length 54–76.5 mm, mean ± s.d. =65.8 ± 4.6 mm, versus 58.5–78.5 mm, mean ± s.d. = 68.7 ± 5.2 mm, respectively; $U_{44,19} = 276.5$, $P = 0.034$).

One or several sneaking males often followed pairs in amplexus, during the pair's search for an appropriate egg deposition site. After eggs had been deposited and the parents had left, these individuals frequently clasped the clutch and released their sperm (Figs 1 and 2; Supplementary Video). This type of behaviour is here termed 'clutch piracy'. In one case a clutch was clasped by a pirate and torn away from the parents at the moment of spawning. In other instances piracy was performed by single males, or by groups of males actively searching the pond for freshly laid clutches. In numerous cases the pirates crawled into the spherical clutch, thereby accessing the eggs in its interior. In 2001, piracy was recorded for 84% of the 119 clutches, by 1–16 males per clutch (mean ± s.d. = 5 ± 4 males). In 45% of the 119 clutches the pirates gained access to the interior eggs. They spent 35–387 s fertilizing a clutch (mean ± s.d. = 96.4 ± 105 s). Piracy usually took place within seconds after the clutches were laid but was also observed for up to 2 h later. *U*-tests of parentals versus pirates, parentals versus all males, and pirates versus all males found no significant size differences among parental males (mean snout–vent length ± s.d. = 66.1 ± 4.2 mm, $n = 27$) and pirates (mean ± s.d. = 65.8 ± 4.9 mm, $n = 28$), or the total male population (mean ± s.d. = 66.3 ± 5.6 mm, $n = 230$) ($P > 0.05$ in all cases). On one occasion, a male performed clutch piracy less than 3 h after having previously acted as a parental male in amplexus with a female. Hence, pirate males in *R. temporaria* are not characterized by a fixed behavioural role, or by a specific age or body size. This differs from examples in other species (mainly fish), in which large bourgeois males coexist with smaller sneakers or satellites[8,9].

To determine the proportion of eggs fertilized by pirates we sampled 16 pirated clutches after monitoring them for 1 h after spawning. Subsequently the parentals and pirates were captured and tissue samples were taken by toe clipping. Molecular paternity analyses detected successful fertilization by pirate males in seven of these clutches (mean ± s.d. = 26.1 ± 36.2% of the eggs). Of the total 319 embryos analysed, 77 (24.1%) resulted from fertilization by pirate males. The highest paternity (100%) of a pirate was recorded in a clutch that had been pirated 1 min after spawning. The second highest pirate paternity (95%) was found in a clutch that had been clasped and carried away by the pirate while the parental male was still releasing his sperm. In four out of the seven pirated clutches, the full pirate paternity could be assigned to the first pirate arriving at the clutch. In the remaining three clutches, another or several pirates succeeded in fertilizing parts of the clutch.

Multiple paternity in frogs has so far usually been attributed to group spawning and simultaneous mating with several males[11,14,21], which can be associated with a reduced overall success of fertilization[13]. Considerable variation in the number of fertilized eggs per clutch has been reported previously for *R. temporaria* (5–100%)[22,23]. By experimentally rearing 25 pirated and 31 non-pirated clutches we found that under specific circumstances clutch piracy helps to increase the number of eggs fertilized per clutch (Fig. 3).

Alternative mating strategies are expected to evolve in the presence of strong sexual selection or whenever some males are excluded from mating[1]. Both conditions might apply to the studied population of *R. temporaria* in the Pyrenees. Only a fraction of all females arrived each day at the pond, significantly skewing the operational sex ratio (OSR) towards a strong male bias (daily OSR: 0.07–0.20 gravid females per sexually active male; mean ± s.d. = 0.13 ± 0.04). Using a conservative approach—that is, assuming that each parental male mated with only one female—the estimated opportunity for sexual selection (the I_{mates} parameter)[1] is higher ($I_{mates} = 1.28$, see Methods) than would be expected if mating were random ($I_{mates} = 1$). Size-assortative mating provides further evidence for the existence of sexual selection in this population. Parental male and female sizes were significantly

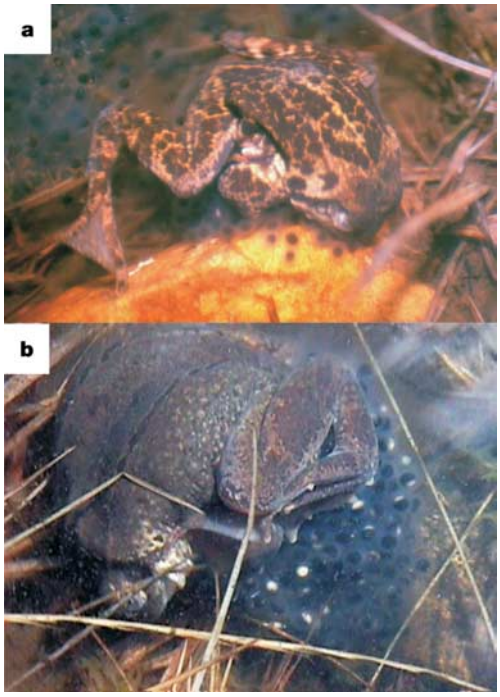

Figure 2 Images of clutch piracy in *R. temporaria*. The pirate male clasps a freshly laid clutch and coils his body over the clutch while fertilizing it. **a**, A male has crawled into the clutch to gain access to the interior eggs. **b**, The male has clasped the clutch at the moment of spawning, while the parental male is still releasing his sperm; in this case, the pirate male succeeded to fertilize 95% of the genotyped eggs.

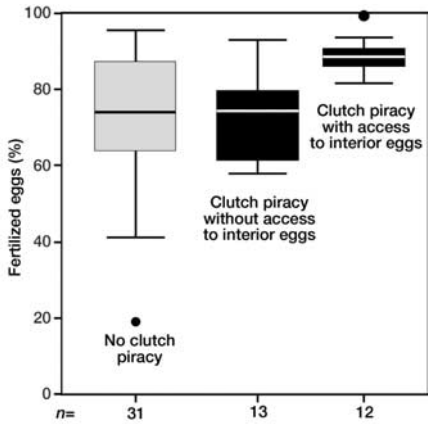

Figure 3 Percentage of fertilized eggs in clutches not exposed (grey) and exposed (black) to clutch piracy. The percentage of eggs of a clutch fertilized in the absence of piracy was 19.2–95.7% (mean ± s.d. = 72.9 ± 18.0%). This percentage was not significantly different to that found for all clutches exposed to piracy ($U_{31,25} = 308$, $P = 0.19$), but in clutches in which the pirate had access to the interior eggs the percentage was higher ($U_{31,12} = 80$, $P = 0.003$). Error bars are 5% and 95% percentiles; 25% and 75% percentiles delimit the box, and the median is shown as a line. Dots represent outliers.

correlated in 2001 (Nonparametric rank correlation, Spearman's correlation coefficient rho = 0.61, $P = 0.0032$, $n = 21$) and over the whole three-year period (rho = 0.49, $P = 0.0004$, $n = 48$, see Supplementary Information D), although males in *R. temporaria* are known to be unselective regarding female size[24]. These results support the hypothesis that clutch piracy evolved in the presence, and probably as a consequence, of the highly male-biased OSR observed in this population.

Clutch piracy can provide direct fitness advantages for individual male and female frogs. Pirates gain the opportunity to fertilize eggs when female availability is low, and females gain offspring due to clutch piracy. Producing offspring sired by different fathers has the further advantages of increasing the genetic diversity and therefore the fitness of the progeny, and reduces potential genetic incompatibilities with individual males[7,17,25]. The effects of clutch piracy on the fitness of parental males is uncertain, except when pirates are able to steal a clutch before it is fertilized by the parent. Males increase their probability of reproduction in the presence of strong selection. This implies that males using this alternative mating behaviour had a fitness exceeding that of males using only the conventional behaviour when invading the population, and have a fitness at least equal to that of conventional males to persist in this population. Estimating the average proportion of time or energy used by the males performing either behaviour would allow these predictions to be rigorously tested[1].

Clutch piracy could result in a highly polygynandrous genetic mating system[3], because males can fertilize eggs in several clutches and eggs in one single clutch can be fertilized by several males. We are not aware of any other instance of multiple paternity caused by similar post-mating behaviours in animals with external fertilization. Egg piracy is known in fishes[26,27] but refers to situations in which eggs are fertilized by other males, then stolen by the pirate and stored in his nest, possibly to attract females, distract predators or serve as food[3]. Brood parasitism usually involves of furtive participation in fertilization during the spawning procedure, but not the active search for clutches and the fertilization of yet unfertilized eggs after the completion of mating. Our paternity analysis gives results comparable to previous indirect studies[12], suggesting that clutch piracy, although not reported before, could be frequent throughout the distribution area of *R. temporaria*. This qualifies the species to be used as a model system for testing further predictions of sexual selection theory in externally fertilizing vertebrates. □

Methods

Clutch sampling

For molecular paternity analyses, a minimum of 15 eggs per clutch (about 4–5% of the total number of eggs) were randomly collected from the upper part, lower part and interior of clutches. Tissue samples were preserved in 99% ethanol. Data from a total of 319 eggs from 16 clutches (14% of the total; 19 ± 4 eggs per clutch) were available for molecular paternity analyses.

Behavioural observations and clutch rearing

Reproductive behaviour was recorded during the whole breeding period of 2001 (2 weeks of permanent observations between 8:00 and 19:00 hours; Supplementary Information A). Behavioural patterns were documented through photography and video. Daily numbers of males, females, couples in amplexus and clutches laid, and the time of spawning were recorded. The daily OSR was calculated as the relative number of females with fertilizable oocytes per sexually active male[20]. To estimate the opportunity for sexual selection, we calculated the I_{mates} parameter[1] as the difference in the opportunity for selection between males (I_{males}) and females ($I_{females}$), minus (OSR–1) × $I_{females}$ (see Supplementary Information B). Fieldwork in 2002 followed the scheme of the previous year but was limited to observations to avoid disturbing the population. For each clutch we counted the number of pirate males attempting to fertilize it, the time that they spent in contact with the clutch (calculated for the whole group in cases of simultaneous piracy by larger numbers of pirates) and whether they gained access to the interior of the clutch. One hour after they had been laid, 25 of these clutches and 31 control clutches that had been isolated from pirates immediately after spawning were taken to the laboratory. Eggs were reared to developmental Gosner stage 25 in small aquaria (22 × 18 × 15 cm) inside the laboratory, to preserve them from direct UV-B radiation that could be a cause of egg mortality. The proportion of fertilized eggs was recorded for each clutch by counting the number of developing embryos that reached the earlier Gosner stages. Every 2 days, part of the water was removed and replaced by clean water from the study pond. The light:dark period in the laboratory was 17:7, similar to field conditions.

Microsatellite paternity analysis

All DNA was isolated from muscle in adults or whole embryos in clutches. We tested a total of ten microsatellites (*RtU4* and *RtU7*; *Rtempμ1*, *Rtempμ4*, *Rtempμ5* and *Rtempμ6*; *Rtμb* and *RtμO*; and *RECALQ* and *RC08604*; ref. 28); five of these (*RtU4, RtU7*, *Rtempμ4*, *Rtempμ6* and *Rtμb*; Supplementary Information C) were successfully amplified by polymerase chain reaction in all individuals and showed a sufficient degree of polymorphism to determine paternity unambiguously. We analysed fragment length with Genotyper 3.7 (Applied Biosystems). Observed and expected heterozygosity and allele frequency for each locus were calculated for the complete adult population with Cervus 2.0 (ref. 29). The probability of detecting multiple mating (P_{DM}) increases with the number of loci, locus polymorphism, number of offspring analysed per clutch and number of specimens genetically contributing to clutch paternity[30]. We used allele frequency data to calculate this variable in our population using the *PrDM* software[30]. This program calculates the P_{DM} using the following: (1) number of loci; (2) the number of alleles at each locus and their population frequencies; (3) the number of sires contributing to a multiple mated clutch; and (4) their reproductive skew. Although the number of observed potential sires in our population ranged from 1 to 17, we used a conservative model of only two sires; one with the mean parental proportion of eggs fertilized (0.73) and the other with the remainder (0.27). The genotype of the parents was not specified in order to calculate the mean P_{DM} for the population[30]. We performed ten replicates of the analysis for 15 offspring samples and calculated the mean P_{DM}. The mean P_{DM} was high ($0.981 \pm 4 \times 10^{-4}$), indicating that the five highly polymorphic loci and samples analysed were sufficient to detect multiple paternity in our population. Paternity of offspring was assigned by direct comparison between their alleles and the alleles present in the parental and pirate males in the five loci analysed. Offspring that were homozygous for at least one allele present in the mother but not in the parental male, or with at least one allele not present in either of the parentals for the five loci analysed, were considered as offspring sired by pirate males.

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Genomic analysis of regulatory network dynamics reveals large topological changes

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Network analysis has been applied widely, providing a unifying language to describe disparate systems ranging from social interactions to power grids. It has recently been used in molecular biology, but so far the resulting networks have only been analysed statically[1–8]. Here we present the dynamics of a biological network on a genomic scale, by integrating transcriptional regulatory information[9–11] and gene-expression data[12–16] for multiple conditions in *Saccharomyces cerevisiae*. We develop an approach for the statistical analysis of network dynamics, called SANDY, combining well-known global topological measures, local motifs and newly derived statistics. We uncover large changes in underlying network architecture that are unexpected given current viewpoints and random simulations. In response to diverse stimuli, transcription factors alter their interactions to varying degrees, thereby rewiring the network. A few transcription factors serve as permanent hubs, but most act transiently only during certain conditions. By studying sub-network structures, we show that environmental responses facilitate fast signal propagation (for example, with short regulatory cascades), whereas the cell cycle and sporulation direct temporal progression through multiple stages (for example, with highly inter-connected transcription factors). Indeed, to drive the latter processes forward, phase-specific transcription factors inter-regulate serially, and ubiquitously active transcription factors layer above them in a two-tiered hierarchy. We anticipate that many of the concepts presented here—particularly the large-scale topological changes and hub transience—will apply to other biological networks, including complex sub-systems in higher eukaryotes.

We began by assembling a static representation of known regulatory interactions from the results of genetic, biochemical and ChIP (chromatin immunoprecipitation)–chip experiments. Figure 1 illustrates the complexity of the resultant network, which contains 7,074 regulatory interactions between 142 transcription factors and 3,420 target genes (interactions can be between transcription factors and non-transcription factor targets, or two transcription factors). To get a dynamic perspective, we integrated gene-expression data for the following five conditions: cell cycle[13], sporulation[14], diauxic shift[12], DNA damage[16] and stress response[15]. From these data, we traced paths in the regulatory network that are active in each condition using a trace-back algorithm (see Methods).

Figure 1b presents the sub-networks active under different cellular conditions, and gross changes are apparent in the distinct sections of the network that are highlighted. Recent functional genomics studies have analysed the dynamics of a few transcription factors[17,18]; however, Fig. 1 represents the first dynamic view of a genome-scale network.

Half of the targets are uniquely expressed in only one condition; in contrast, most transcription factors are used across multiple processes. The active sub-networks maintain or rewire regulatory interactions, and over half of the active interactions (1,476 of 2,476 total) are completely supplanted by new ones between conditions. Only 66 interactions are retained across four or more conditions; these comprise 'hot links'[6] that are always on (compared with the rest of the network) and mostly regulate house-keeping functions.

The large number of changing interactions makes rigorous comparison of active sub-networks impossible visually. Consequently, we introduce SANDY, an approach that combines: standard measures of network connectivity (involving global topological statistics[6] and local network motifs[4]), newly derived follow-on statistics, and comparisons against simulated controls to assess the significance of each observation.

Overall, our calculations divide the five condition-specific sub-networks into two categories: endogenous and exogenous (Fig. 1). This allows us to rationalize the different sub-network structures in terms of the biological requirements of each condition. Endogenous processes (cell cycle and sporulation) are multi-stage and operate with an internal transcriptional programme, whereas exogenous states (diauxic shift, DNA damage and stress response) constitute binary events that react to external stimuli with a rapid turnover of expressed genes.

We begin SANDY by examining global topological measures that quantify network architecture (Fig. 1c)[6]. The view from recent studies is that these statistics are remarkably constant across many biological networks (including regulatory systems)[1,5,6,19,20]. Moreover, most of them remain invariant between randomly simulated sub-graphs of different sizes (Methods).

In fact, we show that topological measures change considerably between the endogenous and exogenous sub-networks. (The probability, P, that their topological measures originate from the same population is $<10^{-4}$; Supplementary Information.) Furthermore, most of the observed measurements differ significantly from random expectation and are insensitive to addition of noise in the underlying network (Methods). The 'in-degree' (k_{in}) is the number of incoming edges per node (that is, the number of transcription factors regulating a target). Its average across each sub-network decreases by 20% from endogenous to exogenous conditions ($P < 5 \times 10^{-3}$). The 'out-degree' (k_{out}) represents the number of