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Genetic evidence for mixed broods and extra-pair matings in a socially monogamous biparental cichlid fish

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Abstract

Mobile young under parental care have a high potential for intermixing with other broods, which potentially increases the costs to the foster parents. Here, we examined for the first time the genetic composition of wild-caught broods of the convict cichlid (*Amatitlania siquia*), a socially monogamous biparental fish, for evidence of brood mixing and adoption. Our microsatellite genotyping data revealed that 79% of broods contained adopted young. Moreover, 25% of broods contained adopted sibsets likely arising from extra-pair matings, a phenomenon hitherto not documented for this species. Overall, adopted foreign fry and host fry in mixed broods were generally different in body length, as would be expected if they have different parents. However, fry from possible extra-pair matings were similar in body length to host fry, suggesting that they are of similar age. Our results are important because they reveal a very high prevalence and degree of brood mixing, and indicate that social monogamy does not necessarily lead to genetic monogamy in the convict cichlid in nature. These findings raise questions about potential brood-mixing mechanisms and the reproductive ecology (especially opportunities for polygamy in nature) of this important model species in the study of animal behaviour.

Keywords

alloparental care, biparental care, brood mixing, cichlid fish, extra-pair mating, microsatellite markers.

1. Introduction

Alloparental care (care of non-descendant young) has been documented in many animal taxa that provide extended biparental care to their young (Riedman, 1982; Wisenden, 1999). This form of care may result from either the misidentification of fostered or 'foreign' young as one's own young, or the active behavioural choice of adopting unrelated young into one's family (Riedman, 1982; Wisenden, 1999). Such care behaviour may be maladaptive if foster parents incur net costs (e.g., energy spent feeding or protecting young, risk of predation) while providing care to unrelated young (Riedman, 1982). Conversely, it may be an adaptive behavioural strategy if parents that care for foreign young gain net fitness benefits from doing so. For example, by adopting foreign young, parents could potentially gain reproductive experience (Riedman, 1982), appear more attractive to, or gain access to, potential mates (Martin, 1989), or exploit foreign young to provide fitness advantages for their own offspring (Nastase & Sherry, 1997). Understanding the evolution of alloparental care is an enduring challenge because it is a complex phenomenon that may depend on reproductive ecology, the condition of parents and young, environmental constraints and other selective pressures (Riedman, 1982; Coleman & Jones, 2011; Sefc, 2011; Wong & Kölliker, 2013). Parental care/investment theory (Clutton-Brock, 1991) predicts that parental effort should decrease with increasing uncertainty of parental relatedness to young under care, which would be the case for genetically mixed broods.

Early work on the ecology of alloparental care largely came from studies on birds (e.g., Eadie et al., 1988; Codenotti & Alvarez, 1994; Nastase & Sherry, 1997; Kalmbach, 2006). Instances of alloparental care of mobile young are found most commonly in bird species with precocial young (Nastase & Sherry, 1997; Kalmbach, 2006). In fishes that exhibit parental care, alloparental care is usually present, particularly so in the Neotropical and African cichlids that care for mobile young (Wisenden, 1999; Coleman & Jones, 2011; Sefc, 2011). These fishes have an extended period of biparental care of their young and can exhibit intra- and inter-specific adoption (McKaye & McKaye, 1977; Wisenden, 1999; Schaedelin et al., 2013). The Neotropical convict cichlid (*Amatitlania siquia*) is putatively one such species. Convict cichlids are substrate brooders that provide extended biparental care of young (6–8 weeks), from the egg stage through to the

exogenous feeding/free-swimming ('fry') stage and until the fry reach independence at a standard body length of 10–12 mm (Keenleyside et al., 1990; Fraser & Keenleyside, 1995; Wisenden, 1995). As fry age and increase in length, the brood and parents become a mobile group, swimming and foraging along the river substratum during the day (Keenleyside et al., 1990; Wisenden, 1995). Parental care in this species primarily involves vigorous defence of young against fish predators by both parents, which is crucial to brood survival and for fry to successfully reach independence (Keenleyside et al., 1990, 2008; Wisenden, 1995).

Previous field behavioural studies on wild convict cichlids have alluded to the occurrence of intraspecific brood-mixing events, where 29% of observed broods contained different size classes of fry consistent with possible brood adoption events (Wisenden & Keenleyside, 1992). Follow-up field manipulation and laboratory experiments demonstrated that acceptance of foreign fry into the brood is an active decision (by both parents and host fry), where body size likely plays a role in the acceptance or rejection of young (Wisenden & Keenleyside, 1992, 1994; Fraser et al., 1993; Fraser & Keenleyside, 1995; Fraser, 1996). Wisenden & Keenleyside (1992) repeatedly visited wild broods and found that the number of young in some broods increased during the parental care period and/or observed the presence of two different size classes of free-swimming young within broods. These observations suggested that parents accepted 'foreign fry' (fry that were not their offspring) into their brood. Their follow-up experimental manipulations indicated that guarding parents generally accepted fry that were of similar size or smaller than their own offspring or 'host fry' (Wisenden & Keenleyside, 1992, 1994). This acceptance of foreign fry into the brood based on body size discrimination could be due to either parental misidentification of foreign fry as their own offspring or selective adoption and/or acceptance of foreign fry into the brood that dilute the risk of brood predation on host young and/or are weaker swimmers than host young and therefore preferential targets for brood predators (McKaye & McKaye, 1977; Wisenden & Keenleyside, 1992, 1994). The size-based method used by Wisenden & Keenleyside (1992, 1994) to identify putative instances of brood adoption indirectly estimated the occurrence of brood mixing at 29%; however, this method is likely conservative because adoption of similar-size young would not have been detected.

Therefore, our main objective in the current study was to quantify more precisely the prevalence and degree of brood mixing using microsatellite genetic markers, and to characterize the body length distribution of young within broods, in wild convict cichlids from the same natural population studied originally by Wisenden & Keenleyside (1992, 1994). We report for the first time strong genetic evidence for extensive brood mixing and associated alloparental care, as well as novel evidence for putative extra-pair matings, in free-ranging convict cichlids. Our results are important because they reveal an extremely high prevalence and degree of brood mixing, and indicate that social monogamy does not necessarily lead to genetic monogamy in the convict cichlid in nature. These findings raise questions about potential brood-mixing mechanisms and the reproductive ecology (especially opportunities for polygamy in nature) of this important model species in the study of animal behaviour.

2. Material and methods

2.1. Field site and brood collections

Our field study site was in the Río Cabuyo, located in the Lomas Barbudal Biological Reserve (10°27′N, 85°20′W), Guanacaste province, Costa Rica (for more details see the Appendix in the online edition of this journal, which can be accessed via http://booksandjournals.brillonline.com/content/journals/1568539x). Under permit and using hand-held aquarium nets (mesh size < 1 mm), we collected 48 complete broods of convict cichlid (totalling 1801 fry) that were under biparental care from 12 pools (surface areas ranging between 200 and 800 m^2) whilst snorkelling in an upstream direction along an approx. 3-km section of this river during the dry season in February 2008 (see Figure 1 for identities of pools and broods). Within each sampled pool, broods that were being guarded by both parents were collected as visually encountered. We defined a brood as a cohesive group of young actively guarded by two parents. Guarding parents were located visually whilst snorkelling, and the entire brood was collected by carefully 'herding' fry into fine-mesh aquarium nets. Each collected brood was preserved in 95% isopropyl alcohol in a separate sealed tube for transportation to Carleton University for later analysis.
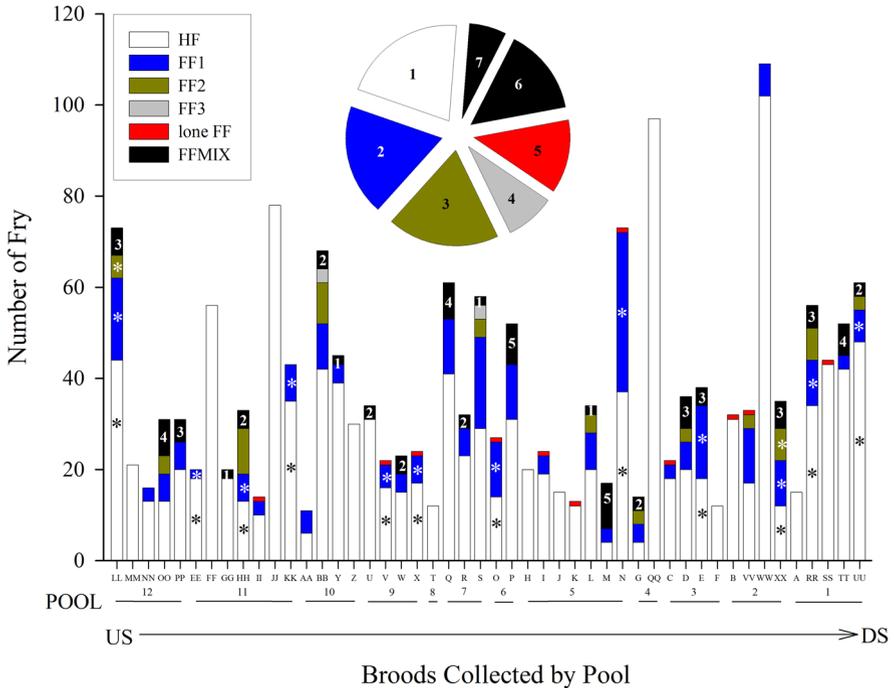
Figure 1. Summary of the genetic composition of wild convict cichlid broods ($N = 48$) collected across 12 pools in the Río Cabuyo, Costa Rica. Each bar depicts the proportion of the brood that comprises a particular genetically-distinct sibset, which are shown in different colours (see inset for their individual codes). The pools (and broods therein) are ordered sequentially from upstream (US) to downstream (DS). HF refers to inferred host fry, FF1–FF3 represent different foreign fry sibsets, lone FF denotes a lone foreign fry, and FFMIX refers to foreign fry that cannot be accurately grouped into a sibset (where the number in this grouping represents the minimum number of 'families' or mated pairs that genetically contributed to these young). *Sibsets that are consistent with having one shared parent (i.e., originating from an extra-pair mating). The pie chart shows the relative frequencies of broods that comprise a different number of families, ranging from 1 family (not a mixed brood) to 2–7 families (i.e., mixed broods). This figure is published in colour in the online edition of this journal, which can be accessed via http://booksandjournals.brillonline.com/content/journals/1568539x.

During brood collections, we did not capture the parents for tissue samples for the following reasons. Attempting to collect parental fish is challenging and time consuming and would have constrained our ability to achieve our study's objective. Our primary goal was to collect broods in their entirety to characterize their individual genetic structure so as to ascertain if brood mixing occurs in the convict cichlid in nature. Our main concerns were to

ensure that we maximized sample size during our limited time in the field and therefore statistical power to detect genetic evidence for brood mixing and minimized disturbance to neighbouring broods and predation on fry when collecting any focal brood, especially when parents are absent (Alonzo et al., 2001).

2.2. *Body size measurements and genetic markers*

The standard body length (from tip of nose to end of the caudal peduncle) of all fry within each brood was measured to the nearest 0.01 mm using image processing software (AxioVision, Carl Zeiss, Jena, Germany) from digital photographs (Canon PowerShot A620, Canon, Tokyo, Japan) taken under a Zeiss Discovery V8 microscope at $10\times$ magnification (for more details see the Appendix).

Because convict cichlid microsatellite markers were not available at the time of our study, we adopted and optimized four polymorphic microsatellite loci (*Acit1*, *Acit2*, *Acit3* and *Acit4*) developed from the Neotropical Midas cichlid, *Amphilophus citrinellus* (Noack et al., 2000), to genotype individual convict cichlid fry and characterize the genetic composition of wild broods. The details of microsatellite marker optimisation, all genetic protocols (DNA extraction, PCRs and microsatellite fragment analysis) and the GenBank accession numbers of the markers are presented in Lee-Jenkins et al. (2011). For 14 fry only (from 5 broods), DNA extractions did not yield successful PCR amplifications, and consequently these individuals were excluded from all analyses (and brood sizes were adjusted accordingly). Although we were able to optimize and use only four microsatellite markers in our study, which may have constrained the likelihood of detecting distinct genetic sibsets, we are nonetheless confident that the allelic diversity in these four loci (ranging from 11 to 36 alleles per locus; Table A1 in the Appendix) provided sufficient resolution to identify cases of brood mixing in wild convict cichlids (as demonstrated in Lee-Jenkins et al., 2011) and allowed us to make conservative inferences on the natural prevalence of brood mixing and putative extra-pair matings in our study population. An extra-pair mating (EPM) represents a case where a mixed brood consists of some fry originating from a mating between one of the guarding parents and another adult not guarding that particular brood. We use the term EPM in lieu of the well-established term 'extra-pair copulation' because convict cichlids are external rather than internal fertilizers.

2.3. Genetic sibsets and groupings

We carried out the analysis of our large sample of wild-caught broods in a systematic 'bottom-up' approach, from a brood-by-brood level to a within-pool comparison of broods, as follows. First, for each brood separately, we used the software KINALYZER (Berger-Wolf et al., 2007; Ashley et al., 2009) to group individuals into genetically-distinct sibsets (= sibling groups) within a brood based on the principle of Mendelian inheritance of alleles of co-dominant genetic markers. KINALYZER is specifically designed to group data into sibsets when no parental genotype information is available (Berger-Wolf et al., 2007; Ashley et al., 2009). We manually double-checked the output of each analysis to ensure that the most conservative groupings were assigned. We then reconstructed for each brood the inferred parental genotypes that would have been necessary to yield the observed sibsets (see Lee-Jenkins et al., 2011 for more details).

Because genetic data were not directly obtained from parents, we could not identify with certainty which fry in a focal brood were the 'host fry' (genetic offspring of the parents guarding the brood) and 'foreign fry' (fry from parent(s) other than those guarding the brood). Consequently, host fry identity was inferred based on the assumption that the sibset that comprised the majority of the brood were the offspring of the guarding parents. This assumption is reasonable given the field observations of Wisenden & Keenleyside (1992) on free-ranging convict cichlid broods under parental care. They reported that, in mixed broods where foreign fry had been adopted, there were distinct body-size class differences between host fry and foreign fry, and that in over 95% of such mixed broods foreign fry comprised less than half of the brood and that in over 50% of the mixed broods foreign fry comprised $\leqslant$15% of the brood. On average ($\pm$SE), mixed broods contained only 11.3 $\pm$ 1.6% of adopted (foreign) fry, a distinct minority. Similarly, Ribbink et al. (1980, cited in Wisenden & Keenleyside, 1992) reported that foreign fry present in mixed broods of several species of Lake Malawi cichlids comprised <5% of the total brood. Although the lack of genetic information from the parents is not ideal, the genetic data on the sampled broods nonetheless unambiguously identifies instances of brood mixing in the Río Cabuyo, where genotype grouping of fry (into sibsets) matched across all four loci (see Lee-Jenkins et al., 2011). In most instances of brood mixing, there was a predominant genotype group (sibset). Where multiple foreign fry ($\geqslant$3 fry) could not be accurately grouped into a sibset, they were placed into

a mixed foreign fry (FF Mix) category. This latter group represented foreign fry that were more than likely not related to one another (or could potentially be related to one other individual in the group) and may potentially represent 'stray' foreign fry. Any remaining lone foreign fry following sibset grouping were placed into a lone foreign fry category (= lone FF).

2.4. Comparison of inferred parental genotypes and possible extra-pair matings

Each constructed sibset was compared to other sibsets within a focal brood at each locus separately, and the genotypes of the inferred parents (for host and foreign sibsets) were compared to determine if extra-pair matings (EPMs) could have occurred. A potential EPM was recorded when two sibsets shared a parental genotype at each of the four microsatellite loci.

We used Monte Carlo randomizations (simulations) to calculate the probability of observing the number of apparent extra-pair matings by chance alone (i.e., in the absence of EPMs) as follows. For each microsatellite locus, we estimated population allele frequencies from the inferred parental genotypes for each brood. As sample sizes were insufficient to estimate allele frequencies for individual pools, pools were combined to provide allele frequencies for the entire sampled population. Each simulated dataset consisted of 33 mixed broods, where 21 broods had 2 sibsets, 10 had 3 sibsets, and 2 had 4 sibsets, as was the case for the observed data (of the 38 mixed broods, 5 broods (namely, GG, U, K, B and SS) were excluded from this analysis because they each had a small number of foreign fry for which parental genotypes could not be ascribed). For each brood and for each microsatellite locus, four alleles were drawn at random using the estimated population allele frequencies. An EPM was inferred for a brood if any of the inferred parental genotypes of two sibsets shared at least two alleles at all four loci. The number of broods with evidence for EPMs was recorded for each simulated dataset. One million such datasets were simulated, generating a null distribution for the number of inferred EPMs that are expected to be observed under random mating. Simulations were carried out in R (R Core Development Team, 2012).

2.5. Comparison of brood genotypes within and among pools

After broods were genotyped and categorized into sibsets (with parental genotypes constructed), we compared sibsets within pools to determine if

mixing events (could have) occurred with fry from other broods collected in the same pool. Within each pool, we first compared all the inferred parental genotypes of host and foreign fry sibsets to any of the genotypes of the 'mixed foreign fry', and then compared all inferred parental genotypes of all brood sibsets within each sampled pool. We then performed the same comparisons among all pools.

2.6. Comparison of fry body sizes

For each brood separately, we calculated the median body lengths of the fry in the inferred host fry (HF) sibset, each foreign fry (FF) sibset, the lone FF group (lone FF) and/or the 'mixed' foreign fry group (FF Mix). We used these median values to compute a 'body size difference score', representing the relative difference between the median body length for a given foreign fry (FF) sibset or group (lone FF or FF Mix) and the median body length of the reference host fry (HF) sibset ($N = 66$ sibsets and groups, originating from 36 broods). Specifically, a difference score (%) = ((median FF body length or median lone FF or FF Mix body length − median HF sibset body length)/median HF sibset body length) × 100. We then used a linear mixed-effect model, with the difference score as the response variable and the corresponding host fry median body length as the predictor variable, to characterize the relationship between the median body length of host fry and that of foreign fry within broods. We included a weighting index (= proportion of foreign fry to host fry used in calculating median body lengths) and brood identity as random factors in the model (lme4 and lmer test packages in R). Foreign fry sibsets that possibly originated from EPMs were analysed in a similar manner, but in a separate linear mixed-effect model ($N = 14$ sibsets, originating from 12 broods). For both analyses, 10 fry from brood N were disregarded because their genotypes were consistent with both the host fry and EPM fry sibsets and consequently could not be accurately placed into a distinct sibset.

3. Results

3.1. Prevalence and degree of brood mixing in nature

Of the 48 wild convict cichlid broods analyzed, 38 were mixed broods comprising two or more distinct sibsets (i.e., groups of genetically-mixed

parentage), representing a 79.2% prevalence of brood mixing in this natural population (Figure 1). The mean ($\pm$ SE) number of fry in mixed broods (37.7 $\pm$ 3.4) and unmixed broods (35.6 $\pm$ 9.7) were similar (t-test, $t_{11.32} = 0.201$, $p = 0.85$, two-tailed).

Mixed broods in the Río Cabuyo contained between 2–7 'families' (i.e., different male and/or female breeding pairs contributing fry to the brood) (Figure 1). Among mixed broods, the degree of mixing varied, ranging from 2% foreign fry in a brood (e.g., brood SS, 1 of 45 fry) to 58% foreign fry (e.g., brood OO, 18 of 31 fry). Overall, there was a significant negative relationship between the median body length of host fry and that of foreign fry within broods (Figure 2a; linear mixed-effects model: $t_{22.6} = -2.422$, $p = 0.024$), such that foreign fry tended to be larger than host fry in broods containing small (<8 mm) host fry and smaller than host fry in broods containing large ($\geqslant 8$ mm) host fry (Figure 2a). We used this 8-mm length criterion to distinguish these two body size categories because the ossification of the skeleton of convict cichlid fry is complete by the time they reach 8 mm standard length, and the ontogenetic transition from a cartilaginous to a bony skeleton coincides with increased swimming performance and predator escape behaviour (Wisenden et al., 2015). The mean body length of fry in unmixed broods ($N = 10$) varied across broods, representing the full range of body lengths of free-swimming convict cichlid fry under parental care (Table A2). However, most (70%) of these broods contained relatively small (<8 mm) fry (Table A2).

3.2. *Extra-pair matings in nature*

We detected possible instances of extra-pair matings in 12 of the 48 broods analysed (25% incidence overall) in the Río Cabuyo, based on the inferred parental genotype of a foreign fry sibset matching at all four loci the genotype of an inferred host parental genotype (Figure 1). Moreover, two of these broods (broods XX and LL) each contained two foreign fry sibsets that shared parental genotypes with the host fry genotype, which is consistent with one shared parent and EPMs that gave rise to three half-sib families. Our Monte Carlo simulation analysis of broods containing foreign fry sibsets revealed that our observation of 12 apparent cases of EPMs in 33 mixed broods was more extreme than any of the one million simulated datasets, in which at most four cases of EPMs were inferred. Therefore, our observed frequency (12 out of 33) of putative EPMs in mixed broods is extremely unlikely to
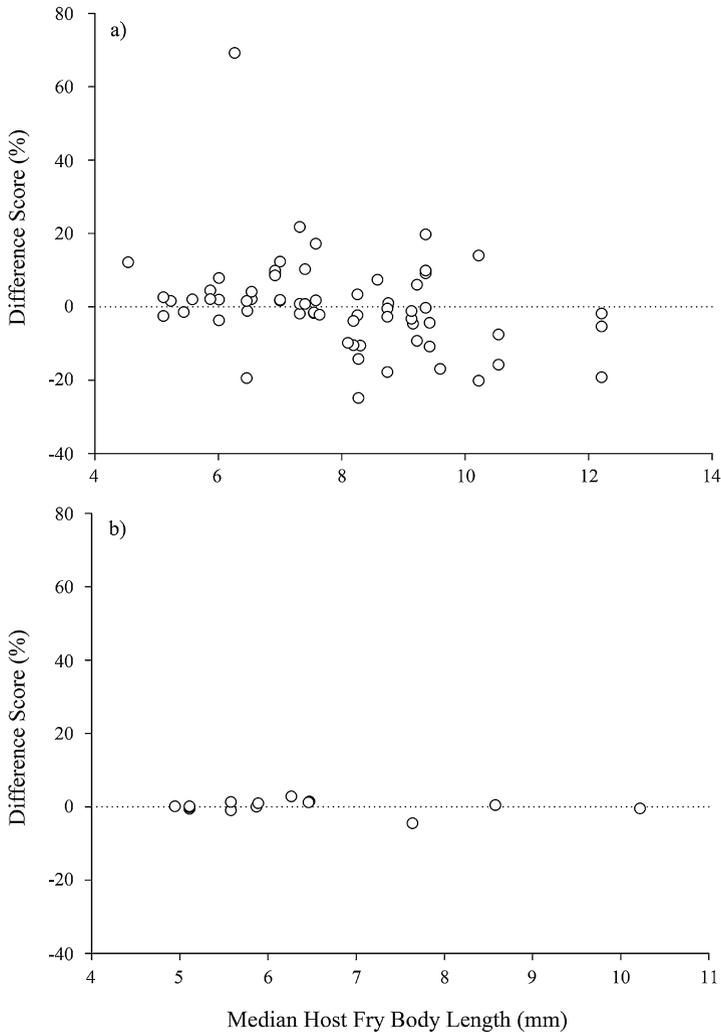
Figure 2. Relationship between the median body length of host fry in a brood and the extent to which the median body length of each foreign fry sibset present in the same brood differs from host fry median body length (represented as a 'difference score'; see Material and methods section for details on how this index score is calculated), for (a) genetically mixed broods ($N = 36$) containing foreign fry sibsets/groups ($N = 66$) not derived from inferred extra-pair matings (EPMs) and (b) mixed broods ($N = 12$) containing foreign fry sibsets ($N = 14$) derived from EPMs. Each data point represents a different foreign fry sibset or fry grouping relative to the host fry in a given brood. A difference score of 0 (the dotted horizontal line) represents equivalence (i.e., the median body length of the foreign fry sibset is equal to that of the host fry sibset in the same brood). Positive and negative difference scores represent foreign fry sibsets/groupings that are larger and smaller than host fry, respectively.

have arisen by chance in a randomly mating population (Monte Carlo randomization test, $p < 10^{-6}$). All foreign fry sibsets originating from possible EPMs in these 12 mixed broods were similar in body length to the inferred host fry (Figure 2b, Table A3; linear mixed-effects model: $t_{7.6} = -0.765$, $p = 0.468$), which would be consistent with the EPM fry and host fry in a given brood being approximately the same age.

3.3. Comparison of brood genotypes within and among pools

We compared the genotypes of broods within each pool and found full siblings occurring in different broods in only seven instances. In three of these cases, individuals identified in the 'mixed' or lone foreign fry group in their focal brood (1 FF lone in brood I; 1 FF lone in brood O; 2 FFMix in brood GG) could be siblings with individuals forming a foreign fry sibset in a neighbouring brood (FFset1 in brood L; FFset1 in brood P, and FFset1 in brood HH, respectively). In one case, a 'mixed foreign fry' (from brood UU) was the full sibling of individuals who were host fry in a different brood (brood RR). These two broods were not the nearest to each other, but rather approximately 9 m apart, at time of collection in Pool 2. A foreign fry sibset of four individuals in brood Y were putative siblings with a foreign fry sibset (of 10 individuals) in brood BB. Moreover, in two cases, a foreign fry found in a brood was the sibling of host fry from another brood in the same pool. In these latter cases, broods OO and PP were both donor and recipient broods, and were neighbouring broods (closest to each other) out of the five broods collected in Pool 12. Taken together, these results indicate that a fry from a given focal brood can find itself in the brood of nearby adults that are not its parents within the same pool. When comparing genotypes across all broods and pools, none of the foreign young were siblings with fry sibsets found in other pools. This is consistent with broods remaining in their natal pool, and not dispersing to other pools, during the parental care period.

On closer examination, four of the cases of siblings occurring in different broods were of individuals that were both categorized as foreign fry in their respective broods. The other three cases were of foreign fry that were siblings to the host fry of an existing brood at the time of sampling. These results suggest that different brood-mixing mechanisms could be at play in this species in nature. In the former four cases, the foreign fry may be from a brood that was no longer present at the time of sampling. In the three latter cases, the foreign fry potentially are individuals that had become separated

or lost from their siblings and parents, who were still in their pool, and joined a neighbouring brood.

4. Discussion

4.1. Prevalence and degree of brood mixing in nature

The current study provides the first genetic evidence for brood mixing, and thus for alloparental care, and the occurrence of putative extra-pair matings in the convict cichlid fish in a natural population. The prevalence of mixed broods (79.2% of 48 sampled broods) observed in the Río Cabuyo population is astounding and substantially higher than the 29% frequency of occurrence inferred from fry body length data alone for this population (Wisenden & Keenleyside, 1992), and, to our knowledge, is among the highest reported incidences of brood mixing in alloparental care species (see Table A4 and review by Coleman & Jones, 2011). Moreover, the degree of brood mixing in our study population varied between broods and was considerable (up to 7 genetically-distinct 'families' were evident within the same brood, Figure 1). As such, the convict cichlid appears to represent an extreme example of brood mixing among vertebrates in the wild.

The high prevalence and high degree of brood mixing in wild convict cichlids suggests that parents may gain fitness benefits (Wisenden, 1999) from caring for mixed broods over unmixed broods. In comparison, the costs of alloparental care in fishes would appear negligible (Wisenden, 1999). Evidence for an indirect fitness benefit accruing to parents alloparentally caring for mixed broods has been reported for the Canada goose (*Branta canadensis*), where 'host' goslings survive longer than adopted goslings in mixed broods and 'host' offspring in mixed broods survive longer than those in unmixed broods (Nastase & Sherry, 1997). Such a survivorship advantage of host young over foreign young in mixed broods may be owing to reduced predation on host young, as mixed broods are often larger than unmixed broods (Codenotti & Alvarez, 1994; Nastase & Sherry, 1997; Wong & Kölliker, 2013) but not always so as in the current study.

In the convict cichlid system, the high prevalence of brood mixing could be a result of intense fish predation pressure in the Río Cabuyo population (Fraser et al., 1993; Wisenden & Keenleyside, 1994; personal observations). The gradual loss of fry through predation is inevitable during the parental care period, even with both parents guarding the brood (Wisenden, 1994).

When the number of young in a brood decreases over time owing to predation, parents could benefit from acquiring/accepting foreign fry into their family to increase brood membership size and thus dilute the risk of predation on their own offspring (safety in numbers mechanism; Krause & Ruxton, 2002). Moreover, by adopting foreign fry that are smaller than their own offspring into their brood, parents could also potentially increase the survival of their offspring (differential predation mechanism; Krause & Ruxton, 2002). Wisenden & Keenleyside (1994) demonstrated such an anti-predation advantage by manipulating convict cichlid broods in the field; they observed that the survival rate of larger (host) fry increased when smaller 'foreign' fry were experimentally placed into a brood. The mechanism by which this occurs coincides with the ossification of the larval skeleton from cartilage to bone between 6 and 8 mm (standard body length) in the Río Cabuyo population (Wisenden et al., 2015). Interestingly, in about 62% of the sibsets across all mixed broods of convict cichlids that we collected from this population and in which host fry had a median body length of >7 mm, the foreign fry were smaller than the host fry (Figure 2a). So, at least for these mixed broods, offspring of the parents (i.e., the host fry) may be more likely to survive predation than those in other mixed and unmixed broods.

Additionally, it would also be advantageous for female convict cichlid parents to accept or actively adopt foreign fry as their brood size declines over the parental care period, given that male parental investment is positively correlated with brood membership size (Wisenden et al., 2008). For example, by adopting foreign fry into their brood, a female may manipulate the level of parental care of her mate and thereby reduce his likelihood of desertion (Wisenden, 1994). In so retaining her mate, a female enjoys male assistance with the defence of her brood against predators; biparental defence is more effective than uniparental defence in the convict cichlid (Wisenden, 1994). Lastly, adopting foreign young may enhance the level of parental care for the entire brood. For example, in the greater rhea (*Rhea americana*), males that adopted unrelated young were found to be 'better parents' because they were more vigilant and protective of young than non-adopting fathers (Codenotti & Alvarez, 1994).

4.2. *Extra-pair matings in nature*

We also provide the first genetic evidence for putative extra-pair matings in the convict cichlid in nature; extra-pair mating in the wild had not been pre-

viously confirmed in this species, although it has been reported for other socially monogamous, biparental, substrate-breeding species of cichlid fishes (Coleman & Jones, 2011; Sefc, 2011). Our finding is somewhat surprising because convict cichlids form socially monogamous pair-bonds and breed in isolated 'caves' within their territories that they form by excavating sand from underneath large rocks (Wisenden, 1995). The female lays adhesive eggs onto the cave's ceiling, the male subsequently enters the cave to fertilize the eggs, and the female then remains in the cave caring for her eggs while the male guards the territory and the entrance to the brooding cave (Keenleyside et al., 1990; Wisenden, 1995; personal observations). For this type of breeding territory, it should therefore be very difficult for a sneaker male to enter the cave to fertilize recently-spawned eggs and, as such, the breeding caves of the convict cichlid are believed to rule out sperm competition (Wisenden, 1995) as they do in other socially monogamous, biparental cichlids (e.g., Langen et al., 2013). Appropriate breeding cave territories are limited in nature and are therefore vigorously guarded by mated pairs throughout the parental care period (Wisenden, 1995).

Previous experimental studies using artificial ponds have alluded to the possibility of short-term male bigamy in convict cichlid populations, particularly when the operational sex ratio is female-biased and predation level is low (Keenleyside, 1985; Keenleyside et al., 1990). A bigamous male shares his time and parental effort (not necessarily equally) with two females within his territory (Keenleyside, 1985; Keenleyside et al., 1990). If male bigamy did occur in our current study, then one of the two females of bigamous males may have 'farmed out' her fry at a young free-swimming stage to the brood of the other female, possibly explaining our observation that some broods contained sets of half-sibs of similar median body size. Clearly more research with microsatellite markers as a tool (to characterize the genotypes of both young and parents) needs to be carried out on natural populations of the convict cichlid to confirm EPMs, and thus polygamy, and their natural prevalence levels in this species, as has been done for other cichlid fish species (Coleman & Jones, 2011; Sefc, 2011).

4.3. Brood-mixing mechanisms

Our novel evidence for the occurrence of genetically mixed broods in wild convict cichlids raises the question of the underlying mechanisms of brood mixing in this species, as well as in other alloparental species (see Table A4

for examples, and Coleman & Jones (2011) and Sefc (2011) for reviews of this phenomenon in cichlid fishes). There are several non-mutually exclusive mechanisms possible.

First, mixed broods could result from extra-pair matings (see above section). Second, mobile young may become separated or lost from their parents and siblings and voluntarily join a nearby family for protection from predation. Indirectly supporting this proposition is our recent finding with wild convict cichlids that lone fry that were experimentally separated from their brood were increasingly less likely to return safely to their family, and more likely to refuge in the pool leaf litter, with increasing separation distance from their family (Lee-Jenkins et al., 2014).

Third, in biparental cichlid fishes in particular, when a female is deserted by her mate she may push her offspring into neighbouring broods ('farming out') because it is very difficult for a lone female to defend her brood successfully in high-predation environments (Yanagisawa, 1985; Keenleyside et al., 1990; Wisenden & Keenleyside, 1992; Wisenden, 1994). Our finding, that most foreign fry differed in body length from the host fry within broods, suggests that the majority of foreign fry in mixed broods originally belonged to other broods that were deserted by one or both of their biological parents.

Fourth, host or foster parents may actively steal or 'kidnap' foreign fry to increase the membership size of their own brood (McKaye & McKaye, 1977; Wisenden, 1999) and thereby gain fitness benefits through reduced predation on their own young (Wisenden & Keenleyside, 1994; Krause & Ruxton, 2002) and/or increased parental effort from their mate (Wisenden & Keenleyside, 1992; Wisenden et al., 2008). When a brood is substantially reduced numerically, parental investment theory predicts that parents should otherwise abandon the remaining young and save their energy for investment in future reproduction (Clutton-Brock, 1991; Wisenden, 1994).

Lastly, through active social partner choice, convict cichlid fry may be responsible for brood-mixing events in nature. Fry that become separated from their parents use chemosensory cues to orient towards conspecifics (Wisenden & Dye, 2009), exhibiting an affinity for parental female cues when they are small and an increased preference for alloparental female cues as they become larger (Wisenden et al., 2014). As fry age (and become larger), they also switch their preference for group mates that are familiar kin (siblings) to socially unfamiliar kin (Lee-Jenkins & Godin, 2013). Taken together, these studies suggest that older young may be more inclined to

stray or be more attracted to neighbouring broods, whereas smaller young (who are more vulnerable to predation) are possibly more motivated to stay closer to their brood mates and guarding parents as there is a survival cost to being distant from guarding parents (Lee-Jenkins et al., 2014).

5. Conclusion

We report the first genetic evidence for brood mixing, and thus alloparental care, and putative extra-pair matings in the convict cichlid fish in nature. At least in the Río Cabuyo population, the prevalence and degree of genetically mixed broods are very high, and indeed among the highest reported for alloparental species in the wild (cf., Table A4; Coleman & Jones, 2011; Sefc, 2011). The high prevalence and degree of mixed broods in this high-predation population suggest that alloparental convict cichlids who care for mixed broods gain some net fitness benefits from doing so, possibly through increased survivorship of their own young. Although the overall degree of brood mixing and the number of donor/foreign families involved in brood-mixing events were high, they did nonetheless vary among mixed broods, suggesting that different mechanisms may underlie brood-mixing events in nature.

Our novel genetic evidence for the occurrence of extra-pair matings in the convict cichlid importantly challenges the hitherto widely accepted notion that this species is monogamous, and indicates that social monogamy does not necessarily lead to genetic monogamy in the wild (cf., Coleman & Jones, 2011; Sefc, 2011). These findings collectively raise questions about underlying brood-mixing mechanisms and reproductive ecology (in particular opportunities for polygamy) in the convict cichlid, an important model species in the study of animal behaviour. As a model species, the convict cichlid presents future opportunities for exploring inter-population variation in the prevalence and degree of brood mixing and prevalence of extra-pair matings (and their respective mechanisms) and to relate such variation to differences in the ecology and demographics of populations.

Acknowledgements

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Appendix: material and methods

A1. Field site

During the dry season, the Río Cabuyo consists of a series of pools interconnected by shallow riffle sections that permit fish movement between pools, which are typically shallow along the shoreline and deeper towards their centre. The substratum of the river varies between pools, but mainly consists of rocks, sand, gravel and some large boulder rock-face shelves. In some pools, the substratum is covered with considerable leaf litter, from the surrounding tree canopy. Fish density and species diversity also vary between pools. Known fish species in this river are the convict cichlid, other cichlids (*Neetroplus nematopus*, *Amphilophus longimanus* and *Parachromis dovii*),

Table A1.
Polymorphisms of the four microsatellite markers used for genotyping convict cichlid fry collected from 12 pools in the Río Cabuyo, Costa Rica.

Locus	Allele size range (bp)	Observed number of alleles	GenBank accession number
Acit1	151–173	11	HQ292647
Acit2	159–231	34	HQ292648
Acit3	141–215	36	HQ292649
Acit4	326–402	27	HQ317442

The size range and number of alleles for each locus are based on data collected from 1801 individually genotyped fry originating from 48 broods under parental care. The characteristics of these microsatellite loci and genetic protocols are further described in Lee-Jenkins et al. (2011).

Table A2.
Mean ± SE (range) of the body length (BL) of fry in unmixed broods (containing no foreign fry), which represented 20.8% of the total number ($N = 48$) of wild convict cichlid broods sampled and genotyped.

Brood ID	Pool ID	No. of broods in pool	No. of fry in brood	BL (mm)	
				Mean ± SE	Min–Max (range)
A	1	5	15	6.90 ± 0.07	6.35–7.42 (1.07)
F	3	4	12	11.26 ± 0.12	10.55–11.48 (1.23)
QQ	4	2	97	5.44 ± 0.02	5.02–5.85 (0.83)
H	5	7	20	8.51 ± 0.07	7.49–8.92 (1.43)
J	5	7	15	8.70 ± 0.07	8.24–9.17 (0.93)
T	8	1	12	7.75 ± 0.05	7.54–8.05 (0.51)
Z	10	4	30	7.99 ± 0.07	6.83–8.62 (1.79)
FF	11	9	56	6.63 ± 0.02	6.03–7.03 (1.0)
JJ	11	9	78	7.02 ± 0.02	6.57–7.38 (0.81)
MM	12	5	21	6.22 ± 0.03	6.04–6.52 (0.48)

Body lengths ranged across the entire developmental period, from newly free-swimming fry (approx. 5 mm) to fry approaching independence (approx. 10–13 mm). Most of these 10 unmixed broods were found in pools containing a relatively high number of broods.

Table A3.
The mean ± SE body length (BL) of host fry (HF) and foreign fry sibsets that originated from potential extra-pair matings (EPMs) in 12 of the 48 broods sampled and genotyped.

Brood ID	Pool ID	No. of fry	HF		EPM fry	
			N	Body length (mm)	N	Body length (mm)
RR	1	56	34	6.46 ± 0.02	10	6.55 ± 0.03
UU	1	61	48	5.86 ± 0.02	7	5.85 ± 0.03
XX	2	35	12	5.53 ± 0.05	10	5.57 ± 0.04
					7	5.60 ± 0.06
E	3	38	18	6.51 ± 0.03	16	6.53 ± 0.05
N	5	73*	32	4.94 ± 0.03	30	4.94 ± 0.03
O	6	27	14	7.63 ± 0.03	12	7.21 ± 0.09
V	9	22	16	6.24 ± 0.04	5	6.35 ± 0.09
X	9	24	17	8.44 ± 0.07	6	8.56 ± 0.15
EE	11	20	18	5.11 ± 0.05	2	5.12 ± 0.16
HH	11	33	13	10.1 ± 0.10	6	10.14 ± 0.10
KK	11	43	35	5.90 ± 0.02	8	6.00 ± 0.05
LL	12	73	44	5.12 ± 0.02	18	5.10 ± 0.04
					5	5.12 ± 0.10

*For brood N, 10 of the fry were not included in the HF or EPM sibsets because their genotypes were consistent with either sibset.

Table A4.
Examples of the frequency of occurrence (%) of brood mixing during the parental care period in the wild in alloparental species that are not cooperative breeders.

Taxon	Percent mixed broods	*N*	Reference
Fishes			
Catfish (spp.)	40	10	Ochi et al. (2001)
African cichlid (spp.)	0–95	7–28	Ochi & Yanagisawa (1996)
African cichlid	59	32	Schaedelin et al. (2013)
African cichlid	14	14	Sefc et al. (2012)
Convict cichlid	79	48	Current study
Bluegill sunfish	89.7	39	Neff (2001)
Birds			
Northern bobwhite	7–67	9–45	Faircloth et al. (2005)
Ring-billed gull	7	268	Brown (1998)
Curlew	60	48	Lanctot et al. (1995)
Canada goose	28	25	Nastase & Sherry (1997)
Insects			
Burrower bug	73	22	Agrawal et al. (2004)
Bee	44	79	Tierney et al. (2002)

These example studies were haphazardly selected form the published literature. *N* denotes the number of broods/families or nests sampled.

characins (*Astyanax aeneus*), catfish (*Rhamdia nicaraguensis*) and large poeciliids (*Poeciliopsis turrubarensis*). All of these fishes are potential predators on convict cichlid fry (personal observations; Keenleyside et al., 1990).

A2. *Fry body length measurements*

To ensure accurate body length measurement, each collected and preserved fry was placed parallel between two sterile glass slides and the top slide was gently pressed to straighten out its body. This process did not alter the fry's body length, which was confirmed by comparing measurements under the microscope with the above procedure and measurements taken separately with callipers during pilot measurements. After being measured, each fish was given a brood-specific individual identification (e.g., A1 represents individual No. 1 from Brood 'A') and transferred to an individual tube ('screw cap' microcentrifuge tube) filled with 95% ethanol for storage until genotyped. For eight fry (B25, B27, D3, QQ36, QQ44, QQ97, WW71, WW108), it was impossible to accurately measure their body length due to body damage. Although these individuals were included in the genotype analysis, they

were excluded from the analysis of fry body length within and between broods.

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