Territorial Males Can Sire More Offspring in Nests with Smaller Doors in the Cichlid Lamprologus lemairii

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To examine how territorial males counter reproductive parasites, we examined the paternity of broods guarded by territorial males using 5 microsatellite loci and factors that determine siring success in a wild population of the Lake Tanganyika cichlid Lamprologus lemairii. Females enter rock holes (nests) and spawn inside, and territorial males release milt over the nest openings. Sneakers attempt to dart into the nests, but territorial males often interrupt the attempt. The body size of territorial males (territorial defense ability) and the size of nest opening (the ability to prevent sneakers from nest intrusions) are predicted to be factors that affect paternity at the premating stage, whereas milt quality traits are factors that affect paternity at the postmating stage. Parentage analyses of 477 offspring revealed that most clutches have few or no cuckolders, and territorial males sired >80% of eggs in 7 of the 10 analyzed clutches. Larger territorial males that spawned in nests with narrower openings had greater siring success. In contrast, none of the milt traits affected the siring success. These suggest that territorial male L. lemairii adopt premating strategies whereby they effectively prevent reproductive parasitism.

Subject area: Reproductive strategies and kinship analysis
Key words: body size, counterstrategy, parentage analysis, reproductive parasitism, sperm competition, substrate-brooding cichlid

Alternative reproductive tactics (ARTs) are widespread among animals, particularly fishes (Oliveira et al. 2008). In species with ARTs, “bourgeois” males that monopolize access to mates try to pair spawn, but they may face the risk of stolen fertilization by males that adopt parasitic tactics (e.g., sneaker males that secretly or rapidly approach the spawning pairs and release sperm). Recent studies have begun to unveil the counterstrategies of bourgeois males, particularly with the understanding of sperm competition (Parker 1998). For example, bourgeois males may make an ad hoc response to the presence of parasitic males and adjust their ejaculate expenditure (e.g., Candolin and Reynolds 2002; Smith et al. 2003). They could also make an a priori response to parasitic males by increasing ejaculate investments, which includes investments in testes and milt quality traits (e.g., Awata et al. 2006a, 2008; Simmons et al. 2007; Ota et al. 2010). Given that male reproductive success is a function of both pre- and postmating episodes, both pre- and postmating competition between reproductive tactics should be considered to understand how bourgeois males counter reproductive parasitism.

Assuming that the investment in milt is costly (Nakatsuru and Kramer 1982), males are expected to allocate their investments between pre- and postmating strategies (Parker 1998; Parker et al. 2013). Bourgeois males are expected to invest in the premating expenditure when they can effectively reduce reproductive parasitism at the premating stages; thus, they reduce reproductive parasitism risks (sperm competition risks). If they cannot succeed in reducing sperm competition risk by increasing premating expenditure, they will allocate their investments mainly to postmating strategies. Premating strategies could include ad hoc responses, for example, territorial defense (Magnhagen 1995; Alonzo and Warner 2000; Zbinden et al. 2003; Scaggage et al. 2005) and reduced courtship rate (Le Comber et al. 2003). However, if bourgeois males detect a risk of reproductive parasitism prior to mating, they may also employ a priori counterstrategies such as constructing nests with a small opening (Svensson and Kvarnemo 2003, but see Svensson and Kvarnemo 2007) and selecting a nest according to the risk of reproductive parasitism (Ota and Kohda 2006a, 2011). Consequently, reproductive parasitism has been recognized to play a considerable role in shaping the phenotypes of bourgeois males. However,
little attention has been paid to male counterstrategies to reproductive parasitism, particularly at the premating stage.

ARTs are widespread in the Lake Tanganyika substrate-brooding cichlid tribe Lamprologini (Sefc 2011), and they have been found in males of the piscivorous cichlid Lamprologus lemairi. This fish is polygynous and displays maternal guarding (Kuwamura 1986; Gashagaza 1991). A territorial male defends a large territory against conspecific males that includes 2–4 separated rock holes (i.e., nests), each of which is defended by a female (Kuwamura 1986; Gashagaza 1991). Intense male–male competition takes place for the holes (Gashagaza 1991), and territorial males are aggressive toward the approach of other males during mating. In the Uvira (3°36′S, 29°10′E) population, the size distribution of mature males is bimodal (Gashagaza 1991), suggesting that sneakers are present. We found behavioral evidence of sneaking in the Wonzye (8°43′S, 31°08′E) population (Ota K, Awata S, personal observations), with small males slowly approaching and darting into nests where spawning was occurring. Lamprologus lemairi females enter the holes and deposit eggs on the inner rock surface. Territorial males, which are much larger than females and thus cannot enter the holes, release milt over the openings of the holes. Females provide brood care alone, which lasts 14–16 days (Gashagaza 1991). Because sneakers have a body size that is as large as or somewhat smaller than that of females (Gashagaza 1991), they are able to enter the nest holes. Such sneaking behavior is common in Lamprologini cichlids, in which spawning takes place in closed objects, such as empty gastropod shells and rock holes (Sato et al. 2004; Ota and Kohda 2006b; Ota et al. 2012).

The aims of this study were to obtain genetic and anatomical evidence of ARTs and examine the counterstrategies of bourgeois “territorial” males against sneakers in the Wonzye population of L. lemairi. More specifically, we first conducted field observations and collected fish to investigate their mating system and phenotypic traits. Second, we analyzed testes size and sperm traits to assess whether they differ between males that use different reproductive tactics. Third, we genetically examined the paternity of captured offspring and putative parents using microsatellites to determine the siring success of males. Finally, we identified factors that explain the siring success of territorial males at both pre- and postmating stages to clarify the counterstrategies of territorial males. The body size of territorial males and nest-opening size were assumed to be possible factors that affect siring success at the premating stage. This is because larger cichlids generally have a better ability to expel rivals than smaller cichlids (Limberger 1983; Brandtmann et al. 1999; Barlow and Lee 2005; Awata et al. 2006b; Ota and Kohda 2011); thus, larger territorial males are expected to be more successful in territorial defense. Also, the possibility that sneakers succeed in entering nests decreases with decreasing size of the nest opening, which would influence the success of preventing sneaking (Sato et al. 2004). Quantity and quality of sperm should be regarded as possible postmating phenotypes because competitive sperms are expected to achieve high fertilization success under sperm competition (Parker 1998; Gage et al. 2004; Snook 2005).

Methods

Field Study

A field study using self-contained underwater breathing apparatus (SCUBA) was conducted during September–October 2006 at Wonzye, near Mbulungu, Zambia. To find brood-caring female L. lemairi and their mates, we searched for females that were immobile at the openings of nest rock holes in a wide range of shallow rocky habitats (5–10 m depth). Once we found a brood caring female, we observed it until their potential mates appeared. In all cases, we identified a male that visited the nest, stayed for a short period, and then left. We assumed these males to be potential mates of the females and labeled them “territorial males.” We then followed the male. Some of the males visited other females that held nests in their home ranges. Consequently, we found a total of 12 territorial males, 10 brood-caring females, and 6 nest-holding (but not caring) females, and we captured them using a fence net. Nests visited by different territorial males were considerably isolated (>10 m), whereas nests held by the same territorial males were located together. After fish capture, we measured the width of the widest nest opening in the nest rock holes using a ruler (to the nearest 1 cm). Ten clutches were sampled from 10 different nests of 7 territorial males. All embryos in the nests were captured for parentage analyses, after releasing an anesthetic (30% clove oil diluted in ethanol) in the nests. A total of 2024 embryos were captured (mean ± standard deviation [SD] = 202.4 ± 161.5 embryos/nest). We also captured L. lemairi within 5 m from the nests (N = 17 nests) using the fence net. We expected that sneakers would be included in them because cichlid sneakers generally reside in the vicinity of nests (Sato et al. 2004; Ota and Kohda 2006b; Ota et al. 2012). We captured a total of 27 individuals in this sampling and labeled mature males among them as “peripheral males.”

Body Measurements and Analyses of Sperm Traits

All captured fish were transported to the laboratory. To determine sex and maturity and to measure body size and sperm traits, the fish were anesthetized with 0.05% of FA100 (10% solution of eugenol, Tanabe Seiyaku Co. Ltd, Tokyo, Japan). We then measured their standard length (SL; to the nearest 0.1 mm) and body mass (BM; to the nearest 0.001 g), and dissected them, except for 6 females, which were sexed by the shape of genital papilla and released at the sampling points the next day after measuring their SL and BM. Fin tissue was clipped from each fish and placed in 99.5% ethanol for parentage analyses. After the dissection, we measured testes mass (TM; to the nearest 0.001 g). Male maturity was determined from the developmental stages of gonads: mature males having white enlarged testes and immature males having small thread-like testes. Immediately after the TM measurement, semen was sampled from the testes near the genital papilla using fine needles and 4 sperm traits (sperm velocity, sperm longevity, flagellum length, and sperm density) were measured (N = 12 territorial males and 11 peripheral males). Details on measuring sperm traits are described in Supplementary Material 1.
Parentage Analyses

Forty-eight offspring that were randomly selected in each clutch (N = 9) were assayed, except for 1 clutch in which all 45 offspring were assayed (N = 10 clutches in total). Genomic DNA was extracted from the fin tissue samples of 22 males and 25 females and tissue samples of 477 offspring. These samples were genotyped at 5 microsatellite loci (Table 1): Adew8 (GenBank Accession No. AB787159, newly developed for a Lake Tanganyika cichlid Astatoreochromis davidii) by H. Munehara using the same method described in Sato et al. [2011]), Chb1 (Munehara et al. 2001), Pzeb1 and Pzeb3 (van Oppen et al. 1997), and UME003 (Parker and Kornfield 1996). Parentage assignment was performed using CERVUS version 3.0.3 (Marshall et al. 1998). The potential mother for each offspring was the female caring for the offspring, and potential fathers for each offspring were territorial and peripheral males (Table 2). COLONY (Wang 2004) was used to infer the number of sires per clutch using the maximum-likelihood estimate method. See Supplementary Material 2 for detailed methods of parentage analyses. In compliance with data archiving guidelines (Baker 2013), we have deposited the genotype data underlying the paternity analysis with Dryad.

Data Analysis

Testes investment was compared according to Tomkins and Simmons (2002). First, we constructed a full linear model (full LM) with log TM as the dependent variable, male type (territorial or peripheral males) as the independent variable, and log soma mass (SM) (BM-TM) as the covariate. Second, because the interaction was not significant (see Results), the effects of male type and log SM on TM were tested after a deletion of the interaction from the model (reduced LM).

Sperm longevity, spermatocrit (estimate of sperm density), and sperm flagellum length were compared between territorial and peripheral males by using LMs. Sperm velocity was compared between the male types by using generalized estimating equation (GEE) with a Gaussian link. This GEE model incorporated time since activation (covariate), sperm longevity did not differ between male types (LM: Wald χ² = 1.61, P = 0.20; male type, Wald χ² = 0.96, P = 0.33; time, Wald χ² = 187.57, P < 0.001; Supplementary Material 3). No significant correlations were observed between sperm traits (TM, sperm longevity, flagellum length, and velocity at 10 s since activation) for each male type (LM: P > 0.078 for all).

Paternity and Maternity

Multiple maternity was detected in 1 clutch (Y01 in Table 2), but it was not detected in the 9 other clutches. In this clutch,

<table>
<thead>
<tr>
<th>Locus</th>
<th>N_a</th>
<th>Size</th>
<th>H_obs</th>
<th>H_exp</th>
<th>PIC</th>
<th>NE1p</th>
<th>NE2p</th>
<th>Fnull</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adew8</td>
<td>23</td>
<td>87–149</td>
<td>0.941</td>
<td>0.932</td>
<td>0.918</td>
<td>0.267</td>
<td>0.155</td>
<td>0.000</td>
</tr>
<tr>
<td>Chb1</td>
<td>19</td>
<td>97–152</td>
<td>0.961</td>
<td>0.933</td>
<td>0.919</td>
<td>0.266</td>
<td>0.154</td>
<td>0.000</td>
</tr>
<tr>
<td>Pzeb1</td>
<td>8</td>
<td>115–134</td>
<td>0.255</td>
<td>0.255</td>
<td>0.247</td>
<td>0.966</td>
<td>0.857</td>
<td>0.001</td>
</tr>
<tr>
<td>Pzeb3</td>
<td>5</td>
<td>324–332</td>
<td>0.373</td>
<td>0.528</td>
<td>0.474</td>
<td>0.855</td>
<td>0.707</td>
<td>0.178</td>
</tr>
<tr>
<td>UME003</td>
<td>20</td>
<td>215–271</td>
<td>0.784</td>
<td>0.814</td>
<td>0.786</td>
<td>0.532</td>
<td>0.358</td>
<td>0.013</td>
</tr>
</tbody>
</table>

The data are based on the CERVUS analysis of 51 fish including 4 young having individual specific alleles. N_a: number of alleles; size: range in base pairs; H_obs: observed heterozygosity; H_exp: expected heterozygosity; PIC: polymorphic information content; NE1p: probability of nonexclusion assuming no parents known; NE2p: probability of nonexclusion assuming 1 parent known; Fnull: estimated null allele frequencies.

Results

Body Size and Nest Size

Of the individuals that were captured around the nests, 42.8% (12 of 28 individuals) were males, and most (11 of 12) males had large developed testes. Nest-holding and brood-caring females (mean ± SD = 104.6 ± 12.7 mm SL, range = 79.3–124.8 mm SL, N = 16) were much smaller than territorial males (175.6 ± 14.2 mm SL, 157.7–205.0 mm SL, N = 12), but they were larger than peripheral males (82.9 ± 7.0 mm SL, 75.1–97.9 mm SL, N = 11) (LM: F1,20 = 196.78, P < 0.001, Tukey Honestly Significant Difference post hoc test, all combinations P < 0.05). Female L. lemairii used rock holes with openings that were 380 ± 80 mm wide (mean ± SD, N = 10 nests) as nests.

Testes Investment and Sperm Traits

Despite a large difference in body size, the absolute TM did not differ between territorial and peripheral males (LM: F1,21 = 0.01, P = 0.94). The LM revealed no significant interaction between male type and SM (F1,19 = 4.22, P = 0.054), but the male type (F1,20 = 26.09, P < 0.001) and the SM (F1,20 = 24.90, P < 0.001) had significant effects on the TM (Figure 1). The spermatocrit, sperm flagellum length, and sperm longevity did not differ between male types (LM: P > 0.27 for all; Supplementary Material 3). The sperm velocity also did not differ between male types (GEE: time × male type, Wald χ² = 1.61, P = 0.20; male type, Wald χ² = 0.96, P = 0.33; time, Wald χ² = 187.57, P < 0.001; Supplementary Material 3).
Determinants of Siring Success in Territorial Males

The siring success of territorial males increased with their SL and decreased with increasing nest-opening width (Figure 3 and Supplementary Material 4), but it did not correlate with any ejaculate traits (Supplementary Material 4). We obtained similar results even if the clutch was omitted from the analyses: the siring success of territorial males increased with male SL and decreased with nest-opening width, although the latter relationship was no longer significant (Figure 3 and Supplementary Material 4).

Discussion

Using microsatellite loci, we examined the paternity in 10 broods of *L. lemairii* that were collected in the field and we obtained genetic evidence that peripheral males sire young. We further showed that the size distribution of mature males around the nests was apparently discontinuous, with smaller peripheral males having a greater investment in testes than larger males, which was consistent with the general patterns of ARTs and theoretical predictions. Our results provide strong evidence that males exhibit ARTs, small sneakers and large territorial males. Although multiple paternities were found in 60% of 10 broods, the siring success of territorial males was highly bimodal among their broods. Consequently, they succeeded in siring more than 70% of total young. A recent review of genetic parentage studies (Coleman and Jones 2011) revealed that multiple paternities are common in male-nesting fishes, and compared with these fish species, the level of cuckoldry in *L. lemairii* is relatively low. Therefore, we can conclude that territorial male *L. lemairii* are highly successful in monopolizing fertilization in the wild.

The best predictor of the siring success of *L. lemairii* territorial males was their own body size, and milt traits did not explain the siring success. This suggests that the high siring success of territorial male *L. lemairii* results from premating strategies. Three nonmutually exclusive explanations exist as to why a larger body size is important at the premating stage. First, large bodies may be advantageous for territorial defense. A large size advantage in male–male competition or territorial defense is known in a variety of taxa, including fishes. In 3-spined sticklebacks, for example, larger males are better able to defend their fertilization against sneaking males (Zbinden et al. 2003). In the Lake Tanganyika cichlid *Telmatochromis vittatus*, larger territorial males more often succeed in nest defense against males trying nest takeover (Ota and Kohda 2011). This is likely the case for *L. lemairii*. Second, sneakers may choose their target nests based on the body size of territorial males. If sneaker avoid trying to steal fertilization opportunities from these successful guardians (i.e., larger territorial males), smaller territorial males are expected to face a high risk of sneaking, resulting in decreased siring success. Third, larger bodies may contribute to an increase in the reliability of paternity through competition for nests.

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**Table 2** Territorial male codes, brood codes, and peripheral male codes

<table>
<thead>
<tr>
<th>Territorial male code</th>
<th>Brood code</th>
<th>Peripheral male code</th>
</tr>
</thead>
<tbody>
<tr>
<td>TR01</td>
<td>Y01</td>
<td>PR01, PR02</td>
</tr>
<tr>
<td>TR02</td>
<td>Y02</td>
<td>PR03</td>
</tr>
<tr>
<td>TR03</td>
<td>Y03</td>
<td>—</td>
</tr>
<tr>
<td>TR03</td>
<td>Y04</td>
<td>—</td>
</tr>
<tr>
<td>TR03</td>
<td>Y05</td>
<td>—</td>
</tr>
<tr>
<td>TR04</td>
<td>Y06</td>
<td>PR04</td>
</tr>
<tr>
<td>TR05</td>
<td>Y07</td>
<td>—</td>
</tr>
<tr>
<td>TR06</td>
<td>Y08</td>
<td>PR05, PR06</td>
</tr>
<tr>
<td>TR06</td>
<td>Y09</td>
<td>PR05, PR06</td>
</tr>
<tr>
<td>TR07</td>
<td>Y10</td>
<td>—</td>
</tr>
</tbody>
</table>

“—” indicates no peripheral males found within 5 m of the nests. TR03 and TR06 visited 3 nests and 2 nests with brood-care females, respectively.
We found that the siring success of territorial males was higher in nests with narrower openings, although the small sample size does not allow strong conclusions (indeed, a statistical significance was detected only when an unsuccessful male was included). The reduced size of nest openings could possibly increase the ability to limit sneaking because it could limit the size of the fish that can enter the nests. This is well supported in the shell-brooding congener cichlid *Lamprologus callipterus* (Sato et al. 2004), but not in the sand goby *Pomatoschistus minutus* (Svensson and Kvarnemo 2007). Particularly in species that build nests on natural substrates, such as *L. callipterus* and *L. lemairei*, it may be important for males to avoid reproductive parasitism by optimizing their nest choice. Similar optimization is found in *T. vittatus*, where territorial males preferred to occupy nests with low risks of parasitism (Ota and Kohda 2006a, 2011). This nest choice strategy would be beneficial for preventing not only sneakers from stealing fertilization but also embryo from being predated upon. However, it might impose some costs on territorial males. Smaller nest openings, for example, may decrease the ventilation efficiency in the nests, thus resulting in a lower survival of eggs/embryos because of a decrease in dissolved oxygen levels (Jones and Reynolds 1999). An experimental study to determine whether nest choice by territorial males changes depending on the risk of reproductive parasitism is required to support this assumption.

The suggested premating strategy of territorial male *L. lemairei* involves contest, which should impose severe costs on
them (Komdeur 2001). Males are expected to allocate their resources to obtain mating (i.e., pre-mating strategies) or to maximize paternity (i.e., post-mating strategies), when they cannot meet both demands (Parker 1998; Alonzo & Warner 2000; Parker et al. 2013). In some gobiid fishes, territorial males well prevent sneaking by increasing efforts for territorial defense, but not by increasing investment in ejaculates (Scagghian et al. 1999, 2005; Rasotto and Mazzoldi 2002; Svensson and Kvarnemo 2005, 2007; Locatello et al. 2007). This was the case in <em>L. lemairii</em> territorial males invested less in testes than sneakers, although no differences were observed in sperm quality traits between them. Overall, territorial male <em>L. lemairii</em> would allocate their energy mainly to pre-mating strategies, so as to secure paternity.

Although female promiscuous mating has been recognized to strongly influence the male phenotypic evolution, particularly with the development of both paternity assessments using microsatellites and an understanding of postmating sexual selection, the effect of reproductive parasitism (an important factor affecting the extent of female promiscuity) on the phenotypes and reproductive strategies of bourgeois males is still not well understood. As shown in recent studies (Parker 1998; Alonzo and Warner 2000; Candolin and Reynolds 2002; Le Comber et al. 2003; Smith et al. 2003; Svensson and Kvarnemo 2003; Awata et al. 2006a, 2008; Ota and Kohda 2006a, 2011; Simmons et al. 2007; Ota et al. 2010) and this study, reproductive parasites play a critical role in shaping phenotypes of bourgeois males. Together with conflicting demands between tactics, this seems to be similar to the role of coevolutionary processes, in which the adoption of 1 tactic (parasitic tactics in the case of ARTs) is expected to change the intensity of selection pressure and/or its regime that acts on another tactic (bourgeois tactics). A study on male counteradaptation would therefore promote our understanding of the evolution of ARTs.

**Supplementary Material**


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**References**


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