

Social composition and relatedness of the cooperatively breeding cichlid *Neolamprologus buescheri* inhabiting the deep water of Lake Tanganyika

Shun Satoh^{1,2*}, Yuki Yoshio², and Masaya Morita³

¹ The Hakubi Center for Advanced Research, Kyoto University, Sakyo-ku, Kyoto, Japan

² Department of Zoology, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto, Japan

³ Sesoko Station, Tropical Biosphere Research Center, University of the Ryukyus, Sesoko Motobu, Okinawa, Japan

Orcid ID

<https://orcid.org/0000-0003-1284-5249> (SS)

<https://orcid.org/0009-0007-1325-2310> (YY)

<https://orcid.org/0000-0002-0170-1226> (MM)

* corresponding author [S.S.]

Email: symphysodondiscus@icloud.com

Tel: +818010497603

Acknowledgments: We thank the members of the Maneno Tanganyika Research Team and the staff of the Department of Fisheries in the Republic of Zambia for their assistance during the field study. We also thank Prof. Satoshi Awata of Osaka Metropolitan University and members of the Animal Ecology Laboratory of Kyoto University for their helpful input into our study.

Author contributions S. S. designed the study. S. S. conducted the field observations. S. S. and Y.Y. conducted the morphological measurements. M.M. conducted the molecular work. S.S. and M. M. analyzed the data. S.S. and M. M. wrote the manuscript with input from all the authors.

Abstract

The cichlids of Lake Tanganyika exhibit remarkable ecological diversity and serve as a model system for studying social evolution. However, the social structures of many species remain poorly understood because they occur in deep-water environments. In this study, we examined the social structure and helping behavior of *Neolamprologus buescheri*, a cichlid species inhabiting deep-water rocky crevices in Lake Tanganyika. Behavioral observations in their natural habitat revealed that breeding groups consisted of up to four subordinates sharing a territory with breeding females. The number of subordinates was positively correlated with the body size of breeding females and negatively correlated with the frequency of territorial defense behavior per individual. This suggests that subordinates may reduce the parental care costs of breeding females, supporting the notion that this species engages in cooperative breeding. SNPs isolated from GRAS-Di confirmed strong kinship between subordinates and dominant breeders, indicating that subordinates are offspring who delayed dispersal from the natal territory. Despite high kinship within a population, we observed a lower inbreeding coefficient and higher heterozygosity compared with other cooperative breeders. This suggests that while kin-based social structures contribute to territory cohesion and defense, the species also maintains genetic diversity at the population level.

Keywords: parental care, cichlids, social system, breeding, aggressive behavior

Introduction

In cooperatively breeding species, offspring receive care not only from their parents but also from other group members, known as helpers or subordinates (Jennions & Macdonald, 1994; Solomon & French, 1997; Koenig & Dickinson, 2004; Taborsky, 2021). These intricate social systems have evolved independently across various vertebrate taxa, making them a model for studying the evolution of cooperative and complex animal societies (Jennions & Macdonald, 1994; Solomon & French, 1997; Koenig & Dickinson, 2004; Taborsky, 2021). While prevalent among mammals and birds, cooperative breeding is rare in fish (Taborsky & Limberger, 1981; Taborsky, 2016; Frommen & Fischer, 2021). While there are a few reports suggesting the cooperative breeding to occur in a riverine cichlid fish (Martin & Taborsky, 1997) and anabantoid fish in Southeastern Asia (Witte & Schmidt, 1992), the best documented evidence of this behavior in fish come from the tribe Lamprologini, a group of cichlids in Lake Tanganyika, East Africa. This group has attracted significant research interest as a model for cooperative and complex societies (Wong & Balshine, 2011; Dey et al., 2017; Tanaka et al., 2018b). However, despite proposals identifying over 20 cooperatively breeding cichlid species, detailed studies exist for only a few (Tanaka et al., 2018b).

In cooperatively breeding cichlids, helpers frequently engage in territorial and brood defense against intruders (Saeki et al., 2022; Satoh et al., 2022; Hidaka et al., 2024), nest maintenance (e.g., excavating and clearing sand from shelters) (Tanaka et al., 2015; Saeki et al., 2022; Satoh et al., 2022), and offspring care (e.g., cleaning and fanning eggs) (Zöttl et al., 2013c; Josi et al., 2019), all of which contribute to offspring survival (Brouwer et al., 2005; Wong & Balshine, 2011; Jungwirth & Taborsky, 2015; Taborsky, 2016). These helping behaviors reduce the costs of parental care (Balshine et al., 2001; Zöttl et al., 2013a; Tanaka et al., 2018a; Josi et al., 2020: 202; Satoh et al., 2022). Additionally, dominant breeders can enforce cooperation through threats or aggressive interaction if a helper fails to contribute (Bergmüller & Taborsky, 2005; Naef & Taborsky, 2020; Zöttl et al., 2023; Hidaka et al., 2024), potentially serving as punishment for neglecting helping behaviors.

Despite superficial similarities in cooperative cichlid societies, social structures vary significantly. For example, in *Neolamprologus pulcher* [Trewavas & Poll, 1952], the relatedness between helpers and dominant breeders exhibits a negative correlation with the age of the helper, and

larger helpers are typically unrelated to the breeders due to the exchange of dominant breeders (Dierkes et al., 2005). While *N. obscurus* [Poll, 1978] exhibits a similar social structure, the relatedness between dominant breeders and helpers is higher than in *N. pulcher* (Tanaka et al., 2015). Additionally, in *Julidochromis ornatus* [Boulenger, 1898], breeding territories typically include a few nonkin helpers, which can sire offspring by assisting dominant breeders in parental care (Awata et al., 2005; Heg & Bachar, 2006).

Kin structure and reproductive skew influence the costs and benefits within cooperative breeding groups, shaping the evolution of this system. Therefore, documenting these aspects in natural habitats is essential (Josi et al., 2021; Jungwirth et al., 2023). However, knowledge of some cooperatively breeding species remains limited due to the challenges of conducting such studies

Neolamprologus buescheri [Staeck, 1983] is a substrate-brooding cichlid endemic to Lake Tanganyika (Staeck, 1982; Ronco et al., 2020). This species inhabits the deep reef zones of the lake, typically at depths greater than 20 m, and constructs breeding territories within small rock crevices. Heg and Bachar (2006) suggested that *N. buescheri* may engage in cooperative breeding, as adult offspring remain near breeding territories and frequently exhibit submissive behaviors toward dominant breeders in water tanks. However, these observations are qualitative or anecdotal, and the species' social structure, ecology, and life history remain poorly understood due to its deep-water, rocky reef habitat.

To determine whether *N. buescheri* engages in cooperative breeding, we conducted field observations in its natural habitat. Underwater cameras were used to assess social structure and document the frequency and type of helping behaviors in breeding groups. We then performed a kin-structure analysis using genotyping by random amplicon sequencing-direct (GRAS-Di) to estimate kinship between dominant breeders and subordinates. Additionally, population genetic analysis was conducted to describe the inbreeding coefficient (F) and heterozygosity (H_e) of *N. buescheri* and compare these metrics with those of related cooperatively breeding cichlid *J. ornatus* at the same population. *Julidochromis ornatus* is a related species (Ronco et al., 2021) that sympatrically occupies breeding habitats similar to those of *N. buescheri*.

Materials and Methods

Study species and fieldwork

Field observations and sampling were conducted via SCUBA diving at Kombe point (8°47'54"S, 31°01'11"E) in southern Lake Tanganyika, Republic of Zambia. While interpopulation variation in body coloration has been documented for this species, individuals at Kombe point display a dark body with white stripes (Fig. 1) (Konings, 1998). The standard length (SL) is approximately 5 cm for breeding males and 7 cm for breeding females (see Results). While these species typically inhabit depths greater than 20 m, they are occasionally observed at depths shallower than 10 m at this location. Before behavioral observations, we established a 75-m guideline within the rocky area of Kombe point (11–22 m depth) from September to December 2022. From September to December 2023, a 55-m guideline (14–26 m depth) was set up to avoid overlapping with the 2022 guideline. All individuals within a 5-m range of the guideline were individually identified based on unique body coloration and numbered. In this study, the home range of breeding females and their subordinates, aggressively defended against intruders, was defined as a territory. Following previous studies (Saeki et al., 2022), individuals within the female territory are referred to as subgroup members, while those within the same harem (defined as the breeding male's territory or home range) are referred to as group members. Note that individuals of this species are readily observable within their territories, as they exhibit strong philopatry and rarely venture beyond their established home ranges.

*Social status definition of *N. buescheri**

Social status was classified into five categories based on body size, behavior, and gonadal characteristics: breeding male, breeding female, subordinate, juvenile, and single male. The largest fish in breeding groups had expansive home ranges encompassing multiple breeding territories. Although not all individuals of this type were captured (see *sampling and measurements*), dissections after the behavioral observation confirmed these individuals as males, so they were classified as "breeding males." The second-largest individuals, which occupied a single territory and displayed aggression toward conspecifics or other species, were designated as "breeding females." Smaller individuals were observed around breeding territories, sharing shelters and territories with breeding

females. Those exceeding 20.0-mm SL were categorized as "subordinates," while those smaller than 20.0-mm SL were classified as "juveniles." Some males exhibited no social interactions with other group members except with male breeders. These individuals were determined to be males by dissection and classified as "single males." The definitions of social rank and the associated behavioral observations (see below) followed the methodology established in previous studies (Saeki et al., 2022; Satoh et al., 2022).

Behavioral observation using video data

Because territories of this species are deeper than the suitable depth for direct visual SCUBA observations, underwater cameras (GoPro Hero 10, 11, GoPro, Inc., USA) were used to collect behavioral data. Upon identifying a territory, we estimated the number of fish around it for approximately 5 min. Cameras were then positioned overhead (1.5-m radius), with a 40 × 40 cm ruler placed nearby for estimating SL and home range area from video recordings. Each territory was recorded for approximately 60 min.

All individuals, except juveniles, were identified based on territory location, size, and natural markings. Behavioral observations focused on breeding females (n = 36 from 26 male territories) and subordinates (n = 41 from 20 female territories and 18 male territories). A 15-min segment was randomly selected from each 60-min recording for analysis. Using video data, we created a topographic map of each territory and traced the swimming tracks of focal individuals over the 15-min observation period. The home range of some individuals could not be measured if they moved beyond the camera frame. Behavioral observations were temporarily suspended when focal individuals moved out of view, and the observation period was extended accordingly to compensate for the interruption.

We recorded aggressive behaviors (frontal display or overt aggression), digging behavior, and submissive behaviors (tail-bending and down-swim) as described in other cooperatively breeding cichlids (Saeki et al., 2022; Satoh et al., 2022). Intruder species attacked by focal individuals were identified whenever possible (Ronco et al., 2020). Behavioral data of focal individuals were excluded if the total duration for which the focal individual remained outside the camera frame (i.e., the time

that would need to be compensated) exceeded one minute. As a result, behavioral data were obtained from 31 breeding females (30 with home range data) and 28 subordinates (25 with home range data). We also recorded the number of territories visits by breeding males within 15 min and their behaviors during visits. For single males, group affiliation was determined using an underwater camera centered on their primary home range or direct observation by S.S. However, detailed behavioral observations of single males were not possible due to their highly variable home ranges.

Sampling and measurements

Before sampling, we reassessed group composition at each territory using video recordings. Since breeding males do not occupy specific territories, we identified them based on video data showing their territory visits. Based on initial group composition, we attempted to capture as many individuals as possible using gill nets and hand nets, aided by 30% clove oil diluted in 70% ethanol. Captured fish were transported to the laboratory at the field station, anesthetized, and euthanized using an overdose of FA100 anesthetic (10% eugenol solution, Tanabe Seiyaku Inc., Japan). We measured body size (SL to the nearest 0.1 mm; wet body weight [BW] to the nearest 0.001 g), dissected specimens, and weighed gonads (GW to the nearest 0.00001 g). Pectoral fins were preserved in anhydrous ethanol for DNA analysis. Juveniles were not sexed, as gonads were undetectable upon dissection.

One juvenile (ID: Nbusc22_38) was identified as an outlier in the kinship analysis (see Supplementary Information S1). At the time of the initial measurements, we identified this juvenile as *N. buescheri*; however, based on the small size of the individual (6.64 mm SL) and the results from kinship analysis, we later determined that this identification was incorrect. Given that a *J. ornatus* breeding nest was near the *N. buescheri* territory, this individual may have mingled with the wrong territory. Consequently, this juvenile was excluded from further analyses.

Although 26 breeding males were identified in the video recordings, only 12 were successfully captured. Similarly, while 41 subordinates and 24 juveniles were observed, we managed to capture only 31 and 13, respectively. Among the 36 breeding females identified, one was not captured. In contrast, all single males ($n = 10$) were successfully collected. For individuals that were not captured, we estimated their approximate size from video recordings and assigned their social

rank based on body size and behavior. However, these individuals were excluded from the body size composition analysis. Additionally, gonad weights could not be determined for nine males and 10 females due to dissection failures.

Isolation of SNPs from Genome-Wide Amplicon Sequences and calculation of Kinship

The kinship structure of all captured fish was determined using SNPs obtained from genome-wide amplicon sequencing (GRAS-Di). DNA was extracted using the Wizard® genomic DNA purification kit (Promega Corporation, Madison, USA), and approximately 100 ng of DNA was used to construct the sequencing library following the method of Furukawa et al. (2024). Sequence quality was assessed with FastQC (Chen et al., 2018), and quality-controlled reads were mapped to the *N. buescheri* genome (JEI5.scf; GCA_015107095.1) (Ronco et al., 2021) using BWA-mem2 and SAMtools. SNP calling was performed with refmap.pl in Stacks 2.65 (Catchen et al., 2013). The resulting VCF files were converted to BED format and filtered in PLINK 1.9 using the following criteria: $\text{geno} \leq 0.05$, $\text{MAF} \geq 0.05$, and $\text{HWE} \leq 0.00001$. Kinship was calculated using KING (Manichaikul et al., 2010). Since the reference genome is scaffold-based and KING supports a limited number of chromosomes, the chromosome number in the BAM file was set to one before analysis. Kinship analysis was conducted under four settings: without degree, degree1, degree2, and degree3. Kinship, social status, sex, territory ID, harem ID, and SL were visualized using a custom Python script, visualize_degree_SL.py (available at GitHub: https://github.com/Masaya0606/Neolamprologus_buescheri_kinship), which graphically represents kinship relationships along with territory ID, harem ID, and SL as an indicator of body size.

The "mean kinship" values in our results are based on the kinship coefficients estimated using the KING algorithm, which differ from Sewall Wright's coefficient of relationship (r) (Manichaikul et al., 2010; Doug & David, 2015). Specifically, KING's kinship coefficient (θ) reflects the probability of two individuals sharing an allele identical-by-descent (IBD) and ranges from 0.5 (self) to 0 (unrelated), with values for parent-offspring or full siblings being ~ 0.25 . In contrast, Wright's r equals 2θ , making KING's estimates systematically half of r . This distinction explains the relatively low mean kinship values.

Heterozygosity (H_e and H_o) and inbreeding coefficient (F) were estimated from the filtered vcf file using VCFtools. The observed (H_o) and expected (H_e) heterozygosity were calculated using the results of the heterozygosity in VCFtool. $H_o = (N_sites - O_HOM)/N_sites$, $H_e = (N_sites - E_HOM)/N_sites$, where N_sites represents the total number of SNP sites, O_HOM is the observed heterozygosity site, and E_HOM is the expected heterozygosity site. To compare the heterozygosity and inbreeding coefficient (F) from another cooperative breeding species, the sympatric *Julidochromis ornatus* was used ($n = 10$ individuals). These individuals were captured using hand nets at the same time in the same study area. The reference genome of *J.ornatus* (GCA_015104695.1) (Ronco et al., 2021) was used, and SNPs were isolated following the above protocols. Heterozygosity (H_o and H_e) and inbreeding coefficient (F) were calculated for *J. ornatus*. This comparison was conducted to assess whether the levels of genetic diversity and inbreeding observed in *N. buescheri* are unique to this species or indicative of broader patterns shared among sympatric cooperative breeders. *Julidochromis ornatus* was chosen as a reference species to establish a baseline for interpreting species-specific patterns of genetic variation within a shared similar ecological framework.

Statistical analysis

All statistical analyses were conducted using R software v 4.3.3 (R Core Team, 2023) with a two-tailed significance threshold of $\alpha = 0.05$. Sample sizes varied between analyses, as not all parameters were measured for every group member. Random effects (year, male territory ID, and female territory ID) were excluded because preliminary analyses indicated their variance was nearly zero in generalized linear mixed models. Consequently, linear models (LMs) were used for continuous data, while negative binomial generalized linear models (GLMs) were applied to count data using the lme4 and MASS packages (Bates et al., 2011). The data used in statistical analyses are available in Supplementary Information S1–S3.

Group structure, body size, and testicle and ovary weight

Differences in body size [SL (mm) and BW (g)] among social ranks were analyzed using LMs, with

body size as the response variable and social rank (breeding males, breeding females, subordinates, single males, and juveniles) as the explanatory variable.

To examine differences in gonad weight among social ranks, LMs were constructed. Specifically, testicle weight was analyzed using a log-transformed response variable, with social rank (breeding males, male subordinates, and single males) and BW as explanatory variables. Ovarian weight was analyzed similarly for females (breeding females and female subordinates).

Additionally, the relationship between the number of subordinates in a breeding territory and the body size (SL or BW) of breeding females was tested using negative binomial GLMs.

Kin structure of the group members

In cooperatively breeding cichlids, substantial interspecific variation exists in kinship between subordinates and dominant breeders (Awata et al., 2005; Dierkes et al., 2005; Tanaka et al., 2015; Josi et al., 2021; Saeki et al., 2022). Consequently, kinship, a measure of pairwise genetic relatedness between individuals, was analyzed based on their social structure. The relationships between individuals were categorized as (i) subgroup members—those sharing the same female territory, (ii) group members—individuals belonging to different female territories within the same male territory, where the same breeding males visited their territories, and (iii) outgroup members—individuals from entirely separate groups. These classifications were incorporated into the LMs to assess their differences. However, behavioral observations and sampling were conducted over two years. Individuals could have immigrated during the interval between field observations. Therefore, the analysis of the total number of individuals collected in the same year was limited to those collected that year, and kinship between individuals from different years was excluded from the round-robin analysis using LMs. Additionally, analyses of kinship degrees between juveniles within the same group and between juveniles and single males were not conducted due to the limited sample size. Since this analysis is exploratory, no adjustments were applied to the p-value. Note that all statistical information was shown in supplementary information S4.

Social behaviors and effects of subordinates on parental care

The difference in home range size (cm²) and the frequency of submissive behaviors during the 15-minute behavioral observation between breeding females and subordinates were tested using LM and negative binomial GLM, respectively.

To assess whether subordinates reduce the parental care costs of breeding females, we conducted an analysis using negative binomial GLM. If subordinates lower parental care costs for dominant individuals, as seen in other cooperative breeding species, the number of subordinates is expected to be negatively correlated with the frequency of attacks by breeding females. The frequency of aggressive behaviors against heterospecific intruders was used as the response variable. The number of subordinates in the territory, social rank (breeding female or subordinate), and the presence or absence of juveniles in the territory were included as explanatory variables. Since digging behavior was observed only once in this study (see Results), no statistical analysis was performed.

Genetic diversity comparisons with other cooperative breeding cichlids

Differences in genetic diversity (H_o and H_e) and inbreeding coefficient (F) between *N. buescheri* and *J. ornatus* were examined using LM under a Gaussian distribution assumption. Since the sample sizes differed, a bootstrap resampling approach was applied 100 times to match the smaller sample size of *J. ornatus* (10 individuals). The median p-values with a 95% confidence interval (CI) were reported.

Results

Group composition, body size, and gonad weight of the group members

Twenty-six breeding males of *N. buescheri* were identified in this study. These males visited multiple female territories, suggesting a harem polygamous mating system. However, the precise number of breeding groups of females within their home range remains undetermined, as their behavioral observations were not conducted.

Thirty-six breeding females, each with a nonoverlapping home range, were observed in the breeding groups. Additionally, 41 subordinates were present in these groups, with home ranges overlapping those of breeding females (Fig. 1). The number of subordinates per female territory ranged from zero to four (median = 1, $n = 36$ female territories), and 41.2% ($n = 15$) of all groups had

no subordinates. Among the captured subordinates, 51.6% (n = 16) were female, and 48.4% (n = 15) were male.

SL differed significantly among breeding males, breeding females, subordinates, juveniles, and single males (LM, $F_1 = 85.078$, $p < 0.001$) (Fig. 2). Breeding males had consistently greater SL (59.60–72.45 mm; mean $\pm$ SD = 66.81 ± 4.14 mm, n = 12) than breeding females (33.95–53.75 mm; 48.29 ± 4.85 mm, n = 35 from 25 male territories), subordinates (20.91–41.40 mm; 30.24 ± 5.31 mm, n = 31 from 17 female territories and 13 male territories), and juveniles (9.20–18.18 mm; 11.42 ± 2.62 mm, n = 13 from 8 female territories and 7 male territories). Single males showed greater variation in body size (33.85–57.12 mm; 46.24 ± 8.56 mm, n = 10 from 7 groups). A similar pattern was observed in BW (LM, $F_1 = 180.73$, $p < 0.001$).

There was a significant correlation between the number of subordinates and body size of breeding females (SL: negative binomial GLM, $\chi^2_1 = 7.0161$, $p = 0.008$; BW: $\chi^2_1 = 9.307$, $p = 0.002$, n = 35 from 25 male territories).

Log-transformed testicle weight was significantly affected by social rank (breeding males, single males, and subordinates) ($\chi^2_2 = 16.870$, $p < 0.001$) and BW ($\chi^2_1 = 8.885$, $p = 0.006$). Similarly, ovary weight was significantly affected by social rank (breeding females and subordinates) ($\chi^2_1 = 36.841$, $p < 0.001$) and BW ($\chi^2_1 = 7.4529$, $p = 0.009$).

Kin structure of the group members

The mean kinship (KING's kinship coefficient (θ)) between breeding males and subordinates within the same breeding male territories (group members) was 0.096 ± 0.104 (n = 18), significantly higher than that between breeding males and subordinates from different male territories (0.018 ± 0.063 , n = 187) (Fig. 3a). This pattern resembled the kinship between breeding males and juveniles but did not extend to associations with single males (Fig. 3a). The kinship between breeding females and subordinates within the same female territories (subgroup members) (0.180 ± 0.053 , n = 28) was higher than that among individuals within the same male territory but from different female territories (0.029 ± 0.066 , n = 33) and those outside the male territory (0.003 ± 0.044 , n = 489) (Fig. 3b). Similarly, the kinship between breeding females and subordinates was higher than that of individuals who neither shared a

female territory nor belonged to the same male territory (Fig. 3b). This gradient pattern was also observed in the kinship between breeding females and juveniles (Fig. 3b), among subordinates (Fig. 3c), and between subordinates and juveniles (Fig. 3c). Consistent with these findings, juveniles within the same female territory had higher kinship than those who neither shared a female territory nor belonged to the same group (Fig. 3d). Additionally, kinship between single males was higher within the same male territory than among those outside the territory (Fig. 3e).

Behaviors of the group members

Breeding females and subordinates typically shared shelters. The home range of breeding females ($n = 30$ from 21 groups) ranged from 1,037.17 to 5,731.59 cm² ($2,702.37 \pm 1,236.02$ cm²), while that of subordinates ($n = 25$ from 19 female territories and 16 male territories) ranged from 239.45 to 3,592.28 cm² ($1,195.78 \pm 750.39$ cm²). The home range of breeding females was significantly larger than that of subordinates (LM, $F_1 = 27.382$, $p < 0.001$).

During the 15-min observation period, breeding females exhibited 0–3 submissive behaviors (median = 1, $n = 31$ from 21 male territories). Of these, 96.4% ($n = 27$) were directed toward breeding males, while 3.6% ($n = 1$) were directed toward subordinates in the same territory. Subordinates exhibited 0–6 submissive behaviors (median = 1, $n = 28$ from 19 female territories and 16 male territories), with 61% ($n = 21$) directed toward breeding females, 29.4% ($n = 10$) toward breeding males, and 8.8% ($n = 3$) toward other subordinates in the same territory. The frequency of submissive behaviors did not significantly differ between breeding female and subordinate (negative binomial GLM, $\chi^2_1 = 0.909$, $p = 0.340$).

Aggressive behaviors toward conspecifics were observed in six breeding females, but not in subordinates. Three of the breeding females attacked a conspecific intruder that had entered their territory once during the 15-minute observation period. The other three breeding females directed aggression toward subordinates within the same subgroup. Additionally, one instance of aggressive interaction between juveniles was recorded on video; however, the exact frequency of such interactions remains unknown, as juveniles spend most of their time within shelters.

Breeding males rarely visited territories, with visit frequencies ranging from 0 to 6 per

territory during the 15-minute period (median = 2, n = 31 female territories from 21 male territories). During visits, they swam around breeding females, entered shelters, and displayed aggression toward heterospecific intruders near the focal territory six times. The breakdown of aggressive interactions by breeding males was as follows: *Lepidolamprologus elongatus* [Boulenger, 1898] (n = 2), *Gnathochromis pfefferi* [Boulenger, 1898] (n = 1), *Synodontis* sp. (n = 2), and a school of juvenile *Cyprichromis* sp. (n = 1).

Effects of the number of subordinates

Both breeding females and subordinates actively participated in territorial and brood defense (Fig. 4). The frequency of aggression toward heterospecific intruders was significantly influenced by the number of subordinates and social rank, with a marginal effect from the presence of juveniles (Table 1). Breeding females exhibited higher aggression than subordinates (breeding females: range = 0–14, median = 4 attacks; subordinates: range = 0–6, median = 1 attack per 15 minutes). However, aggression in both declined as the number of subordinates increased (Fig. 4). Breeding females attacked 17 intruder species, while subordinates attacked 10 (Table 2). The relative contribution to aggressive defense against potential intruders—calculated as the number of aggressive defense behaviors directed at each intruder by either breeding females or subordinates, divided by the total number of aggressive defense behaviors by both classes—was greater in breeding females (Fig. 4; Table 2). Nevertheless, subordinates also proactively engaged in aggressive defense, particularly against intruders such as *J. ornatus* and juvenile *Cyprichromis* sp. (Table 2).

Sand-digging behavior, a known indicator of cooperative behavior in cichlids, was rarely observed. Only one subordinate exhibited digging behavior during field observations during the 15-min behavioral observation.

Genetic diversity in N. buescheri

Kinship analyses revealed a high degree of relatedness among individuals within the territory. However, the genetic diversity of this species, represented by the heterozygosity measures (observed heterozygosity *Ho* and estimated heterozygosity *He*), was significantly higher than that of the other

cooperative breeding species, *J. ornatus* (Fig. 5), which was obtained from the same population in this study. Bootstrap resampling was performed 100 times, and the median p-values with a 95% CI were reported (*He*: median 3.63×10^{-24} , CI: 4.24×10^{-27} – 7.87×10^{-22} , *Ho*: median 4.27×10^{-9} , CI: 7.57×10^{-11} – 9.63×10^{-4}). The inbreeding coefficient (*F*) of *N. buescheri* was also significantly lower than that of *J. ornatus* (Fig. 5c, F-values median 74.5, CI 7.23–112.1, p-values median 1.93×10^{-9} , CI: 9.71×10^{-11} – 1.64×10^{-3}). Although the kinship among members of the male and female territory was high (Fig. 3; Supplementary information S5), the mechanisms to maintain genetic diversity are likely present.

Discussion

This study reveals that in *N. buescheri*, breeding females allow subordinates to remain within the breeding territory. Furthermore, subordinates actively participate in territorial defense by exhibiting aggression toward intruders. The number of subordinates was negatively correlated with the frequency of aggression exhibited by female breeders toward intruders, supporting the hypothesis that subordinate helping behaviors may reduce the workload of breeding females during territorial defense and parental care, benefiting them by conserving energy (Tanaka et al., 2018c, 2018a). However, our observations do not rule out an alternative explanation—namely, that larger female breeders may have both more subordinates and a territory with lower territorial defense costs. In this scenario, the observed correlation between the number of subordinates and aggression frequency may be spurious. Experimental manipulations, such as subordinate removal, would be necessary to rigorously assess these possibilities (Zöttl et al., 2013b; Tanaka et al., 2018c).

In contrast to territorial defense in this species, subordinates did not engage in territory maintenance behaviors, such as digging from the shelter, which is considered an indicator of helping behavior in other cooperatively breeding cichlids (Sato et al., 2022). However, breeding females also did not exhibit these behaviors, and there was not much sand in the territory (supplementary S6), suggesting that territories in this species may require minimal maintenance rather than indicating a lack of subordinate involvement. In the substrate-brooding cichlids *N. obscurus* and *N. bifasciatus* [Büscher, 1993], closely related cooperative breeders of *N. buescheri* (Ronco et al., 2021), subordinates are exclusively engaged in nest maintenance as well as territory defense (Tanaka et al.,

2015, 2018a; Satoh et al., 2022). This may represent evolutionary flexibility in task division among cooperatively breeding cichlids. Indeed, such behavioral flexibility has been experimentally demonstrated in other cooperative breeding cichlids (Bruitjes & Taborsky, 2011).

In examining the evolutionary dynamics of cooperative breeding, relatedness between dominant and subordinate individuals is recognized as a crucial factor influencing various behavioral and life-history traits (Kingma 2017; Riehl, 2017; Zöttl et al. 2023; but see Quinones et al. 2016). Indeed, there are substantial interspecific variations in the degree of relatedness between dominant breeders and subordinates across numerous cooperatively breeding vertebrates (Koenig & Dickinson, 2004; Riehl, 2013), including cichlid fish (Awata et al., 2005; Tanaka et al., 2018c; Josi et al., 2021; Saeki et al., 2022). For instance, in *J. ornatus*, subordinates and dominants exhibit minimal genetic relatedness (Awata et al., 2005), whereas in *N. obscurus* and *N. meeli* [Poll, 1948], they are highly likely to be closely related (Saeki et al., 2022). Such variation in relatedness suggests that evolutionary pressures shaping cooperative breeding systems may differ significantly among species. Notably, variations in relatedness across many vertebrates, including cichlid fish, can potentially affect reproductive skew (Awata et al., 2005; Heg et al., 2008; Riehl, 2017) and the extent of helping behavior (Reyer, 1984; Kingma, 2017; Zöttl et al., 2023).

In the present study, we found that subordinates are genetically related to both breeding males and females. Given the body size differences between subordinates and breeding females, it is reasonable to infer that subordinates are predominantly the offspring of breeding females. Interestingly, our kinship analysis also showed that relatedness between females and smaller individuals (juveniles or helpers) is relatively high. This finding suggests that juveniles in this species delay dispersal from the natal nest and assume helper roles, a phenomenon well-documented in other cooperatively breeding cichlids, such as *N. pulcher* (Dierkes et al., 2005; Jungwirth et al., 2023), *N. savoryi* (Josi et al., 2021), *N. obscurus* (Tanaka et al., 2015), and *N. meeli* (Saeki et al., 2022). In contrast, we found that the kinship between females and juveniles is not higher than between females and subordinates. This finding is particularly noteworthy in light of previous reports documenting breeder replacement and helper immigration in other cooperatively breeding cichlid species (Dierkes et al., 2005). Despite superficial resemblances to other cooperative breeders, this species may possess

a relatively rigid social organization, with limited individual mobility or social turnover.

This species inhabits deep-water rock crevices (Konings, 1998), where suitable nesting sites may be limited. Indeed, when breeding females were captured and removed from their territories during sampling, other female individuals promptly emerged and began defending the territory, exhibiting behavior characteristic of former breeding females (Satoh, personal observation). This suggests that competition for access to breeding territories is intense, potentially driving delayed dispersal among subordinates. By remaining in their natal territory, subordinates may gain fitness benefits, such as priority access to shelter and food resources, particularly in a saturated environment with limited dispersal opportunities. Simultaneously, dominant individuals may reduce the energetic costs of territorial defense with the help of subordinates (Jungwirth et al., 2023).

Moreover, kinship variance between subordinates and breeding males was substantially greater than that observed with breeding females, a pattern reported in other cooperatively breeding cichlids (Dierkes et al., 2005; Josi et al., 2021; Saeki et al., 2022). This discrepancy may result from greater variability in harem social structures compared with the relatively stable female group (= subgroup) composition within breeding groups (namely, higher replacement rate of breeder males), sex-specific dispersal dynamics, or reproductive parasitism (Taborsky, 2016). Additionally, in *N. multifasciatus* [Boulenger, 1906], a species recognized as a cooperative breeder or a family-living species (Schradin & Lamprecht, 2002; Bose et al., 2022), multiple sexually mature males coexist within the same breeding group. If *N. buescheri* exhibits a similar social structure, relatedness between breeding males and subordinates (or juveniles) within the same group would be lower, similar to cases where harem structures are unstable. Since this study did not allow for detailed behavioral observations of breeding males, further research is necessary to elucidate *N. buescheri*'s social structure, particularly the role of breeding males. Notably, we documented an instance in which a breeding male and another male (with sex inferred from body size) swam side by side near a breeding female's territory (Supplementary Information S6). However, this qualitative observation alone is insufficient to determine whether multiple breeding males exist within a single group or whether it reflects heightened intramale conflict and harem instability in this species.

We found that kinship between breeding females and subordinates and among subordinates

within the same male territory was higher than that between different territories. This tendency was also observed among juveniles and single males. These findings suggest that this species forms gradual kin-based social groups, regardless of subordinates. This kin structure pattern is akin to the case reported by *N. pulcher* (Jungwirth et al., 2023). However, it remains unclear whether these kin-based social groups, extending beyond the breeding group (female group), arise from dispersal patterns and spatial distribution or from nepotism by dominant breeders. Additionally, our kinship analysis using KING revealed that, unlike *J. ornatus*, the *N. buescheri* population exhibits a high frequency of kin relationships (many individuals have kinship within degree 3; Supplementary Information S5). In contrast, *N. buescheri* exhibited higher heterozygosity (H_e and H_o) and a lower inbreeding coefficient (F) than *J. ornatus*. Studies in other populations have shown that kinship between dominant breeders and subordinates in *J. ornatus* is remarkably low (Awata et al., 2005), a trend also observed in this population (M.M., personal observation). Given the social structure and kinship patterns in these two cooperatively breeding cichlids, one would expect reduced genetic diversity in *N. buescheri*. However, contrary to this expectation, the opposite trend was observed. Although the exact mechanisms remain unclear, potential factors such as occasional extragroup mating or periodic dispersal events may help maintain genetic diversity, warranting further investigation.

Conclusion

Although *N. buescheri* has been proposed as a cooperative breeder based on anecdotal reports and aquarium observations (Heg & Bachar, 2006), detailed behavioral studies in natural environments are scarce due to its life-history traits, as it inhabits deep-water rock crevices. In this study, subordinates genetically related to dominant breeders actively participated in territorial and brood defense against heterospecific intruders, potentially reducing the parental care burden of breeding females. These findings provide strong evidence that *N. buescheri* operates within a kin-structured cooperative breeding system. As a key model for studying social evolution beyond mammals and birds, the cichlid fishes of Lake Tanganyika have attracted significant attention from evolutionary biologists. Therefore, understanding the social structure and life-history traits of cooperatively breeding cichlids in their

natural habitats is crucial for advancing knowledge of cooperative societies in vertebrates.

Funding This work was financially supported by the Hakubi Project of Kyoto University and the KAKENHI program sponsored by the Japan Society for the Promotion of Science (grant numbers No. 23KK0131, 23H03868, 21K06346 to S.S, and 21H05304, 23H03868 to M.M.).

Data availability The raw data supporting the findings of this study are available in the Supplementary Materials.

Statements and Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This study was conducted with permission from the Department of Fisheries of the Republic of Zambia. All animal procedures were followed by the Animal Care and Use Committee of the Kyoto University.

References

- Awata, S., H. Munehara, & M. Kohda, 2005. Social system and reproduction of helpers in a cooperatively breeding cichlid fish (*Julidochromis ornatus*) in Lake Tanganyika: field observations and parentage analyses. *Behavioral Ecology and Sociobiology* 58: 506–516. <https://doi.org/10.1007/s00265-005-0934-6>.
- Balshine, S., B. Leach, F. Neat, H. Reid, M. Taborsky, & N. Werner, 2001. Correlates of group size in a cooperatively breeding cichlid fish (*Neolamprologus pulcher*). *Behavioral Ecology and Sociobiology* 50: 134–140. <https://doi.org/10.1007/s002650100343>.
- Bates, D., M. Maechler, & B. Bolker, 2011. lme4: linear mixed-effects models using Eigen and R syntax. R package version 0.999375-39.
- Bergmüller, R., & M. Taborsky, 2005. Experimental manipulation of helping in a cooperative breeder: helpers 'pay to stay' by pre-emptive appeasement. *Elsevier. Animal Behaviour* 69: 19–28.
- Bose, A. P. H., J. Dabernig-Heinz, L. Koch, J. Grimm, S. Lang, B. Hegedűs, T. Banda, L. Makasa, A. Jordan, & K. M. Sefc, 2022. Parentage analysis across age cohorts reveals sex differences in reproductive skew in a group-living cichlid fish, *Neolamprologus multifasciatus*. *Molecular Ecology* 31: 2418–2434. <https://doi.org/10.1111/mec.16401>.
- Brouwer, L., D. Heg, & M. Taborsky, 2005. Experimental evidence for helper effects in a cooperatively breeding cichlid. Oxford University Press. *Behavioral Ecology* 16: 667–673.
- Bruintjes, R., & M. Taborsky, 2011. Size-dependent task specialization in a cooperative cichlid in response to experimental variation of demand. *Animal Behaviour* 81: 387–394. <https://doi.org/10.1016/j.anbehav.2010.10.004>.
- Catchen, J., P. A. Hohenlohe, S. Bassham, A. Amores, & W. A. Cresko, 2013. Stacks: an analysis tool set for population genomics. *Molecular Ecology* 22: 3124–3140. <https://doi.org/10.1111/mec.12354>.
- Dey, C. J., C. M. O'Connor, H. Wilkinson, S. Shultz, S. Balshine, & J. L. Fitzpatrick, 2017. Direct benefits and evolutionary transitions to complex societies. Nature Publishing Group UK London. *Nature Ecology & Evolution* 1: 0137.
- Dierkes, P., D. Heg, M. Taborsky, E. Skubic, & R. Achmann, 2005. Genetic relatedness in groups is sex-specific and declines with age of helpers in a cooperatively breeding cichlid. *Ecology Letters* 8: 968–975. <https://doi.org/10.1111/j.1461-0248.2005.00801.x>.
- Doug, S., & B. David, 2015. Relatedness in the post-genomic era: is it still useful? *Nature reviews genetics* 16: 33–44.
- Frommen, J. G., & S. Fischer, 2021. Proximate and Ultimate Mechanisms of Cooperation in Fishes. In *The*

Cambridge Handbook of Animal Cognition. pp. 272–294.

Heg, D., & Z. Bachar, 2006. Cooperative Breeding in the Lake Tanganyika Cichlid *Julidochromis ornatus*. *Environmental Biology of Fishes* 76: 265–281. <https://doi.org/10.1007/s10641-006-9032-5>.

Heg, D., E. Jutzeler, D. Bonfils, & J. S. Mitchell, 2008. Group composition affects male reproductive partitioning in a cooperatively breeding cichlid. *Molecular Ecology* 17: 4359–4370. <https://doi.org/10.1111/j.1365-294X.2008.03920.x>.

Hidaka, R., S. Sogawa, M. Kohda, & S. Awata, 2024. Punishment from dominant breeders increases helping effort of subordinates in a cooperatively breeding cichlid. *Elsevier. Animal Behaviour* 211: 99–109.

Jennions, M. D., & D. W. Macdonald, 1994. Cooperative breeding in mammals. *Trends in Ecology & Evolution* 9: 89–93. [https://doi.org/10.1016/0169-5347\(94\)90202-X](https://doi.org/10.1016/0169-5347(94)90202-X).

Josi, D., D. Heg, T. Takeyama, D. Bonfils, D. A. Konovalov, J. G. Frommen, M. Kohda, & M. Taborsky, 2021. Age- and sex-dependent variation in relatedness corresponds to reproductive skew, territory inheritance, and workload in cooperatively breeding cichlids. *Blackwell Publishing Inc Malden, USA. Evolution* 75: 2881–2897.

Josi, D., M. Taborsky, & J. G. Frommen, 2019. First field evidence for alloparental egg care in cooperatively breeding fish. *Ethology* 125: 164–169. <https://doi.org/10.1111/eth.12838>.

Josi, D., M. Taborsky, & J. G. Frommen, 2020. Investment of group members is contingent on helper number and the presence of young in a cooperative breeder. *Elsevier. Animal behaviour* 160: 35–42.

Jungwirth, A., & M. Taborsky, 2015. First- and second-order sociality determine survival and reproduction in cooperative cichlids. *Proceedings of the Royal Society B: Biological Sciences* 282: 20151971. <https://doi.org/10.1098/rspb.2015.1971>.

Jungwirth, A., M. Zöttl, D. Bonfils, D. Josi, J. G. Frommen, & M. Taborsky, 2023. Philopatry yields higher fitness than dispersal in a cooperative breeder with sex-specific life history trajectories. *Science Advances* 9: eadd2146. <https://doi.org/10.1126/sciadv.add2146>.

Kingma, S. A., 2017. Direct benefits explain interspecific variation in helping behaviour among cooperatively breeding birds. *Nature Publishing Group UK London. Nature Communications* 8: 1094.

Koenig, W. D., & J. L. Dickinson, 2004. *Ecology and Evolution of Cooperative Breeding in Birds*. Cambridge University Press.

Konings, A., 1998. *Tanganyika Cichlids in Their Natural Habitat*. Cichlid Press, Miami.

Manichaikul, A., J. C. Mychaleckyj, S. S. Rich, K. Daly, M. Sale, & W.-M. Chen, 2010. Robust relationship

inference in genome-wide association studies. Oxford University Press. *Bioinformatics* 26: 2867–2873.

Martin, E., & M. Taborsky, 1997. Alternative male mating tactics in a cichlid, *Pelvicachromis pulcher* : a comparison of reproductive effort and success. *Behavioral Ecology and Sociobiology* 41: 311–319. <https://doi.org/10.1007/s002650050391>.

Naef, J., & M. Taborsky, 2020. Commodity-specific punishment for experimentally induced defection in cooperatively breeding fish. *Royal Society Open Science* 7: 191808. <https://doi.org/10.1098/rsos.191808>.

R Core Team, 2023. R: A language and environment for statistical computing. R Foundation for Statistical Computing.

Reyer, H.-U., 1984. Investment and relatedness: a cost/benefit analysis of breeding and helping in the pied kingfisher (*Ceryle rudis*). Elsevier. *Animal Behaviour* 32: 1163–1178.

Riehl, C., 2013. Evolutionary routes to non-kin cooperative breeding in birds. *Royal Society. Proceedings of the Royal Society B: Biological Sciences* 280: 20132245. <https://doi.org/10.1098/rspb.2013.2245>.

Riehl, C., 2017. Kinship and Incest Avoidance Drive Patterns of Reproductive Skew in Cooperatively Breeding Birds. The University of Chicago Press. *The American Naturalist* 190: 774–785. <https://doi.org/10.1086/694411>.

Ronco, F., H. H. Büscher, A. Indermaur, & W. Salzburger, 2020. The taxonomic diversity of the cichlid fish fauna of ancient Lake Tanganyika, East Africa. Elsevier. *Journal of Great Lakes Research* 46: 1067–1078.

Ronco, F., M. Matschiner, A. Böhne, A. Boila, H. H. Büscher, A. El Taher, A. Indermaur, M. Malinsky, V. Ricci, & A. Kahmen, 2021. Drivers and dynamics of a massive adaptive radiation in cichlid fishes. Nature Publishing Group UK London. *Nature* 589: 76–81.

Saeki, T., S. Satoh, J. G. Frommen, M. Kohda, & S. Awata, 2022. Kin-structured cooperatively breeding groups due to limited dispersal in the obligate shell-brooding cichlid *Neolamprologus meeli*. *Behavioral Ecology and Sociobiology* 76: 89. <https://doi.org/10.1007/s00265-022-03201-w>.

Satoh, S., T. Saeki, M. Kohda, & S. Awata, 2022. Cooperative breeding in *Neolamprologus bifasciatus* , a cichlid fish inhabiting the deep reefs of Lake Tanganyika. *Ecology of Freshwater Fish* 31: 640–649. <https://doi.org/10.1111/eff.12658>.

Schradin, C., & J. Lamprecht, 2002. Causes of Female Emigration in the Group-Living Cichlid Fish *Neolamprologus multifasciatus*. *Ethology* 108: 237–248. <https://doi.org/10.1046/j.1439-0310.2002.00775.x>.

Solomon, N. G., & J. A. French, 1997. Cooperative Breeding in Mammals. Cambridge University Press.

Staeck, W., 1982. *Lamprologus buescheri* n. sp. from the Zambian part of Lake Tanganyika (Pisces: Cichlidae). *Senckenbergiana Biologica* 63: 325–328.

Taborsky, M., 2016. Cichlid fishes: A model for the integrative study of social behavior. In *Cooperative breeding*. Cambridge University Press, Cambridge. pp. 272–293.

Taborsky, M., 2021. Cooperative Breeding. In Shackelford, T. K., & V. A. Weekes-Shackelford (eds) *Encyclopedia of Evolutionary Psychological Science*. Springer International Publishing, Cham. pp. 1473–1476.

Taborsky, M., & D. Limberger, 1981. Helpers in fish. *Behavioral Ecology and Sociobiology* 8: 143–145. <https://doi.org/10.1007/BF00300826>.

Tanaka, H., J. G. Frommen, L. Engqvist, & M. Kohda, 2018a. Task-dependent workload adjustment of female breeders in a cooperatively breeding fish. *Oxford University Press UK. Behavioral Ecology* 29: 221–229.

Tanaka, H., J. G. Frommen, S. Koblmüller, K. M. Sefc, M. McGee, M. Kohda, S. Awata, M. Hori, & M. Taborsky, 2018b. Evolutionary transitions to cooperative societies in fishes revisited. *Ethology* 124: 777–789. <https://doi.org/10.1111/eth.12813>.

Tanaka, H., D. Heg, H. Takeshima, T. Takeyama, S. Awata, M. Nishida, & M. Kohda, 2015. Group composition, relatedness, and dispersal in the cooperatively breeding cichlid *Neolamprologus obscurus*. *Behavioral Ecology and Sociobiology* 69: 169–181. <https://doi.org/10.1007/s00265-014-1830-8>.

Tanaka, H., M. Kohda, & J. G. Frommen, 2018c. Helpers increase the reproductive success of breeders in the cooperatively breeding cichlid *Neolamprologus obscurus*. *Behavioral Ecology and Sociobiology* 72: 152. <https://doi.org/10.1007/s00265-018-2566-7>.

Witte, K.-E., & J. Schmidt, 1992. Betta brownorum, a new species of anabantoids (Teleostei: Belontiidae) from northwestern Borneo, with a key to the genus. *Ichthyological Exploration of Freshwaters* 2: 305–330.

Wong, M., & S. Balshine, 2011. The evolution of cooperative breeding in the African cichlid fish, *Neolamprologus pulcher*. *Biological Reviews* 86: 511–530. <https://doi.org/10.1111/j.1469-185X.2010.00158.x>.

Zöttl, M., S. Fischer, & M. Taborsky, 2013a. Partial brood care compensation by female breeders in response to experimental manipulation of alloparental care. *Elsevier. Animal behaviour* 85: 1471–1478.

Zöttl, M., S. Fischer, & M. Taborsky, 2013b. Partial brood care compensation by female breeders in response to experimental manipulation of alloparental care. *Animal Behaviour* 85: 1471–1478. <https://doi.org/10.1016/j.anbehav.2013.03.045>.

Zöttl, M., D. Heg, N. Chervet, & M. Taborsky, 2013c. Kinship reduces alloparental care in cooperative cichlids where helpers pay-to-stay. *Nature Publishing Group UK London. Nature communications* 4: 1341.

Zöttl, M., T. Schreier, & M. Taborsky, 2023. Coercion promotes alloparental care in cooperative breeders. *Oxford University Press UK. Behavioral Ecology* 34: 363–372.

Table and legends

Table 1. Factors affecting the frequency of aggressive behavior toward heterospecific intruders in the substrate-brooding cichlid *Neolamprologus buescheri* estimated by negative binomial GLM.

Explanatory Variables	Estimate ± SE	df	χ^2	P
Intercept	2.2055 ± 0.1683	—	—	—
Number of subordinates	-0.4858 ± 0.1086	1	21.8586	< 0.001
Social rank	—	1	8.3504	0.0038
Breeding female	Reference	—	—	—
Subordinate	-0.6701 ± 0.2350	—	—	—
Existence or absence of juveniles	—	1	3.7540	0.0526
Absence	Reference	—	—	—
Existence	-0.3670 ± 0.1897	—	—	—

Table 2. List of intruder species attacked by breeding females and subordinates of substrate-brooding cichlid *Neolamprologus buescheri*. We also showed relative contribution to aggressive defense against potential intruders by female breeders (calculated as the number of aggressive behaviors directed at each intruder by breeding females/ total number of aggressive behaviors by breeding female and subordinates).

Intruders	Breeding females (n=31)		Subordinates (n= 28)		Percentage of attacks initiated by breeding females against each intruding heterospecific
	Events	Percentage	Events	Percentage	
<i>Chalinochromis brichardi</i> [Poll, 1974]	5	4.6	1	3.0	83.3 (n = 6 events)
<i>Cyprichromis</i> sp.	8	7.4	10	30.3	44.4 (n = 18 events)
<i>Gnathochromis pfefferi</i> [Boulenger, 1898]	1	0.9	0	0.0	100 (n = 1 event)
<i>Julidochromis ornatus</i> [Boulenger, 1898]	12	11.1	6	18.2	66.6 (n = 18 events)
<i>Lamprologus lemairii</i> [Boulenger, 1899]	1	0.9	0	0.0	100 (n = 1 event)
<i>Lepidiolamprologus elongatus</i> [Boulenger, 1898]	5	4.6	2	6.1	71.4 (n = 7 events)
<i>Lobochikotes labiatus</i> [Boulenger, 1898]	4	3.7	0	0.0	100 (n = 4 events)
<i>Mastacembelus</i> sp.	6	5.6	0	0.0	100 (n = 6 events)
<i>Neolamprologus fasciatus</i> [Boulenger, 1898]	6	5.6	2	6.1	75.0 (n = 8 events)
<i>Neolamprologus mustax</i> [Poll, 1978]	3	2.8	1	3.0	75.0 (n = 4 events)
<i>Neolamprologus obscurus</i> [Poll, 1978]	1	0.9	0	0.0	100 (n = 1 event)
<i>Neolamprologus sexfasciatus</i> [Trewavas & Poll, 1952]	1	0.9	0	0.0	100 (n = 1 event)
<i>Neolamprologus</i> sp. "Kombe"	6	5.6	1	3.0	85.7 (n = 7 events)
<i>Perissodus microlepis</i> [Boulenger, 1898]	6	5.6	1	3.0	85.7 (n = 7 events)
<i>Synodontis</i> sp.	38	35.2	8	24.2	82.6 (n = 46 events)
<i>Telmatochromis vitatus</i> [Boulenger, 1898]	4	3.7	1	3.0	80.0 (n = 5 events)
<i>Xenotilapia spilopterus</i> [Poll & Stewart, 1975]	1	0.9	0	0.0	100 (n = 1 event)
unidentified species	45	—	9	—	—

Figure legends

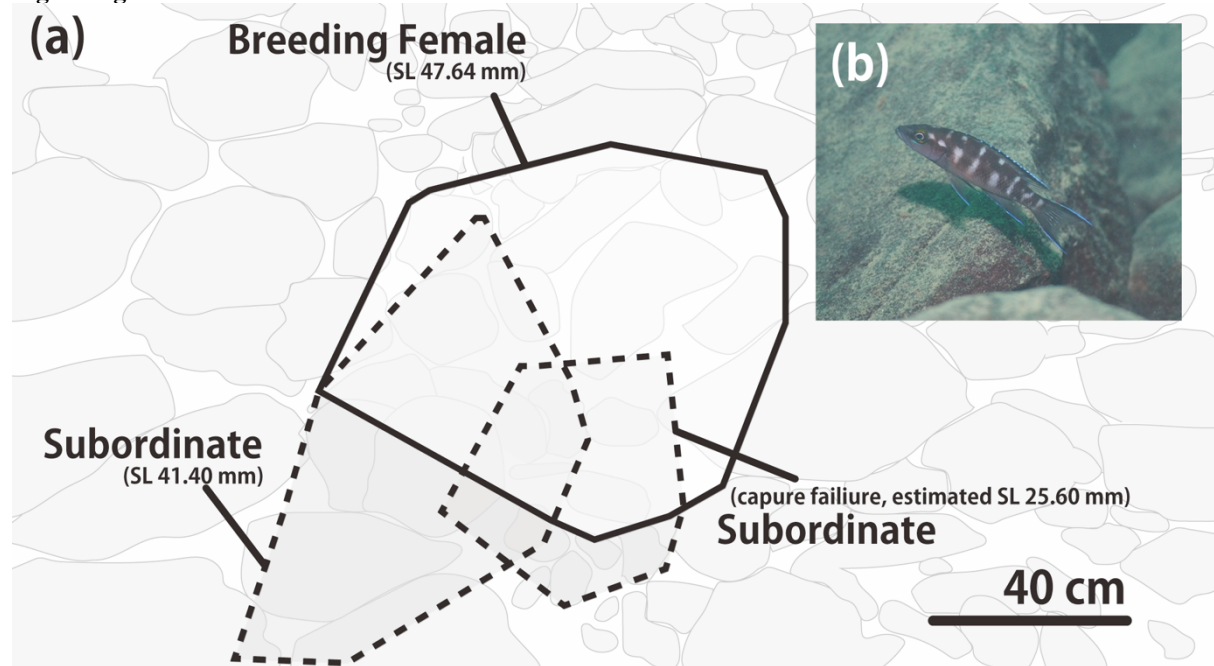


Figure 1. a) Example of home ranges in breeding female and subordinate *Neolamprologus buescheri* at the Kombe point of Lake Tanganyika. The area enclosed by a solid line indicates the home range of a breeding female, while the area enclosed by a dashed line indicates the home ranges of subordinates. b) Photograph of the breeding female in her territory.

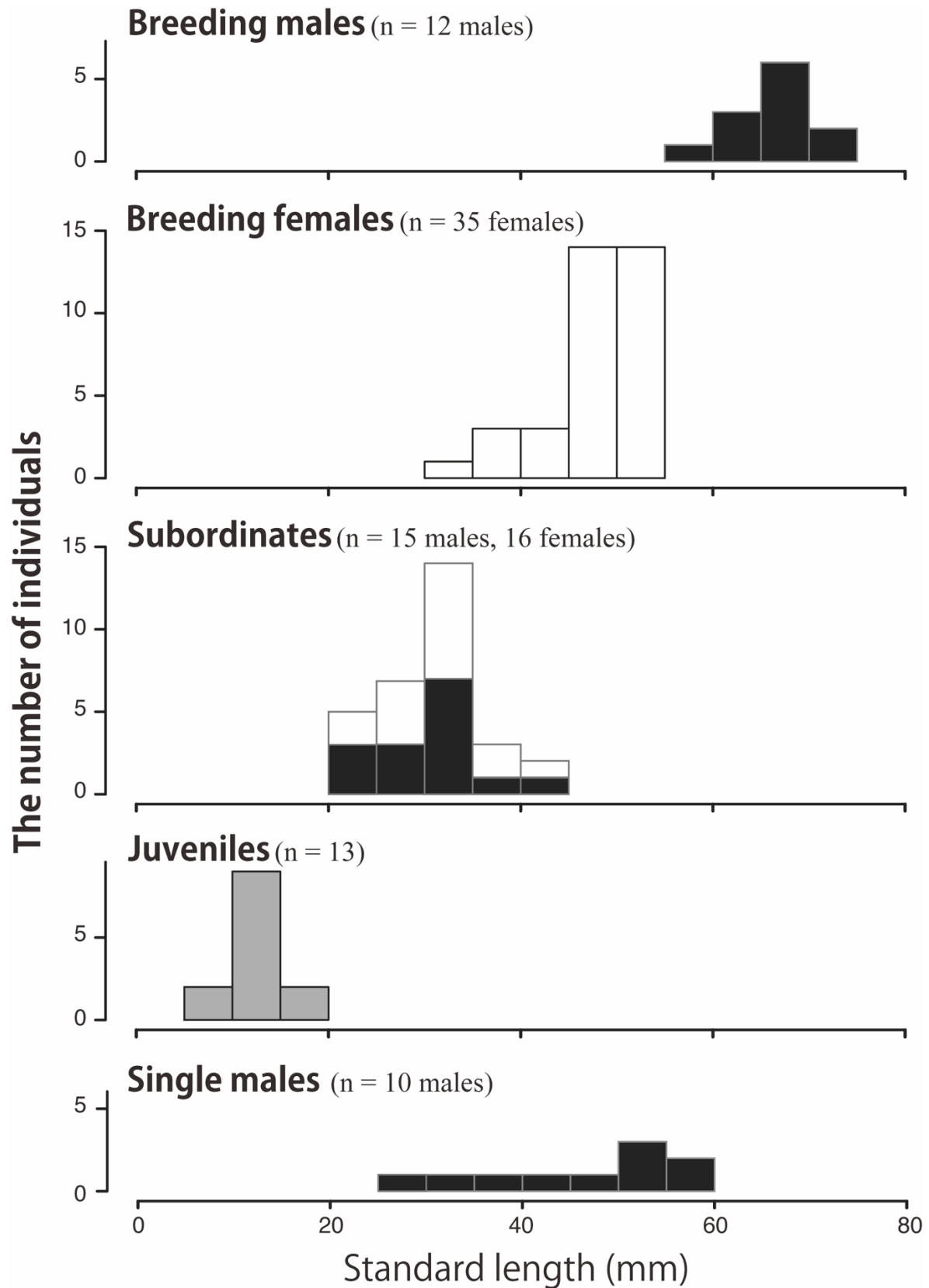


Figure 2. Body size distribution of breeding males, breeding females, subordinates, juveniles, and single males in the substrate-brooding cichlid *Neolamprologus buescheri*. Black and white bars show male and female, respectively. With regard to juveniles (smaller than 20 mm), sexes were not determined. Sample sizes are presented above the bars.

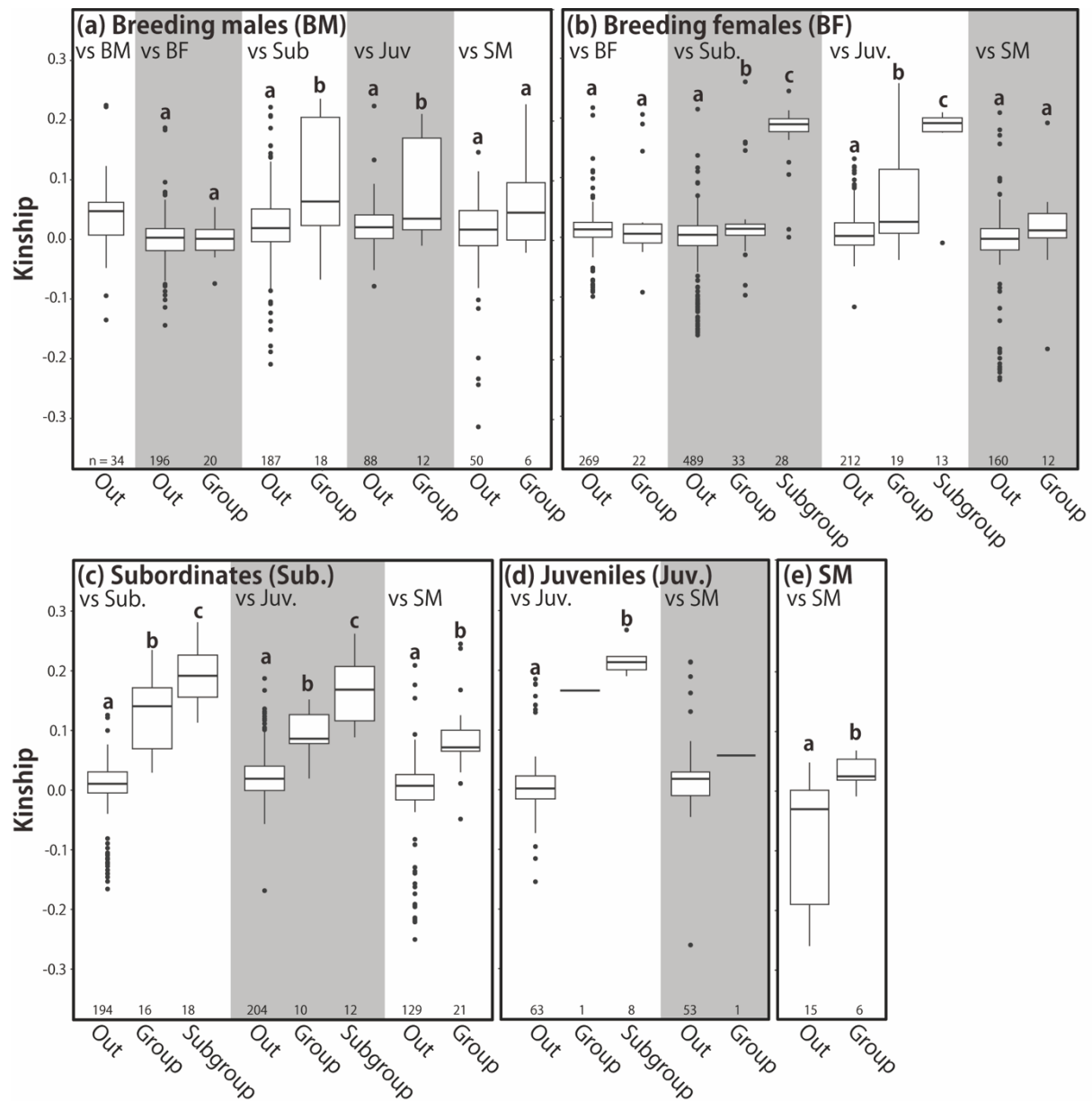


Figure 3, Kinship coefficient (θ) estimates derived from GRAS-Di for a) breeding males (BM), b) breeding females (BF), c) subordinates (sub.), d) juveniles (Juv.), and e) single males (SM) in the substrate-brooding cichlid *Neolamprologus buescheri* in relation to fish from the outgroup (out), members of different female territories within the same male territory (group), and members of the same female territories (subgroup). The KING's kinship coefficient (θ) reflects the probability of two individuals sharing an allele identical-by-descent (IBD) and ranges from 0.5 (self) to 0 (unrelated), with values for parent-offspring or full siblings being ~ 0.25 . Distinct letters indicate significant differences by LMs with sequential Bonferroni correction ($P < 0.05$). Statistical analysis was not conducted for the kinship between juveniles and SM (d) since the sample size consisted only of one individual. The data for kinship between members of different female territories within the same juvenile group were excluded from the statistical analysis, (d) as the sample size was also limited to one individual. Sample sizes are presented below the boxplots.

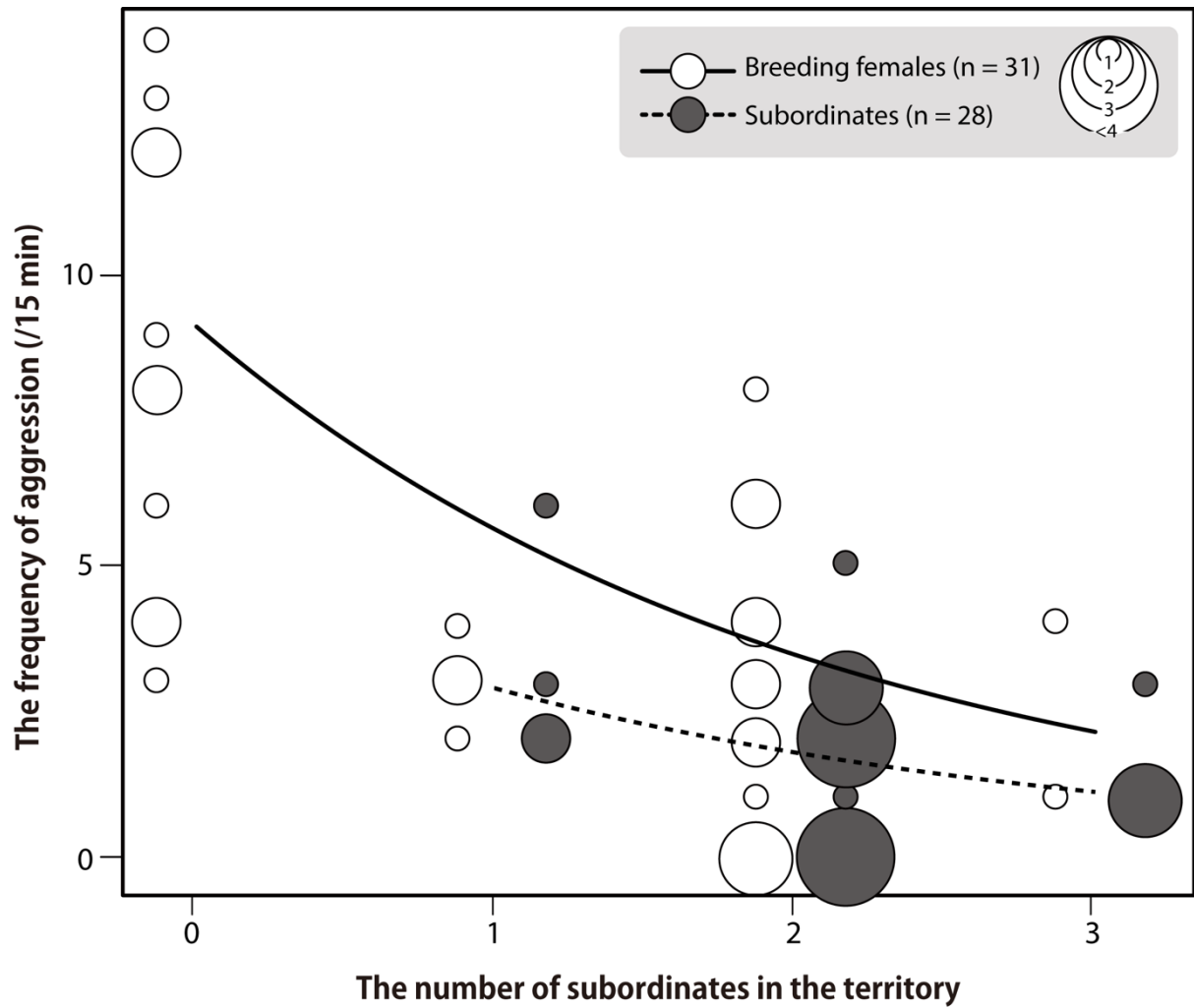


Figure 4. Relationship between the number of subordinates and per capita frequency of aggressive behavior toward heterospecific intruders in the substrate-brooding cichlid *Neolamprologus buescheri*. White plots with solid lines show breeding females, and gray plots with dashed lines show subordinates. The regression lines were estimated by negative binomial GLM (detailed statistical information shown in Table 1). The size of the circles indicates the number of individuals.

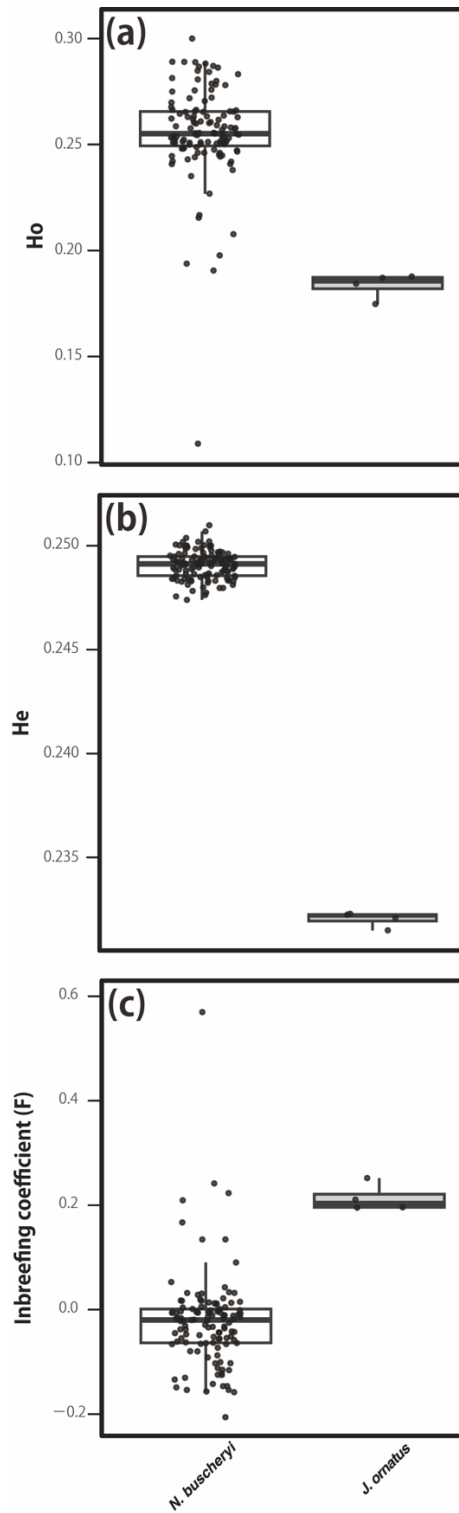


Figure 5. Genetic diversity represented by the heterozygosity and inbreeding coefficient of *Neolamprologus buscheri* and *Julidochromis ornatus* at the Kombe point. a) Observed heterozygosity (H_o), b) expected heterozygosity (H_e), and c) inbreeding coefficient (F). Because the sample size differed between *N. buscheri* ($N = 102$) and *J. ornatus* ($N = 10$), bootstrap subsampling was performed 100 times, adjusting the sample size to match *J. ornatus* before conducting statistical analyses using a LM. The median of P-values with 95% CI was indicated as follows; H_e : median 3.63×10^{-24} . CI: 4.24×10^{-27} – 7.87×10^{-22} , H_o : median 4.27×10^{-9} , CI: 7.57×10^{-11} – 9.63×10^{-4} ; and F : median 1.93×10^{-9} . CI: 9.71×10^{-11} – 1.64×10^{-3} .

Supplementary Information

Supplementary information S1: Kinship and heterozygosity data among subgroup members, group members, and individuals outside the group, as estimated by GRAS-Di, in *N. buescheri*.

Supplementary information S2: Data on body size and various life-history traits associated with social rank in *N. buescheri*.

Supplementary information S3: Behavioral data of breeding females (n = 31) and subordinates of *Neolamprologus buescheri*.

Supplementary information S4: All statistical information of kin-structure analysis describing Figure 3.

Supplementary information S5: Network of kinship among individuals in this study. Individuals related within the third degree of kinship were connected by lines and placed near each other by group.

Supplementary information S6: Video footage capturing two male individuals of *Neolamprologus buescheri* swimming side by side near a breeding territory.