Sympatric and allopatric *Alcolapia* soda lake cichlid species show similar levels of assortative mating

Michael R. M. Lawson[1]*, Michael G. B. Hayle[2],
Asilatu H. Shechonge[3], Wanja Dorothy Nyingi[4],
Antonia G. P. Ford[5], Joseph I. Hoffman[6,7], Julia J. Day[8],
George F. Turner[2] and Kanchon K. Dasmahapatra[1]

[1]Department of Biology, University of York, Heslington, United Kingdom, [2]School of Natural Sciences, Bangor University, Bangor, United Kingdom, [3]Tanzania Fisheries Research Institute, Dar es Salaam, Tanzania, [4]National Museums of Kenya, Nairobi, Kenya, [5]Department of Life Sciences, Whitelands College, University of Roehampton, London, United Kingdom, [6]Department of Animal Behaviour, Bielefeld University, Bielefeld, Germany, [7]British Antarctic Survey, Cambridge, United Kingdom, [8]Department of Genetics, Evolution and Environment, University College London, London, United Kingdom

Characterizing reproductive barriers such as mating preferences within rapid evolutionary radiations is crucial for understanding the early stages of speciation. Cichlid fishes are well-known for their adaptive radiations and capacity for rapid speciation and as such we investigate assortative mating among *Alcolapia* species; a recent (<10,000 years), small adaptive radiation, endemic to the extreme soda lakes, Magadi (one species) and Natron (three species), in East Africa. In seminatural aquarium conditions, we observed both courtship and mate choice (tested by microsatellite paternity analysis) to be significantly assortative among the three sympatric Natron species in a three-way choice experiment. This was also the case between allopatric species from Natron and Magadi, as found in a two-way choice experiment. However, the proportion of disassortative matings was substantial in both of these experiments, with hybrids comprising 29% of offspring in sympatric species and 11.4% in allopatric species comparisons. Previous work suggests that the Natron/Magadi split might not be much older than the radiation within Natron, so the similar rate of hybridization in the allopatric comparison is surprising and inconsistent with predictions of reinforcement theory, which predicts a faster rate of accumulation of premating isolation in sympatry. The relatively weak assortative mating in sympatry suggests that additional reproductive barriers, such as microhabitat preferences or spatial structuring may contribute to genetic isolation in nature.

KEYWORDS

adaptive radiation, speciation, reproductive isolation, hybridization, behavior

1. Introduction

Speciation can be best understood by studying the emergence of reproductive barriers within a previously interbreeding population (Coyne and Orr, 2004). As speciation progresses, gene flow between the diverging taxa diminishes as a consequence of the strengthening of existing barriers, and/or the accumulation of other barriers, eventually

leading to complete reproductive isolation (Kulmuni et al., 2020). In sexually reproducing organisms, reproductive barriers can be prezygotic or postzygotic. Postzygotic barriers can be extrinsic, and associated with adaptation to divergent environments, or intrinsic and dependent upon genetic incompatibilities that occur irrespective of ecology (Coyne and Orr, 2004). The degree of reproductive isolation changes as speciation proceeds, with the order and appearance of reproductive barriers varying at different stages of the speciation continuum (Drès and Mallet, 2002; Stankowski and Ravinet, 2021). Therefore, characterizing reproductive barriers at different stages along this continuum is important for creating a complete picture of speciation, and mechanisms initiating speciation are best studied by focusing on taxa at the earliest stages along the continuum (Coyne and Orr, 1997; Butlin et al., 2012).

Classical models of speciation focused on the role of geographic barriers in the formation of new species, where populations become physically separated and diverge under the effects of local adaptation and genetic drift (Mayr, 1947; Turelli et al., 2001). Sympatric or parapatric speciation, which occurs in the absence of physical barriers and in the presence of gene flow, generally arises when premating isolation becomes associated with a trait under divergent selection (Coyne and Orr, 2004; Servedio et al., 2011; Smadja and Butlin, 2011). Reproductive character displacement, where there is a greater divergence of reproductive traits in sympatry compared to allopatry, can occur due to reinforcement, whereby selection strengthens premating barriers that reduce hybridization rates (Dobzhansky, 1940; Pfennig and Pfennig, 2009), or via reproductive interference among fully isolated species (Templeton, 1981). However, premating isolation can emerge in allopatry if adaptation to different ecological pressures is associated with reproductive barrier traits (Servedio et al., 2011), as a by-product of sexual selection (Fisher, 1958; Mendelson and Safran, 2021), or through mutation-order effects (Mani and Clarke, 1990). Therefore, to provide evidence of the mechanisms driving speciation under gene flow, it can be informative to include contrasts with recently separated allopatric populations (Coyne and Orr, 1997; Funk, 1998).

Cichlid fishes are well-known for their striking adaptive radiations in the East African Great Lakes, where hundreds of species have evolved from a single or handful of closely related ancestral species often over very short timescales (Turner et al., 2001; Salzburger and Meyer, 2004). Many mechanisms drive speciation in cichlids, including ecologically-mediated processes such as diet or habitat depth (Albertson et al., 2003; Terai et al., 2006) and sexually-mediated processes such as assortative mate choice (e.g., Knight et al., 1998; Seehausen and van Alphen, 1998). Further factors influencing diversification include introgressive hybridization (Salzburger et al., 2002), the reassembly of old genetic variants into new combinations (Meier et al., 2017; Marques et al., 2019) or geographic isolation (Sturmbauer et al., 2001). Additionally, multiple processes may operate together, an example being sensory drive (Seehausen et al., 2008). Between sympatric cichlid species, isolating mechanisms are more often prezygotic, with female choice generally being the ultimate barrier to mating (Kocher, 2004; Henning and Meyer, 2014).

Understanding the emergence of reproductive barriers in larger and/or older systems such as the East African Great Lakes is often difficult due to the complications of historic lake level fluctuations combined with the complex evolutionary history of ancestral lineages, with past periods of gene flow and extensive incomplete lineage sorting making inferences more difficult (Malinsky et al., 2015; Svardal et al., 2021). Therefore, recent and smaller radiations from isolated lakes make for more tractable study systems, where it may be possible to disentangle both the order of emergence and relative contribution of different reproductive barriers (Barluenga et al., 2006; Malinsky et al., 2015; Kautt et al., 2018; Poelstra et al., 2018). Direct tests of the levels of assortative mating in such simple systems have thus far only been reported between Midas cichlids from Nicaraguan crater lakes (Elmer et al., 2009; Machado-Schiaffino et al., 2017).

In this study, we investigate the magnitude of assortative mate choice among the four closely-related *Alcolapia* species which comprise a young, and isolated adaptive radiation endemic to the East African soda Lakes Magadi, in Kenya, and Natron, in Tanzania (Seegers and Tichy, 1999). Although widely referred to as a separate genus (Kavembe et al., 2014; White et al., 2020), the *Alcolapia* clade is in fact nested within the genus *Oreochromis* (Seegers et al., 1999; Ford et al., 2019). Magadi and Natron are volcanic, alkaline lakes dominated by large areas of thick sodium hydrogen carbonate precipitates, with very shallow (<1 m) lagoons, streams, and hot springs interspersed around the lake margins (Kaufman et al., 1990; Seegers and Tichy, 1999). *Alcolapia* are the only fishes found in these lakes and have evolved several unique adaptations to thrive in extremes of pH, temperature, salinity, UV light and oxygen levels (Trewavas, 1983; Narahara et al., 1996; Walsh et al., 2001; Wood et al., 2012, 2016; White et al., 2020). The much larger and deeper (∼55 m) paleolake Orolonga, which comprised part of rift lake network intermittently connected by rivers, contracted and split to form Natron and Magadi ∼8 Ka. Orolonga itself had more freshwater conditions, and the current highly alkaline and hypersaline conditions are thought to have developed ∼7 Ka (Roberts et al., 1993; Dommain et al., 2022). Therefore, both the adaptive evolution and the speciation of *Alcolapia* has been extremely rapid.

Three *Alcolapia* species are described from Lake Natron, *Alcolapia alcalica*, *A. ndalalani*, and *A. latilabris*. A single species, *A. grahami*, is known from Lakes Magadi and Little Magadi (Coe, 1966; Seegers et al., 2001). The species differ in morphology, size and male nuptial coloration (Seegers and Tichy, 1999; **Figure 1**). The Natron species have different head and mouth shapes which likely relate to fine-scale niche specialization toward different forms of herbivory (Ford et al., 2016). *Alcolapia* populations are distributed across the springs, lagoons and small streams around the perimeters of Natron and Magadi. While the distribution of the three species around Lake Natron is uneven, there are sites in the south of the lake where all three species can be found swimming alongside each other at high densities (Ford et al., 2015; **Figure 1**). Genomic data revealed evidence of ongoing gene flow between all the sympatric Natron species and extremely low genomic differentiation between species (Ford et al., 2015), even when compared to other cichlid radiations (Svardal et al., 2021). Despite this gene flow, these species are genetically distinct (**Figure 1**).

In *Alcolapia*, high population density, conspicuous male nuptial coloration, presence of leks and male-biased sex ratios are all predictors of high levels of sexual selection (Seegers and Tichy, 1999; Ford et al., 2016; Maina et al., 2019). Differences in
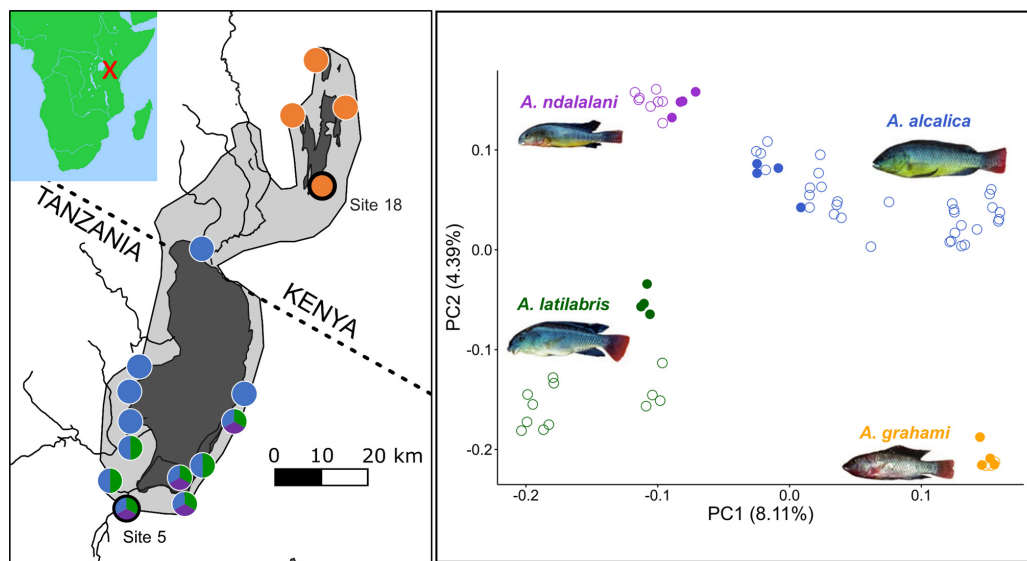
FIGURE 1
The *Alcolapia* spp. radiation of lakes Natron and Magadi. **(Left panel):** Map showing the distribution of the species from sites that have been sampled. The light gray area shows the approximate maximum extent of Palaeolake Orolonga (∼700 Ka) (Williamson et al., 1993). The dark gray area represents the current expanse of the lakes. Colored circles correspond to species present in the right panel with black-ringed circles denoting the populations used in this study. Photos and site information are from Ford et al. (2015). **(Right panel):** Principal Component Analysis (PCA) of a published *Alcolapia* RADseq dataset (Ford et al., 2015) demonstrates that the species form distinct genetic clusters even in sympatry. **(Left panel):** Colors correspond to species. Filled circles represent the populations used in this study (site five for sympatric species and site 18 for *A. grahami*, the allopatric species); empty circles are individuals from other sites. The covariance matrix was created using PCAngsd (Meisner and Albrechtsen, 2018) using a genotype likelihood file output from ANGSD (Korneliussen et al., 2014).

male coloration between the three sympatric Natron species suggest that prezygotic assortative mate choice could be an important factor in their reproductive isolation. Since a comparison between sympatric and allopatric taxa can be valuable in understanding the mechanisms driving speciation with gene flow, we use semi-natural aquarium conditions to quantify the levels of assortative mate choice (male courtship behavior and paternity of embryos) both among sympatric Natron *Alcolapia* species (*A. alcalica*, *A. latilabris*, and *A. ndalalani*) and also between allopatric species (Natron *A. alcalica* and Magadi *A. grahami*). We use this data to test the hypothesis that sympatric *Alcolapia* exhibit stronger assortative mate compared to the allopatric species *A. grahami*.

2. Materials and methods

2.1. Fish collection and husbandry

The three Natron species were collected from site 5 in July 2017 (Ford et al., 2015; White et al., 2020), and *A. grahami* collected from Lake Magadi site 18 (Ford et al., 2015) in March 2019 (**Figure 1**). Fishes were kept in separate single-sex stock tanks at Bangor University. Appropriate water chemistry was maintained (pH = 9.0, GH = 180 ppm, KH = 180 ppm, specific gravity = 1.005) by the addition of $NaHCO_3$ (0.5 g/L), Na_2CO_3 (0.07 g/L), $MgSO_4.7H_2O$ (1.5 g/L) and Instant Ocean® Sea Salt (0.5 g/L). Water conditions were maintained using a continuously recirculating filtration system with the daily addition of a buffer solution. The tanks were kept at 31°C under a 12:12 light:dark cycle. Fish were fed daily with spirulina flake food.

2.2. Experimental setup

To quantify the degree of assortative mating in *Alcolapia* and to determine whether this is affected by geographic context, we carried out two separate mate choice experiments in aquarium setups; among species that are found in sympatry, and between species that have allopatric distributions. The strength of assortative mating was assessed through observations of courtship behavior and paternity analysis of resulting offspring.

Behavioral observations and brood collection for the sympatric mate choice experiment involving the three Natron species, *A. alcalica*, *A. latilabris*, and *A. ndalalani*, was carried out between February and March 2019. For the allopatric experiment involving *A. alcalica* and *A. grahami*, behavioral observations were carried out between January and February 2020. Broods from the allopatric experiment were collected between March and April 2020. The sympatric mate choice experiment was carried out in a single 8 m (L) × 0.6 m (W) × 0.7 m (D) tank. The allopatric mate choice experiment was carried out in the same tank, but reduced to a length of 6 m, to maintain approximately the same density of fish. An 8 cm layer of silica sand was used as a substrate, with shelter provided by evenly placing five clay pots for every 2 m of tank length.

Fully mature males were selected for both experiments and size-matched with a range in standard length (SL) of no more than 12 mm within species. For the sympatric experiment, fish were all first-generation laboratory-bred and consisted of 10 females and six males of each of the Natron species derived from the wild-caught parents. In the sympatric experiment, males had the following mean SL ± SD: *A. alcalica* 76.2 ± 4.9 mm, *A. latilabris*

68.7 ± 4.0 mm, *A. ndalalani* 65.8 ± 3.0 mm. The larger SL of *A. alcalica* males relative to the other sympatric species reflects inherent size differences of the species observed in the wild (Seegers and Tichy, 1999). In the allopatric experiment, fish consisted of 12 females and seven males each of first-generation laboratory-bred *A. alcalica* and wild-caught *A. grahami. Alcolapia alcalica* was selected as the allopatric Natron species because it is the most widely distributed and forms the basal lineage (Ford et al., 2015). *A. alcalica* also possesses similar trophic morphology to *A. grahami,* with both sharing a terminal mouth (Seegers and Tichy, 1999; Ford et al., 2016). Mean male SL was 72.1 ± 3.7 mm for *A. alcalica* and 71.1 ± 4.0 mm for *A. grahami.*

For both experiments, males and females were kept in single-sex stock tanks for at least a month before being added simultaneously to the experimental setup. While males of all the *Alcolapia* species and female Natron species are easily differentiated by their coloration and unique mouth morphology, female *A. grahami* and *A. alcalica* are difficult to differentiate. Therefore, the different species of females in the allopatric experiment were made visually distinguishable by caudal fin clips. *A. grahami* females were fin-clipped along the dorsal section of the caudal fin, whereas *A. alcalica* were fin-clipped along the ventral section. Every 2 weeks, one species of female was fin-clipped after the fin section had almost re-grown, and the species that was clipped was subsequently alternated.

2.3. Courtship behavior measurements

Mating preferences in *Alcolapia* and other *Oreochromis* are primarily displayed by females (Baerends and Baerends-van Roon, 1950; Seegers et al., 2001). While the identification of focal males was possible using reference photos of their unique scale markings along the intersection of the tail and caudal fin, focal observations of females were not possible as we were unable to differentiate between individuals across observation periods. Therefore, behaviors of randomly selected individual males were scored during daily, 5 min focal observations between 10:00 and 13:00 over a period of 15 days. Since individual identification was not possible for females or for male opponents with whom interactions were too rapid, only the species, sex and relative size of the interacting fish was recorded. These behavior measurements provide an assessment of a combination of female and any male courtship preferences.

Since many of the courtship behaviors in *Alcolapia* were found to be similar to those described for other *Oreochromis* species (e.g., tilting, circling, and quivering), a reduced ethogram of behaviors was created (**Supplementary Table 1**) based on the descriptions by Baerends and Baerends-van Roon (1950). Courtship was scored and its duration was recorded if the male performed any courtship behaviors directed at a female within a distance of two SL of the focal male. Courtship directed toward multiple females of different species was recorded, but not assigned a species. For the allopatric experiment, focal observations were video recorded and were carried out blind with respect to the identity of the female species (females were scored as either fin-clipped or non-fin-clipped). Courtship behavior was then scored using Solomon Coder v. 19.08.02 (Péter, 2011) using a custom ethogram.

2.4. Paternity testing

Alcolapia are maternal mouthbrooders and females were checked visually each day for brooding. The partially developed broods were removed from females, euthanised and counted. Each time a brood was removed from a female, a sample of the female's DNA was obtained by swabbing the fish along the body using sterile cotton swabs (Breacker et al., 2017). All males were photographed, measured (SL) and swabbed for DNA before being placed into the setup. Swabs and embryos were stored in 95% ethanol at –20°C until required for DNA extraction. In the sympatric experiment, 12–13 separate broods were collected from each species during the same period when courtship measurements were taken. While 15 separate broods were collected per species in the allopatric experiment, these were collected after the period during which the courtship measurements were made.

Swab DNA was extracted following the protocol outlined in Breacker et al. (2017). To assess paternity, DNA was extracted from 2 to 3 mm sections of tissue from up to six randomly selected embryos per brood. For most embryo DNA extractions, a modified version of the swab protocol was used by replacing the swab with dissected tissue. For a few embryos, a DNeasy Blood and Tissue Kit (Qiagen Inc., Hilden, Germany) was used. Extracted DNA was diluted 1:5 using ddH$_2$O for use in PCR.

Dinucleotide microsatellites were detected in the *Oreochromis niloticus* genome (O_niloticus_UMD_NMBU; Conte et al., 2017) using SciRoKo (Kofler et al., 2007), after which 2–3 primers pairs per chromosome were designed using Primer-BLAST (Ye et al., 2012). Initial screening of 53 dinucleotide-repeat microsatellites for polymorphism was carried out using a panel of eight individuals of the three Natron species using FAM-labeled M13-tailed primers (**Supplementary Table 2**; Schuelke, 2000). Seven loci exhibiting within and among species polymorphism were selected for use in paternity testing. Microsatellite DNA loci were amplified in two separate multiplexes of fluorescent-tagged primers using the Type-IT Microsatellite PCR Kits (Qiagen Inc.) in 10 μl PCR reaction volumes using the manufacturer's standard protocol (**Supplementary Table 2**). The PCRs were run with an initial 95°C denaturation stage for 5 min before conducting 32 cycles: 95°C for 30 s, 60°C for 90 s and 72°C for 30 s, and a final stage of annealing at 60°C for 30 min. PCR products were diluted 1:5 before being analysed on an ABI 3730xl DNA Analyzer. Allele sizes were scored automatically using GeneMarker v. 2.6.2 (SoftGenetics, LLC., State College, PA, USA). All traces were manually inspected and corrected where necessary to ensure high genotype data quality.

Microsatellite genotypes were manually scored using Microsatellite Analysis Software (MSA) (Thermo Fisher). First, to identify which swabbed females had matching genotypes, an identity analysis was carried out using *Cervus* v.3.0.7 (Kalinowski et al., 2007) using all seven loci (**Supplementary Table 2**). In addition, sequencing failed for one female (*A. ndalalani* 10) and therefore to check if this genotype matched with any of the other females, genotype reconstruction was performed in *Colony2* using its known offspring and all candidate males (Jones and Wang, 2010).

The clustering of individual males and females to assigned species was visualized using STRUCTURE v. 2.3.4 (Pritchard et al., 2000). All seven loci were used with a default

allele frequency parameter (λ = 1). STRUCTURE was run 20 times separately for allopatric and sympatric species using values of *k* of 2 and 3, respectively with a burn-in of 10,000 and 100,000 iterations.

Parentage analysis was carried out for both the sympatric and allopatric experiments separately using *Cervus*. Allele frequencies were generated using all the parental genotypes (Flanagan and Jones, 2019) and a simulation of paternity was run with 100,000 offspring using all possible candidate fathers. A threshold minimum of four typed loci was used for paternity assignment. Paternity was assigned based on the LOD score of offspring and parent trios. The trio LOD score is calculated using the genotypes of offspring, known mothers and candidate males while accounting for potential mistyping errors. First, all offspring were assigned a single compatible male if the trio LOD score had assignment confidence of at least 95%. For the remaining offspring, candidate fathers were removed if they had more than one mismatch in either pairwise (offspring-candidate father) or trio comparisons. For the single female with no genotype information, only candidate fathers with a positive pairwise LOD score were kept.

2.5. Statistical analysis

Courtship preference toward different taxa was modeled using Generalized Linear Mixed Models (GLMMs) using two different response metrics: (1) the total amount of time spent courting and (2) courtship frequency, or the total number of courtship behaviors directed toward females. Both response measures were the sum of behaviors carried out over a 5 min focal observation. For both models, fixed effects included the species of male and species of female involved in courtship, whereas individual male ID and date of observation were modeled as random factors, correcting for pseudoreplication. In addition, to account for potential temporal variations in courtship, time of observation was modeled as an additional random effect with times of day split into 5 min intervals. Models with and without the time of day term were selected depending on the Akaike Information Criterion (AIC). Courtship time and courtship frequency behaviors were modeled with a separate model for each experiment. For the courtship time models, the response had a heavily right-skewed distribution with many zeros, therefore models used a zero-inflated gamma distribution with a log link. We allowed zero-inflation to vary within each level of the fixed effects. As courtship frequency consists of count data, these models used a Poisson distribution and log link, but due to overdispersion in the allopatric experiment, a negative binomial distribution (*nbinom2*) with a log link was used instead.

Assortative mate preferences were also tested using the offspring paternity data. Broods were aggregated for each individual female and offspring were then scored as being either of conspecific or heterospecific paternity. Mating preference toward conspecifics was modeled using a Generalized Linear Model (GLM) with the *cbind* function and a beta-binomial distribution to account for overdispersion. Due to insufficient data, it was not possible to account for variance in individuals by using a mixed model design. The proportion of conspecific broods was modeled as the response variable and the species of the mother as the independent variable. In addition, differences in total brood size between species were tested using a GLMM with a Poisson distribution and individual female ID modeled as a random effect.

For all models, estimates and post-hoc contrasts were generated using the *emmeans* package (Lenth et al., 2018). For each species, estimates of their overall courtship propensity towards any species and different species were obtained. The significance (*p*-values) of differences in courtship between taxa was obtained through pairwise comparisons, with Tukey adjustments to account for multiple contrasts. To obtain estimates of the degree of assortative mating from offspring paternity data, the predicted probabilities of mating with a conspecific male were obtained from the output of the GLM. To test for the significance of assortative mate choice for each taxon, *p*-values were obtained by testing if the predicted probability of mating with conspecifics was significantly different from the expected proportion of conspecific matings under random mating: 0.5 in the allopatric experiment (two-way choice) and 0.33 in the sympatric experiment (three-way choice). In addition, to test for significant differences in the probability of conspecific mating between species, pairwise tests were carried out using *emmeans*.

Mating assortativity within and between each experiment was investigated using a network-based approach. Newman's assortativity coefficients were calculated for each weighted network using the R package *assortnet* (Farine, 2014). To test whether assortativity was statistically significant, these values were compared to a null distribution of assortativity coefficients, which was generated from 10,000 permutations where species identity was randomly shuffled across nodes without replacement. To test for differences in assortativity between the sympatric and allopatric experiments, the *t*-statistic was calculated by comparing their assortativity coefficients. The statistical significance of differences in assortativity was obtained by comparing this observed *t*-statistic with a null distribution of *t*-statistics generated from the 10,000 permuted assortativity coefficients from each experiment (Heathcote et al., 2016).

All statistical analysis were carried out using R version 4.1.2 (R Core Team, 2013). The packages *lme4* (Bates et al., 2014) and *glmmTMB* (Brooks et al., 2017) were used to generate models, while *DHARMa* (Hartig, 2020) was used to test model assumptions and the fit of each model. To visualize parental-offspring relationships, a network was created with the R package *tidygraph* v1.2.1 (Pedersen, 2022a) using additional code maintained by James Ward[1] to create a node layout using the Fruchterman-Reingold algorithm. The package *ggraph* v.2.0.5 (Pedersen, 2022b) was used to plot networks while *ggplot2* v3.3.6 was used to generate all other plots (Wickham, 2016).

3. Results

3.1. Observations of territoriality and courtship in *Alcolapia*

Males were highly active and performed courtship and territorial behaviors soon after their introduction to the experimental setup. Between courtship and aggressive behaviors, dominant males spent a significant amount of time constructing bowers, simulating the lekking areas found in the wild (Coe, 1969).

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1 https://jmw86069.github.io/multienrichjam/

While individual males often held the same bower for multiple days, bower ownership also changed frequently over the course of the experiments.

Courtship was common, but spawning was observed less frequently. While some females were observed to mate with the same male on multiple occasions, other females were observed to spawn with several males within a single brood. In a small number of cases potential sneak mating was observed, where spawning was interrupted by a rival male as the female released an egg. In sympatric species, brood size ranged from 5 to 31 with a mean ± SD of 20.1 ± 7.9 *A. alcalica*, 9.8 ± 4.4 *A. latilabris* and 15.7 ± 6.6 *A. ndalalani*. *A. latilabris* had significantly smaller brood sizes than both *A. alcalica* (GLMM, post-hoc, $p < 0.001$) and *A. ndalalani* ($p < 0.006$). However, comparisons between *A. alcalica* and *A. ndalalani* were not significant (GLMM, post-hoc, $p < 0.14$) (**Supplementary Table 4**). In the allopatric experiment, brood size ranged from 3 to 51 and *A. alcalica* had a larger mean (±SD) brood size of 28.2 ± 12.6 compared to 19.1 ± 13.4 in *A. grahami*, but the difference was marginally non-significant (GLMM, post-hoc, $p < 0.064$) (**Supplementary Table 4**).

3.2. Assortative courtship behavior

Male courtship behaviors are based on 1,050 and 1,350 minutes of observation data for the allopatric and sympatric experiments, respectively. In the allopatric experiment, males of both species spent significantly more time courting conspecific than heterospecific females (GLMM post-hoc, *A. alcalica*: $p < 0.001$; *A. grahami*: $p < 0.0001$, **Figure 2A** and **Supplementary Table 6**). Additionally, overall courtship time with any species did not differ between males or females of each species (males: $p = 0.54$, females: $p = 0.65$, **Supplementary Table 6**). Contrastingly, *A. alcalica* males had a higher courtship frequency (number of courtship attempts) with conspecific females, while *A. grahami* males did not (*A. alcalica*: $p < 0.001$, *A. grahami*: $p = 0.18$, **Figure 2B** and **Supplementary Table 7**). This was despite there being no differences in overall courtship frequency (courtship toward any species) between males of both species ($p = 0.27$, **Supplementary Table 7**). Likewise, *A. alcalica* females were courted by conspecific males significantly more often, while *A. grahami* females were not (*A. alcalica*: $p = 0.002$, *A. grahami* $p = 0.23$, **Supplementary Table 7**); however, *A. alcalica* females were courted more often overall compared to *A. grahami* females ($p = 0.015$, **Supplementary Table 7**).

In the sympatric experiment, *A. latilabris* males spent significantly longer courting *A. latilabris* females than *A. alcalica* females ($p = 0.001$) and *A. ndalalani* females ($p < 0.001$), but there was no significant difference in time spent courting between both heterospecific species ($p = 0.32$, **Figure 2C** and **Supplementary Table 9**). *A. ndalalani* males spent significantly longer courting conspecific females compared to *A. alcalica* females ($p < 0.001$) and *A. latilabris* females ($p < 0.001$), but there was no difference in courtship time in the two heterospecific comparisons ($p = 0.21$, **Figure 2C**). In contrast, there was no significant difference in the amount of time spent courting between any of the species by *A. alcalica* males (*A. alcalica–A. latilabris*: $p = 0.96$, *A. alcalica–A. ndalalani*: $p = 0.73$, *A. ndalalani–A. latilabris*: $p = 0.52$,

Figure 2C). There were no significant differences in overall courtship time between males (*A. alcalica–A. latilabris*: $p = 0.99$, *A. alcalica–A. ndalalani*: $p = 0.63$, *A. ndalalani–A. latilabris*: $p = 0.57$); however, courtship was directed toward *A. ndalalani* females for significantly longer overall compared to *A. alcalica* females ($p = 0.018$; **Supplementary Table 9**).

The number of courtship events largely reflected these results with *A. latilabris* males courting conspecific females significantly more often than heterospecifics (*A. latilabris–A. alcalica*: $p = 0.003$, *A. latilabris–A. ndalalani*: $p < 0.001$, *A. ndalalani–A. alcalica*: $p = 0.18$, **Supplementary Table 11** and **Figure 2D**). Similarly, *A. ndalalani* males also spent had a higher courtship frequency with conspecifics compared to heterospecifics (*A. ndalalani–A. alcalica*: $p = 0.002$, *A. ndalalani–A. latilabris*: $p = 0.003$, *A. latilabris–A. alcalica*: $p = 0.97$, **Supplementary Table 11** and **Figure 2D**). There was no significant difference in courtship frequency between any of the species by *A. alcalica* males (*A. alcalica–A. latilabris*: $p = 0.79$, *A. alcalica–A. ndalalani*: $p = 0.24$, *A. ndalalani–A. latilabris*: $p = 0.56$, **Supplementary Table 11** and **Figure 2D**). There were no significant differences in overall courtship frequency between males of each species (*A. alcalica–A. latilabris*: $p = 0.19$, *A. alcalica–A. ndalalani*: $p = 0.16$, *A. ndalalani–A. latilabris*: $p = 0.99$); however, *A. alcalica* females were courted more often overall compared to the other species (*A. alcalica—A. latilabris*: $p = 0.01$, *A. alcalica—A. ndalalani*: $p = 0.03$, *A. latilabris—A. ndalalani*: 0.91, **Supplementary Table 11**). Full model outputs and associated test statistics can be found in **Supplementary Tables 3–11**.

3.3. Assortative mate choice

STRUCTURE analysis confirmed that individual males and breeding females predominantly clustered to their respective assigned species (**Supplementary Figure 2**).

In the allopatric experiment, a total of 140 offspring from 31 broods were successfully genotyped with paternity assigned to an individual male in all cases. The total number of breeding females for each species was 10 for *A. alcalica* and 11 for *A. grahami* (**Figure 3A**). The proportion of conspecific offspring was 88.6% (124/140) for all the offspring in the allopatric experiment. Both *A. alcalica* (62 of 68 offspring) and *A. grahami* females (62 of 72 offspring) showed a significant preference toward conspecifics (GLM *post-hoc* test, *A. alcalica*: *t.ratio* = 3.0, $df = 18$ $p = 0.007$; *A. grahami*: *t.ratio* = 2.8, $df = 18$, $p = 0.012$; **Figure 3B**). There were no significant differences in the probability of conspecific mating between females of each species (GLM, $z = 0.98$, $p = 0.33$) and mating was significantly assortative overall ($r = 0.77$, $p < 0.001$).

In the sympatric experiment, a total of 193 offspring from 36 broods were successfully genotyped with paternity assigned to at least the species level. The total number of breeding females for each species was eight for *A. alcalica*, nine for *A. latilabris*, and ten for *A. ndalalani* (**Figure 3C**). Of the successfully genotyped offspring, 89.6% (173/193) were assigned to individual males. The proportion of offspring assigned to individual males differed among species; 74.6% (44/59) in *A. alcalica*, 91.9% (57/62) in *A. latilabris* and 81.4% (57/70) in *A. ndalalani* (**Figure 4B**). The proportion of conspecific offspring was 71.0% (137/193) for all the offspring in the
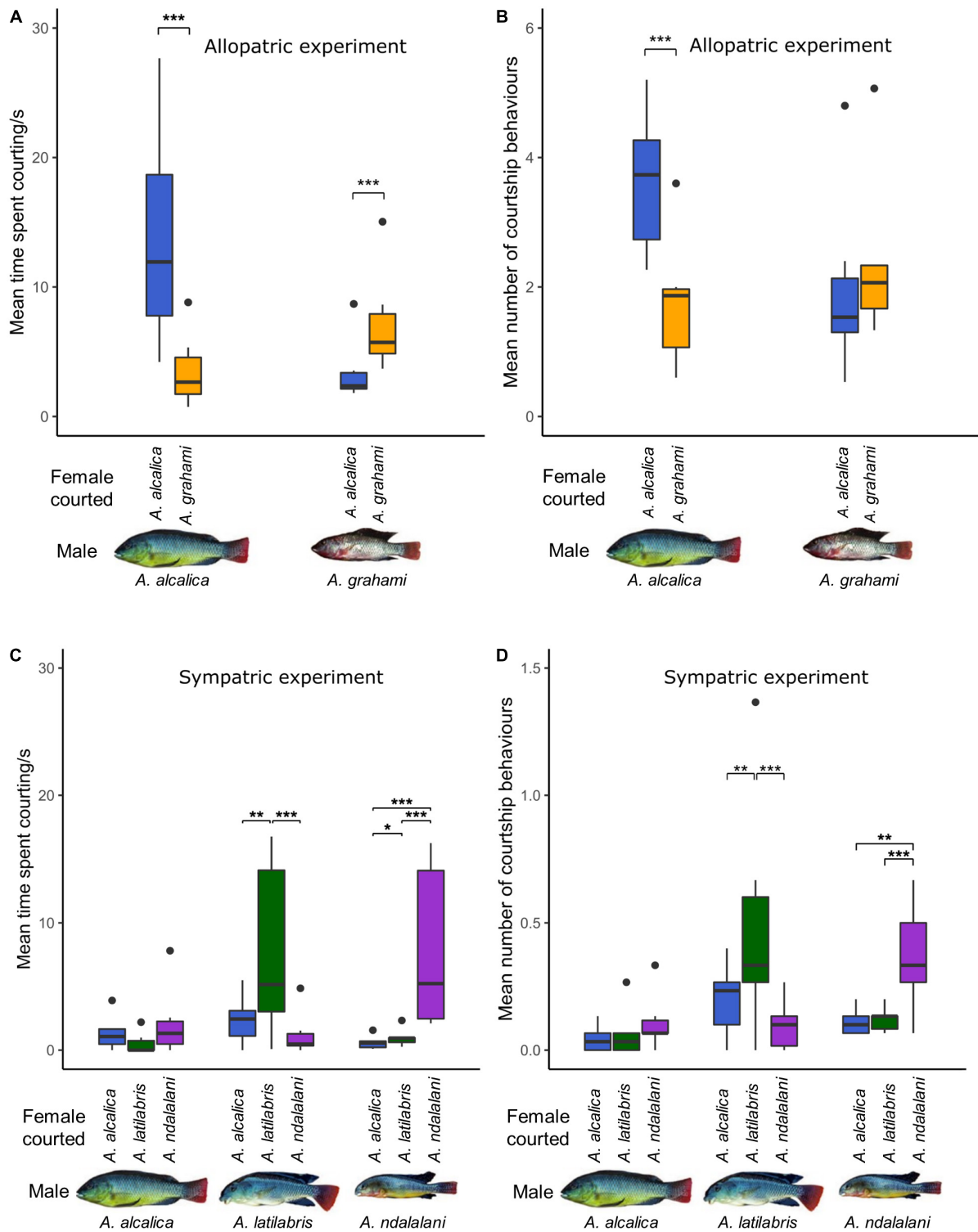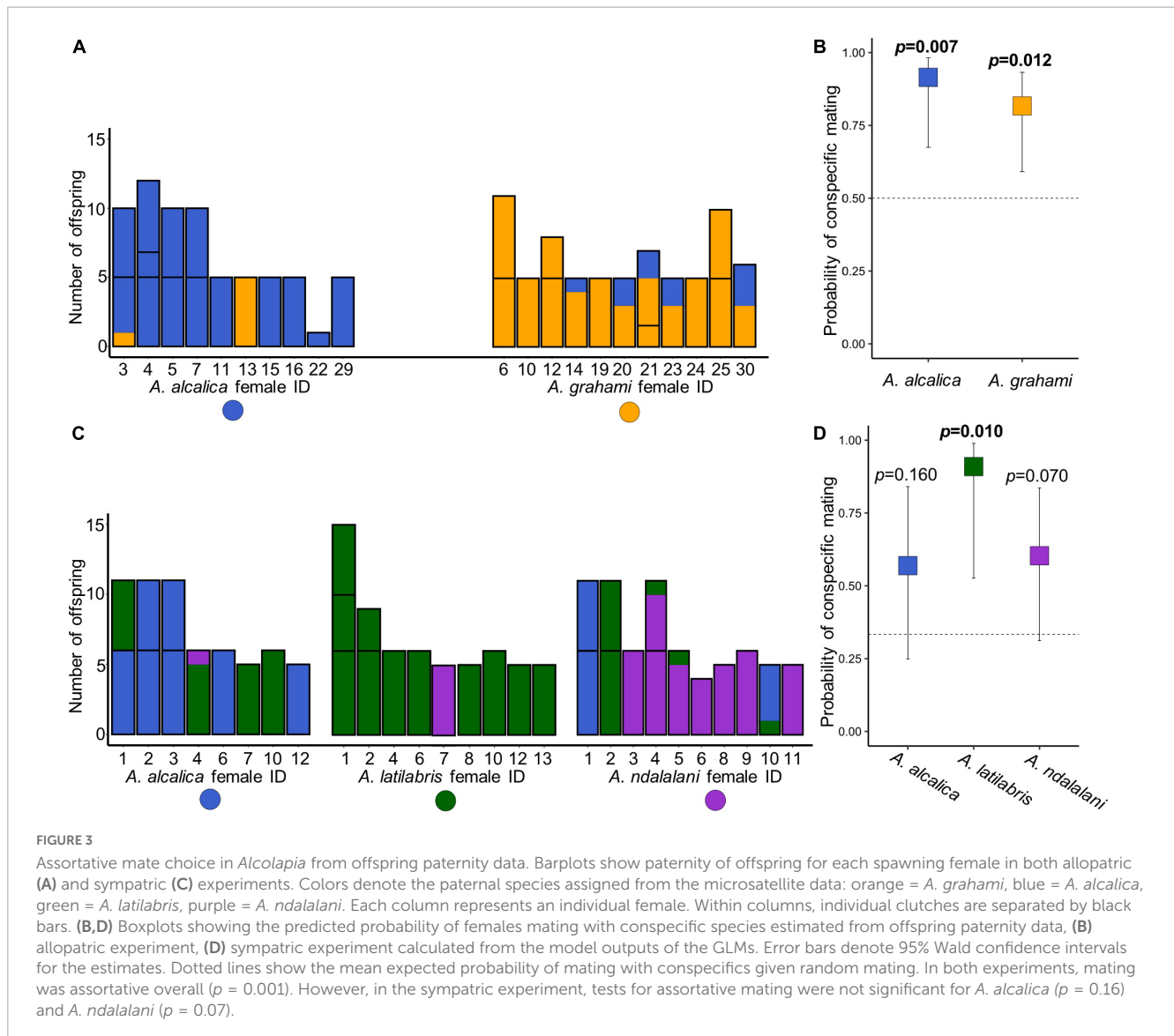
FIGURE 2
Male courtship with conspecific and heterospecific females in *Alcolapia*; **(A,C)** time spent courting and, **(B,D)** number of courtship attempts for each male during 5 min focal observations. Courtship results are shown for both the allopatric **(A,B)** and sympatric experiments **(C,D)**. Colors denote the species of female courted with: blue = *A. alcalica*, green = *A. latilabris*, purple = *A. ndalalani*, orange = *A. grahami*. Large outliers (35.4 and 57.6 s) were removed from panel **(C)** for clarity (see **Supplementary Figure 1** for full figure). Asterisks denote significant levels of pairwise contrasts extracted from GLMMs: *** < 0.001, ** < 0.01, * < 0.05.

sympatric experiment: 63.9% (39/61) in *A. alcalica* females, 98.4% (57/62) in *A. latilabris* females, and 58.6% (41/70) in *A. ndalalani* females (**Figure 3C**). Overall, females spawned with conspecific males significantly more than expected given random mating (GLM *post-hoc* test, *t.ratio* = 3.65, *df* = 23, *p* = 0.001; **Supplementary Table 5**) and mating was significantly assortative overall (*r* = 0.56,

FIGURE 3
Assortative mate choice in *Alcolapia* from offspring paternity data. Barplots show paternity of offspring for each spawning female in both allopatric (**A**) and sympatric (**C**) experiments. Colors denote the paternal species assigned from the microsatellite data: orange = *A. grahami*, blue = *A. alcalica*, green = *A. latilabris*, purple = *A. ndalalani*. Each column represents an individual female. Within columns, individual clutches are separated by black bars. (**B,D**) Boxplots showing the predicted probability of females mating with conspecific species estimated from offspring paternity data, (**B**) allopatric experiment, (**D**) sympatric experiment calculated from the model outputs of the GLMs. Error bars denote 95% Wald confidence intervals for the estimates. Dotted lines show the mean expected probability of mating with conspecifics given random mating. In both experiments, mating was assortative overall (*p* = 0.001). However, in the sympatric experiment, tests for assortative mating were not significant for *A. alcalica* (*p* = 0.16) and *A. ndalalani* (*p* = 0.07).

$p < 0.001$). While *A. latilabris* females had a significant preference toward conspecifics (GLM *post-hoc* test, *t.ratio* = 2.82, *df* = 23, *p* = 0.010; **Figure 3D**), *A. alcalica* (*t.ratio* = 1.45, *df* = 23, *p* = 0.160; **Figure 3D**) and *A. ndalalani* females did not (*t.ratio* = 1.90, *df* = 23, *p* = 0.070; **Figure 3D**). There were no significant differences in the probability of conspecific mating between females of each sympatric species [GLM *post-hoc* test, *F.ratio* = 1.45, *df* = (2,23), *p* = 0.255]. There were no significant differences in mating assortativity between the sympatric and allopatric experiments (*t* = 2.37, *p* = 0.25).

Multiple mating was relatively common, with 61.3% (19/31) and 69.4% (25/36) of broods showing multiple paternity in the allopatric and sympatric experiments, respectively (**Figure 4**). These represent the minimum level of multiple paternity as we only genotyped a maximum of six offspring per brood (brood sizes varied from 3 to 51), and we also could not distinguish between all males in the sympatric experiment. Individual mating success also varied among males. For instance, in the allopatric experiment, one *A. grahami* male sired at least one offspring with every breeding *A. grahami* female, but none with any *A. alcalica* females

(**Figure 4A**). By contrast, a single *A. grahami* male and *A. alcalica* male were not assigned parentage to any of the offspring in the allopatric and sympatric experiments, respectively (**Figures 4A, B**).

4. Discussion

4.1. Main findings

We demonstrate that *Alcolapia* species show evidence of assortative mating in comparisons both between sympatric and allopatric species in a semi-natural aquarium set-up. Hybrid offspring were generated between all species pairs that were tested, indicating that in our experimental setup, reproductive isolation is incomplete. Together with results from previous studies showing differences in trophic morphology between sympatric species (Ford et al., 2016), our findings lend support to the theory that speciation in sympatry is more likely when there is a combination of assortative mating and ecological divergence
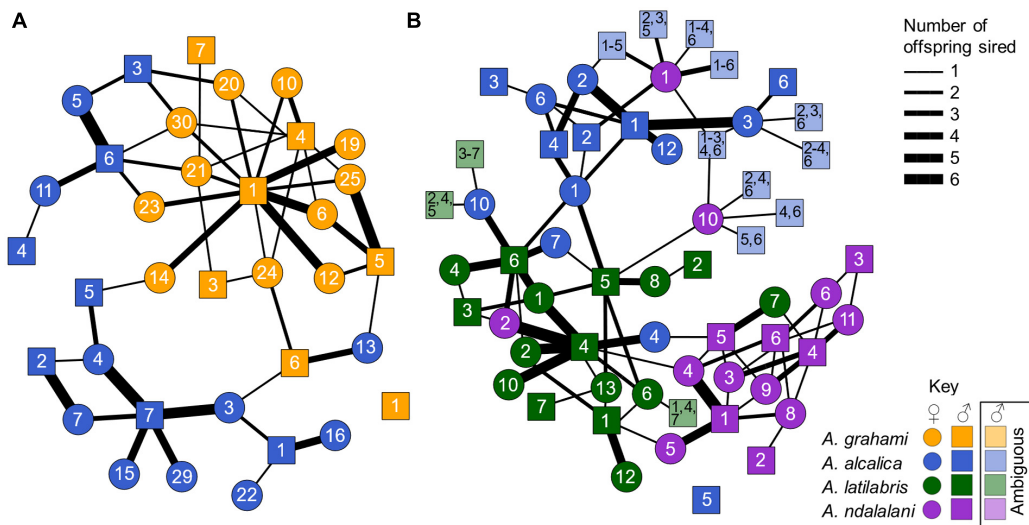
FIGURE 4
Network showing parent-offspring relationships in the allopatric experiment **(A)** and sympatric experiment **(B)** using offspring paternity data. Colors indicate species: blue = *A. alcalica*, orange = *A. grahami*, green = *A. latilabris*, purple = *A. ndalalani*. Circles = females and squares = males. Numbers correspond to unique male or female ID. Line thickness denotes the number of offspring sired between parents. Lighter colors denote offspring with ambiguous paternity that were not assigned to a single compatible male. Paternity could be assigned unambiguously in 94.0% of offspring. 88.6% (two-way choice) and 71.0% (three-way choice) of offspring in the allopatric and sympatric experiments resulted from conspecific matings. Mating was significantly assortative in both the allopatric ($r = 0.77$, $p < 0.001$) and sympatric ($r = 0.56$, $p < 0.001$) experiments. There were no significant differences in assortativity between both experiments ($t = 2.37$, $p = 0.25$).

(Dieckmann and Doebeli, 1999). These findings correspond with other studies of recent and small cichlid adaptive radiations such as Lake Ejagham in Cameroon and the crater lakes of Nicaragua (Martin, 2013; Machado-Schiaffino et al., 2017).

4.2. The evolution of assortative mating in allopatry and sympatry

The forces driving speciation may vary depending on the geographic context and amount of gene flow between populations. In general, it is expected that prezygotic barriers will be stronger among sympatric compared to allopatric taxa as there is selection to avoid heterospecific matings in sympatry, but not in allopatry (Butlin, 1987; Coyne and Orr, 1997). Contrary to these expectations, our results cannot detect a difference in the degree of assortative mating between allopatric species and sympatric species.

While reinforcement may be a key driver of premating isolation in some taxa (Yukilevich, 2012), premating barriers are also predicted to increase with genetic distance (Zouros, 1973; Coyne and Orr, 1989). Allopatric *A. grahami* females showed strong premating isolation, but they are also more genetically distinct compared to the sympatric Natron species (**Figure 1**; Ford et al., 2015). On the other hand, phenotypic rather than genetic distance is often a better predictor of assortative mate choice (McPeek and Wellborn, 1998). For example, in *Pseudocrenilabrus* spp. of Lake Mweru and Bangweulu, less closely related but more phenotypically similar species have stronger premating isolation than more distantly related but less phenotypically similar species (Stelkens and Seehausen, 2009). *Alcolapia grahami* are more phenotypically similar to *A. alcalica* in terms of trophic morphology (head and mouth shape) compared to the other sympatric Natron species

(Ford et al., 2016), but they do differ in male coloration, which is typically a primary cue used by females for mate selection (Seehausen et al., 1997; Selz et al., 2014). A meta-analysis of cichlid premating isolation studies found only slight differences in the levels of assortative mating between sympatric and allopatric species (Rometsch et al., 2020); however, they also found greater variance in premating isolation among allopatric species, and lower levels when comparing allopatric and sympatric populations, suggesting an important role in the duration of the population split. Nevertheless, strong assortative mate choice can evolve among cichlids over relatively short periods of allopatry (Knight and Turner, 2004; Genner et al., 2007), especially if there are divergent ecological pressures resulting from habitat differences (Tyers et al., 2014).

Fossil and geological data date the Magadi-Natron split to approximately 8 Ka (Dommain et al., 2022), and the lakes are currently situated approximately 25 km apart by a topographic sill (Williamson et al., 1993; **Figure 1A**). Using nuclear genetic data, the *A. grahami* (Magadi)—Natron *Alcolapia* species split has been estimated at 0.007–1.55 Ma (95% HPD) (Ford et al., 2019), which suggests that *A. grahami* populations in Magadi have been separated for at least 7 Ka and may have diverged before the lakes split (Williamson et al., 1993; Dommain et al., 2022). Therefore, our results indicate that strong mate discrimination can either evolve or persist in allopatry without recent (<7 Ka) reinforcement.

The weak species-assortative mating observed in sympatric *A. alcalica* and *A. ndalalani* in this experiment differs from other cichlid studies which generally find strong premating isolation among sympatric species (Knight et al., 1998; Plenderleith et al., 2005; Machado-Schiaffino et al., 2017). However, levels of assortative mating can be lower and more variable among more recently separated sympatric cichlid species

or populations (Jordan et al., 2003; Selz et al., 2016; Nyalungu and Couldridge, 2020). For instance, Selz et al. (2016) found varying levels of assortative mating between *Pundamilia nyererei* populations in two-way choice experiments, but mate choice was strongly assortative when females were provided with a choice of closely related *P. igneopinnis* (96–100%) (Selz et al., 2016). Premating isolation in sympatric *Alcolapia* may therefore be more comparable to that observed between populations or sub-species in other cichlid systems. Incomplete assortative mating in *Alcolapia* may be unsurprising given that the radiation is extremely recent (Ford et al., 2015) and that the degree of genetic differentiation is among the lowest measured across all cichlid radiations (Svardal et al., 2021). Moreover, asymmetry in mating preferences or species discrimination may be common among recently diverging cichlids (Nevado et al., 2011; Malinsky et al., 2015; Van Steenberge et al., 2022) and hence it may be unsurprising that some species exhibit weaker assortative mate choice.

4.3. Factors influencing the strength of assortative mate preferences

The levels of hybridization among *Alcolapia* species reported in our experiments (Figure 3) are high enough that species would likely hybridize to the point of becoming indistinct within a few generations (Irwin and Schluter, 2022). Yet both genetic (Figure 1) and morphological data indicate that hybrids are comparatively rare in most wild populations (Ford et al., 2015; Ford et al., 2016), suggesting that premating isolation is either stronger in the wild or is accompanied by strong selection against hybrids during early life stages.

Assortative mate choice may be influenced by extrinsic factors such as spatial, temporal and environmental components, some of which may not be present in an aquarium setup. For example, spatially-mediated size-assortative mating has been observed in the cichlid *Eretmodus cyanostictus*, where larger males dominate high-quality habitats while smaller, subdominant males occur more frequently in low-quality environments (Taborsky et al., 2014). In the wild, *Alcolapia* may exhibit some spatial separation which could affect encounter rates and influence levels of assortative mating. For instance, Seegers et al. (2001) recorded a higher abundance of *A. latilabris* in the upper courses of streams. During field collections, we observed that *A. latilabris* and *A. ndalalani* mainly occurred in upstream sections and in more rocky habitats, whereas downstream sections with fine-grained substrates were often dominated by *A. alcalica*. On the other hand, observations of breeding leks in Lake Natron were found to be comprised of multiple different species (Seegers and Tichy, 1999). Furthermore, the occurrence of multiple allopatric sites containing only *A. alcalica* indicates that its area of sympatry is less extensive compared to *A. latilabris* and *A. ndalalani*, which have a significant overlap in their distribution (Figure 1A).

A multitude of ecological and environmental factors may influence premating isolation beyond the primary cues used for mate choice. While previous studies on the sensory cues used by cichlids in assortative mate choice have usually found visual cues such as male coloration to be the primary premating cues (Seehausen et al., 1997; Selz et al., 2014), single cues alone seldom control all premating isolation (Plenderleith et al., 2005; Blais et al., 2009; Rometsch et al., 2020). Instead, sensory cues may be multimodal, with each cue contributing to premating isolation to different degrees (Houck and Verrell, 1993; Rafferty and Boughman, 2006; Keller-Costa et al., 2015; Mérot et al., 2015). Due to the lack of shade from terrestrial vegetation, the shallow depths and the high clarity of the water column, the light environment of Natron and Magadi is extremely bright (Johannsson et al., 2014). This bright visual environment may not be fully replicated in the aquarium setup and subsequently, visual cues involved in mate choice may become less salient (Maan et al., 2010; Wright et al., 2018). In line with this hypothesis, the breakdown of reproductive barriers *via* the loss of visual cues may already have occurred in *Alcolapia*, with a high turbidity site on the eastern shore of Natron supporting a potential hybrid population with intermediate morphology (Ford et al., 2015).

Olfactory cues have also been shown to be a component of mate choice in some cichlid species (Plenderleith et al., 2005; Blais et al., 2009). Partitioning of diet among *Alcolapia* species could potentially promote the differentiation in olfactory cues used in mate choice (Kavembe et al., 2016; Ford et al., 2016). As species were fed an identical diet in these experiments, any diet-related odor discrimination would be eliminated. The combination of ecologically mediated premating cues may have an additive effect, where assortative mating is relatively weak with only primary mate choice cues but strong in the presence of additional factors (Tinghitella et al., 2020). Future studies may investigate the relative contribution that different cues (visual, olfactory etc.) play in *Alcolapia* reproductive isolation with aquarium experiments (e.g., Knight and Turner, 1999; Selz et al., 2014). Other factors relating to population density and spatial structuring that may also influence mate choice should be considered.

5. Conclusion

The adaptive radiations of cichlids in the East African great lakes are model systems in speciation research (Kocher, 2004; Seehausen, 2006), but can be challenging when studying early speciation due to their size and complex evolutionary histories. Small and young cichlid radiations are more tractable, but few studies have characterized the reproductive barriers between emerging species. Here, we present evidence of weak to moderate assortative mating both between sympatric and allopatric *Alcolapia* species. These findings are consistent with most study systems at the early stages of divergence, where premating rather than postmating or postzygotic barriers tend to play a greater role in speciation and reach completion at faster rates (Coyne and Orr, 1997; Grant and Grant, 1997; Seehausen et al., 2014). The similar degree of assortative mate choice in allopatry compared to sympatry observed here suggests that assortative mating can accumulate in allopatry, perhaps through divergent sexual selection. The high rates of hybridization observed between species suggest additional factors such as ecological divergence may also be an important component of their reproductive isolation.

Data availability statement

The original contributions presented in this study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

This animal study was reviewed and approved by Bangor University Animal Welfare and Ethical Review Body (AWERB).

Author contributions

AS, WDN, AF, JD, and GT carried out field collections in Kenya and Tanzania. ML, KD, JD, and GT designed the study. ML, MH, and GT carried out the aquarium experiments. ML and KD carried out laboratory work. JH and KD designed microsatellite markers and assisted with the analysis. ML led the data analysis and wrote the manuscript with input from all authors. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2023.1150083/full#supplementary-material

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