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Partner cues and individual variation underlie sex-reversed parental care in poison frogs

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Flexible parental care strategies are widespread in nature and factor into conflict between the sexes and the realization of sex roles. While adaptive explanations abound, the mechanisms underlying flexible 'sex-reversal' of care are less clear. We enlist a biparental frog (*Ranitomeya imitator*) with flexible parental care to investigate the extrinsic and intrinsic mechanisms underpinning parental decisions. Using mate removal experiments in the laboratory, we show that members of the primary caregiving sex (males) show less variation than the flexible sex (females) in their propensity to provide care and that care propensity in females is affected by extrinsic partner cues as well as individual variability. Indeed, individual repeatability in parental effort is high in both typically caregiving and flexible parents. To investigate the underpinnings of differences in care propensity, we sequenced RNA from whole brains of caregiving and non-caregiving frogs of both sexes. While actively caregiving females showed minimal differential gene expression compared to actively caregiving males, females that failed to provide care showed distinct patterns of gene expression. Our findings offer an initial glimpse into the environmental and genetic regulation of individual variation in sex-reversed parental care.

1. Introduction

Cross-sexual transfer, a process by which traits ancestrally expressed in only one sex appear in the opposite sex in descendant lineages [1], provides a powerful framework for studying the evolution of behaviour, including parental care [2,3]. While parenting systems are often characterized by a high degree of task specialization in one or both sexes (i.e. 'sex roles') [4–8], there is also inherent flexibility required for rearing offspring [9]. Selection may capitalize on the mechanisms mediating flexible expression of sex-atypical behaviours within species to drive the evolution of different care strategies between species via cross-sexual transfer [2,3]. While studies across taxa suggest that males and females rely on largely shared molecular 'toolkits' for care [10–13], the conditions that lead to their activation may differ. Thus, understanding the conditions that promote evolutionary reversal of parental sex roles requires probing the mechanisms underlying both their maintenance and plasticity. Because the capacity for sex-reversed care varies among individuals, contexts and species, investigations that incorporate individual behavioural variation will provide new insights into the extrinsic factors and underlying mechanisms that promote cross-sexual transfer of parental care.

Neotropical poison frogs (family: Dendrobatidae) offer an exciting system for investigating cross-sexual transfer of parental behaviour owing to the diversity of care modes among closely related species combined with sex-reversed parental care within species [3,14–17]. While there is tremendous diversity in who expresses specific behaviours, care follows a similar sequence across species: (1) parents care for and guard terrestrial eggs; (2) hatched tadpoles are transported 'piggyback' style to water to complete metamorphosis; and in some species, (3) mothers return to lay unfertilized, trophic eggs for tadpoles to consume (reviewed in [18]). Based on ancestral state reconstructions in this group, West-Eberhard [1] and others [19,20] have theorized that uniparental male care probably gave rise to uniparental female care via biparental care, with female flexibility as a stepping stone. If so, then sex-specific plasticity in parental behaviour lays the foundation for cross-sexual transfer of care.

As in many biparental species, biparental poison frogs subdivide care tasks, with one sex serving as the primary caregiver [21–25]. In the monogamous mimic poison frog, *Ranitomeya imitator*, males and females share the duty of egg care [25], and females facultatively provision tadpoles with trophic eggs [24,26]. Although mothers associate closely with male partners throughout care and feed tadpoles, tadpole transport is performed overwhelmingly by males, who also coordinate egg feeding by alerting females to offspring need [22,24,27]. Yet recent work in a related species and our own observations confirm a capacity for females to take over tadpole transport, especially in the absence of their male partners [12,24,28]. Based on field observations of *Allobates femoralis*, Ringler *et al*. [28] proposed that male acoustic signals may play a key role in informing female takeover decisions. In *R. imitator*, males produce distinct calls to advertise and defend territories, court females and solicit trophic egg feeding [27,29,30], and there is some evidence that reliance on acoustic communication for effective caregiving is associated with the evolution of individual vocal recognition and partner preference [31]. Nevertheless, the extrinsic cues that facilitate recognition and communication among parents are probably multimodal [27].

While the propensity for parental care differs between the sexes, underlying mechanisms may be shared in males and females [10–13]. Recent investigations into the hormonal and neurogenomic basis of care in poison frogs have revealed similar patterns of glucocorticoid response and neural activity in transporting males and females [12,32]; however, the mechanisms mediating behavioural plasticity in the non-typically transporting sex remain unclear. Interestingly, work in the uniparental male species, *Dendrobates tinctorius*, has shown that observing female parents exhibit hormone and brain transcriptome—but not neural activity—profiles paralleling those of their actively transporting male partners [12]. One possible explanation for this pattern is that, in addition to 'priming' for possible takeover of care duties through activation of genes associated with transport, observing parents recruit additional gene sets to monitor the progression of care directed by their partner and make decisions as to whether to takeover. In biparental *R. imitator*, non-transporting females even exhibit similar patterns of neural induction to their actively transporting partners, suggesting the existence of a shared neurogenomic 'caring state' that endures across the entire parental cycle and is independent of specific behaviours [32].

We investigated the mechanisms of sex-reversed tadpole transport in biparental *R. imitator*. We began with mate removal experiments to characterize sex and individual variation in transport behaviour and determine the relative contributions of male visual and acoustic signals to female takeover decisions. We predicted that males and females would differ in their propensity for, and performance of, a male-biased parental task (tadpole transport) but not shared tasks (egg care), and that individual variation in propensity to transport would be inflated in the typically non-transporting sex (females). Furthermore, we anticipated that partner social cues (male presence and isolated acoustic and visual signals) would impose strong effects on female transport decisions, but that male transport would not differ in the presence versus absence of females. To explore underlying mechanisms associated with behavioural differences, we then compared hormones and brain gene expression profiles of males and females that flexibly provide care to females that did not. We expected that both sexes would show changes in glucocorticoid circulation and gene expression associated with the activation of transport behaviour, and that variation between observing, transporting and non-transporting females would pinpoint mechanisms underlying the capacity for sex-reversed care.

2. Methods

(a) Frog husbandry

Frogs for this study were from a captive colony maintained at the University of Illinois Urbana-Champaign (UIUC). Tanks (12 inch × 12 inch × 18 inch glass terraria with mesh lids (Exo Terra, Mansfield, MA, USA)) were located in temperature- (22.05 ± 2.47°C) and light- (12 : 12 h cycle) controlled rooms and ambient humidity (84.95 ± 3.08%) was maintained via a misting system (Mist King, Ontario, Canada). Terraria contained live plants, driftwood, sphagnum moss, leaf litter and film canisters: two horizontal canisters positioned approximately 10″ above the ground for egg laying and two vertical, water-filled canisters on the ground for tadpole deposition. In captivity as in the wild, once a pair bond is established, females lay 1–4 terrestrial eggs as frequently as every 1–2 weeks ([22], pers. obs.). Breeding pairs were monitored daily for breeding activity (e.g. eggs and/or tadpoles). Additionally, tanks were continuously monitored using a video surveillance system (RLC-510A, Reolink, New Castle, DE, USA). Frogs were fed flightless *Drosophila* fruit flies dusted with vitamin supplements three times weekly.

(b) Behaviour

(i) Experimental overview

To investigate the role of social stimuli in mediating sex-reversed tadpole transport, we conducted six types of trials. The first trial served as a behavioural baseline (control), wherein male and female behaviour was observed in the absence of any manipulation. In subsequent trials, either the male or the female was removed to observe the behaviour of single parents. To determine whether a female's propensity to takeover tadpole transport is affected by cues of male presence (visual and/or auditory), we also conducted trials in which males were removed and females were presented with a 3D-printed male dummy painted to match their mate's colouration and pattern (electronic supplementary material, appendix A, figure S1), playback of their mate's advertisement calls (electronic supplementary material, appendix B) or both. Trial order was randomized following the control (baseline) trial. Only pairs that had successfully produced eggs and transported tadpoles on at least two occasions were used in this experiment. Full life-history details for focal individuals are in electronic supplementary material, table S1.

(ii) Data collection and monitoring

Trials took place from October 2021 through June 2023. Trials were initiated on the 8th day (day 8) following the discovery of a clutch (day 0) in the home terrarium. For mate removal trials (male removal and female removal), either the male or the female was moved to temporary housing in a non-adjoining room to prevent any acoustic communication between partners. For visual only trials, the focal male was removed and replaced with his corresponding dummy (electronic supplementary material, appendix A), which was positioned immediately adjacent to the egg canister. For playback only trials, a speaker (Mod1 Orb speaker, Orb Audio, New York, NY, USA) was suspended with its woofer facing down approximately 2 inches above the mesh lid of the terrarium. Recorded calls from the focal male (electronic supplementary material, appendix B) were stored on individual USB drives such that at the end of each 2 h sequence, the track restarted from the beginning and looped for the duration of the trial. Calls were broadcast through an amplifier (MAMP1, MouKey, Solihull, UK) at 60–70 dB, which approximates the sound pressure level of advertising males measured from a distance of approximately 30 cm. The frequency response of the playback system was flat ±2.5 dB over the range of interest (2–6 kHz). Acoustic signals were only broadcast during daytime hours when diurnal *R. imitator* are active (06.00–18.00). To initiate playback + visual trials, dummies and playback were presented as described above but simultaneously. Video/audio recording of daylight hours (06.00–18.00) began on day 9.

From day 9 onwards, focal clutches were inspected daily for hatching. Hatch dates were recorded as the day on which the first tadpole hatched (average 12.9 ± 2.6 days post-oviposition). Eggs hatch asynchronously (up to 24 h apart) and tadpoles are transported individually. Failure to hatch (i.e. due to moulding or desiccation) resulted in termination of the trial and retrial. From the time of hatching, egg canisters and deposition pools were checked daily for evidence of transport and tanks were visually inspected for transporting frogs. Based on our preliminary data, we concluded that hatched tadpoles typically desiccate within approximately 4 days if not transported. Therefore, if no transport occurred within 5 days (excluding hatch day), the trial was ended, and we recorded the outcome as failure to transport. Transport activity was recorded from the time a hatched tadpole disappeared from the egg canister, was visually observed on a frog, and/or appeared in a pool. The disappearance of all hatched tadpoles paired with their failure to appear on a parent or in a pool within 3 days prompted us to end the trial and record the outcome as a failure to transport. If a frog was detected with a tadpole on its back, the individual was given as many days as needed to deposit the tadpole in a pool (successful transport) or until they lost the tadpole (failure to transport). As soon as the first transported tadpole was detected in a pool, we noted the time and location and ended the trial. Though multiple tadpoles may be transported in succession, we only scored the outcome of the first transport event per clutch. At the end of a trial, all stimuli were removed from the tank and mates returned. After pairs were reunited, subsequent trials were conducted on newly laid clutches, such that all pairs were afforded at least 8 days between the completion of one trial and the initiation of another.

(iii) Video scoring

Videos were reviewed to confirm the identity of the transporter (in the case of control trials) and to refine the time window for transport. For trials involving two focal individuals (i.e. male and female pair), individuals were differentiated based on their distinct colour patterns. To determine whether pre-hatching parental behaviours predict transport, we screened video from the last full 12 h light cycle before hatch day. For each focal individual, we quantified the number of times they visited the focal egg canister (defined as full body inside of the canister) and the duration of each visit (minutes), as well as the number and duration of visits to deposition pools (defined as full body inside the pool).

From hatch day onwards, videos were screened for evidence of tadpole transport. Because overhead cameras did not offer views inside of egg canisters, we recorded the timing of tadpole pickup as the last time the focal frog entered the egg canister before it emerged with a tadpole on its back. Transporting frogs were observed until they entered a pool and exited without a tadpole or otherwise appeared in frame without a tadpole on their back, whichever came first. Timing of deposition was recorded as the last time the focal frog entered a deposition pool before emerging without a tadpole. Trials in which frogs failed to transport based on direct inspection of tanks were reviewed for evidence of tadpole pickup.

(iv) Audio scoring

We quantified male call rates across three distinct periods of parental care: (1) the last full (12 h) day before hatch day or *pre-hatching*; (2) the day of hatching up to the moment of tadpole pickup as identified from the video analysis or *pre-transport*; and (3) the time that the tadpole was in transit (i.e. pick-up to drop-off) as identified from the video analysis or *transport*. We predicted that if calling functions primarily within pair bonds to communicate changes in offspring state, then males should reduce both the effort they expend on calling (i.e. overall call rate) and degree to which they modulate calling (i.e. variation in call rate across care) when females are removed. To test this, we selected a subset of 10 trials (n = 5 control and n = 5 females removed) with known hatch dates and transport times to evaluate the context-specificity of calling behaviour. We selected trials involving males with repeated measures in each trial type to provide a direct test of individual plasticity in calling behaviour. We did not score audio tracks for every trial as the scrutiny required for accurate call enumeration rendered this prohibitive at scale (electronic supplementary material, appendix C).

Scoring was conducted blind to treatment groups and sampling periods. We made no distinctions between call 'types' due to strong overlap in acoustic properties [27]. Call rate was quantified by dividing the total number of calls by the duration of the trimmed and filtered track (hours).

(v) Data analysis

All statistical analyses were performed in R v. 4.4.2 (http://www.R-project.org/). To investigate the factors driving variation in transport success across trials, we fit Bayesian mixed-effects models with transport success/failure as a Bernoulli-distributed dependent variable using the package 'brms' [33]. We chose a Bayesian approach for this analysis as classical frequentist models failed to converge, likely due to low rates of transport failure in sex-typical trials. We separately tested the fixed effects of transporting sex and trial type on trial outcomes, including transporter ID and trial order number as random effects in all models. To address whether females became more likely to takeover transport with experience, we additionally fit a model of transport success in trials 2–4 where transport success in trial 1 (the first mate removal trial) was specified as a fixed effect. For all models, we ran four chains of 10 000 iterations for each model, with a burn-in of 1000 iterations. Default priors were used, and convergence was assessed for each model parameter using the Rhat value (all potential scale reduction statistics approx. 1.00). We tested specific hypotheses by extracting posterior draws of the parameter estimates for each level of interest, creating posterior distributions of the differences between selected pairwise contrasts (e.g. male removal versus playback) and computing the highest posterior density interval (HDI) of the distribution for each contrast. If the 95% credible interval (CI) of the difference contained zero, we concluded that there was no evidence for a difference in transport success in the selected contrast. Individual repeatability in transport success, adjusted for the fixed effect of trial type, was assessed using the rptBinary function in the R package, 'rptR' with 1000 parametric bootstraps [34].

In addition to exploring differences in trial outcomes, we compared specific parenting behaviours between the sexes and across trial types. To evaluate sex differences in the latency to transport tadpoles, we fit Cox proportional hazard regression models using the package 'survival' [35]. Other behaviours were modelled using a series of linear mixed models (lmm) and generalized linear mixed models (glmm) with the package, 'lme4' [36]. Specifically, tadpole transit time (i.e. from tadpole pickup to tadpole drop-off) was modelled as a gamma regression with transporting sex as a fixed effect and transporter ID as a random effect. Male call rates were modelled as a Poisson regression with treatment (female present and female removed), parental stage (pre-hatching, hatch day and transport) and their interaction as fixed effects and male ID as a random effect. Pairwise post hoc testing was carried out using the 'emmeans' package with Tukey adjustment [37].

To investigate variation in pre-hatching behaviours, we inspected the distribution of egg and pool visitation rates (0–16) and their cumulative duration (0–12 240 s) in the last full day before egg hatching. The duration of egg and pool visits was log-transformed to meet assumptions of normality, and individual repeatability in each behaviour was assessed using the rptPoisson or rptGaussian functions in 'rptR', as appropriate. We modelled variation in the number of visits to eggs and pools using glmms with Poisson links. Log-transformed durations of egg and pool visits were modelled using lmms. Effects of sex and trial type on each behaviour were assessed by specifying these variables as fixed effect in the models, with the latter being carried out separately for each sex. We next tested whether any pre-hatching behaviours would predict transport propensity among females by fitting a series of binomial regressions with the behaviour as a fixed effect. Finally, we examined the effect of pre-hatching behaviour on latency to transport and log-transformed tadpole transit time among frogs that successfully transported tadpoles. Latency to transport (in integer days) and transit time (hours) were modelled using glmms with Poisson links and lmms, respectively, and pre-hatching behaviour, sex and their interaction were included as fixed effects. In all models, individual ID was included as a random effect.

(c) Hormone and gene expression analyses

(i) Tissue collection

A subset of frogs (n = 21 females and n = 10 males) was selected for whole brain gene expression and hormone analysis. Based on observed individual repeatability in female propensity to takeover tadpole transport when their male partners are removed, we categorized females *a priori* as either flexible (i.e. transported tadpole in a male removal trial) or inflexible (i.e. failed to transport tadpole in a male removal trial). For terminal sampling trials, focal clutches were monitored daily and male partners were removed on day 9 in the sex-reversed social condition.

Frogs were sacrificed at three timepoints: (1) during egg care, but before hatching (9 days post-oviposition); (2) after eggs hatched and tadpole transport was initiated (within 1 day of hatching); and (3) after tadpoles had been hatched for 3 days without transport. Based on behavioural trials, 82% of tadpole transport occurs within 2 days of eggs hatching. Thus, 3 days ensured that tadpoles were still alive and available for transport (i.e. offspring cues still present) but the likelihood that frogs were sacrificed before they had the opportunity to transport was low. This sampling scheme yielded four behavioural groups: egg care (i.e. males (n = 5) and females (n = 5) performing egg care only), sex-typical (i.e. male transporting (n = 5) and female observing (n = 5)), sex-reversed-flexible (i.e. female transporting in absence of male (n = 5)) and sex-reversed-inflexible (i.e. non-transporting female in absence of male (n = 5)). Because hatching times varied between clutches, timing of sampling varied slightly across behavioural groups (sex-typical pairs: days 10–15; sex-reversed females: days 14–20).

Immediately upon capture, frogs were rapidly decapitated and trunk blood was siphoned with heparinized capillary tubes. Blood was centrifuged at 4°C to separate plasma, which was stored frozen at −20°C until further processing. Due to *R. imitator*'s small body size, plasma collection was not possible in all cases. Whole brains were dissected simultaneously with blood collection. Tissue was added to cryotubes pre-filled with ceramic beads (Omni International, Kennesaw, GA, USA) and flash-frozen in liquid nitrogen. Brain tissue was stored at −80°C until further processing. This full process took <5 min.

(ii) Hormone quantification and analysis

Plasma samples were assayed for cortisol using a competitive ELISA immunoassay kit (K003-H; Arbor Assays, Ann Arbor, MI, USA). Our research group recently demonstrated that cortisol is the more prevalent glucocorticoid present in waterborne hormone samples for *R. imitator* [38] and is associated with parental behaviour in *D. tinctorius* [12]. Each sample was run in duplicate by adding equal volume of dissociation reagent to the plasma and incubating at room temperature for 5 min before resuspension in assay buffer. Samples with 1 µl of plasma (n = 2), 2 µl of plasma (n = 7), 4 µl of plasma (n = 1) and 5 µl of plasma (n = 17) were resuspended in 198, 196, 392 or 390 µl of assay buffer, respectively. Assays were carried out following manufacturer instructions, with the exception of using X065 assay buffer to allow for direct comparison with other cortisol and corticosterone measurements in our lab. Absorbance was measured at 450 nm on a BioTek 800 TS plate reader. Final concentrations (ng ml^{-1}) were calculated from the standard curves on MyAssays.com and adjusted for dilution factor before being compared among groups using ANOVA followed by Tukey post hoc contrasts.

(iii) RNA extraction

RNA was extracted using Qiagen RNeasy Plus Mini kits (Qiagen, Venlo, The Netherlands) following standard protocols for purification of RNA from animal tissues. Briefly, we added 350 µl of 0.5% Reagent DX-RLT lysis buffer to cryotubes containing frozen brains and homogenized tissue using a beadmill homogenizer (TissueLyser, Qiagen, Venlo, The Netherlands). We followed manufacturer instructions to elute RNA in 30 µl RNase-free water. Total RNA concentrations were quantified on a Qubit (Invitrogen, Waltham, MA, USA), and RNA was stored at −80°C until further processing.

(iv) Transcriptome assembly, annotation and transcript quantification

Quality control, RNA library construction and sequencing were performed at the Roy J. Carver Biotechnology Center at UIUC. Libraries were prepared with the Kapa Hyper Stranded mRNA library kit (Roche Diagnostics Corporation, Indianapolis, IN, USA). Sequencing was carried out on an Illumina Nova X Plus platform with v. 1.0 sequencing kits and a 150 nt paired-end protocol, yielding on average >65 M reads per sample. Transcriptome assembly and processing was performed on the TinkerCliffs computing cluster supported by the Advanced Research Computing unit at Virginia Tech University (electronic supplementary material, appendix D). Briefly, a de novo assembly was constructed and annotated from corrected paired-end reads following the Trinity-Trinotate pipeline [39,40]. On average, 93.44% of reads mapped back to the final, filtered assembly. Transcript abundances for each sample were aggregated into a single count matrix at the gene level using Trinity's gene_counts_matrix, which yielded a total 859 656 genes of which 43 820 were annotated.

(v) Differential expression analysis

We compared gene expression across sexes and behavioural groups using DESeq2 [41] in RStudio v. 3.386. For each group, low-count genes (<10 in at least half of samples) were filtered prior to analysis, yielding 65 000–75 000 genes per group. To visualize variation between groups, raw expression data was transformed using the variance stabilizing transformation and plotted using the plotPCA function. Principal component scores obtained from this analysis were compared between behavioural groups with ANOVA followed by Tukey post hoc comparisons. We estimated log-fold changes in expression using the 'ashr' shrinkage estimator [42] with a false discovery rate cutoff of $\alpha = 0.05$ applied to all p-values.

To investigate the function of transcriptomic differences across behavioural groups, we queried sets of genes differentially expressed at $\alpha = 0.1$ in each pairwise comparison for enriched GO terms. We used topGO to search for annotations in all three GO categories: biological processes, molecular function and cellular component [43]. The nodeSize parameter was set to 10 to remove GO terms with fewer than 10 annotated genes and only terms with more than 1 significant gene were retained. Significance was estimated using a Fisher's exact test.

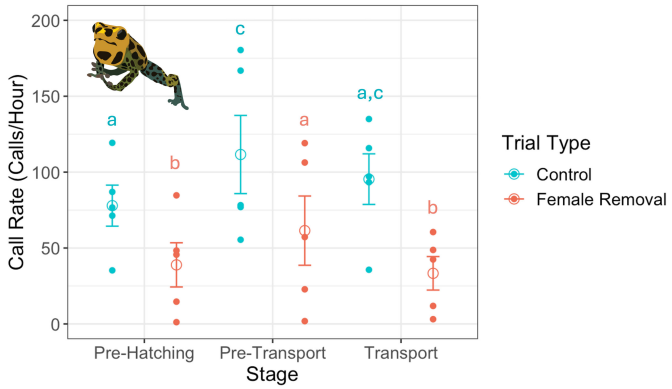

Figure 1. 12 Variation in male call rates by trial type (female present, i.e. 'control' or female removed). Letters distinguish statistically significant differences between groups in pairwise comparisons.

3. Results

(a) Male call rates vary across parenting cycle and between social conditions

Males called at significantly higher rates in trials where females were present (94.96 ± 42.51 calls h^{-1}) compared to trials where females were removed (44.56 ± 37.13 calls h^{-1}; $\chi^2 = 62.013$, $p < 0.0001$; electronic supplementary material, table S2; figure 1). Call rates also varied across stages of parenting ($\chi^2 = 29.567$, $p < 0.0001$), increasing after eggs hatched but prior to tadpole transport in both treatments (female removal: Tukey HSD = 4.959, $p < 0.0001$; control: Tukey HSD = 5.436, $p < 0.0001$), and remaining elevated during tadpole transport when both partners were present (Tukey HSD = 2.987, $p = 0.0336$). In female removal trials, call rates decreased between the pre-transport and transport stages (Tukey HSD = 6.336, $p < 0.0001$).

(b) Sex and treatment differences in transport success

Tadpole transport success was influenced by both transporting sex (electronic supplementary material, table S3) and trial type (electronic supplementary materials, tables S4 and S5; figure 2). Males transported tadpoles in >95% of sex-typical trials and success did not differ in the presence versus absence of female partners (HDI_{mean} [CI] = −12.8 [−54.6 to 14.9]). In trials where males were removed and transport could only be performed by females, a far greater proportion of trials (≥40%) resulted in failure (HDI_{mean} [CI] = −5.77 [−10.4 to −1.67]). Among male removal trials, we predicted that exposing females to acoustic and visual cues emulating their partner would lead to lower transport rates. Although we found no difference in female transport success between male removal trials (i.e. male visual and acoustic cues absent; 58%) and trials involving visual (60%; HDI_{mean} [CI] = 0.459 [−2.99 to 4.17]) or acoustic playback cues alone (40%; HDI_{mean} [CI] = 2.55 [−0.877 to 6.24]), when females were exposed to both male acoustic and visual cues simultaneously, transport success declined to 20% (HDI_{mean} [CI] = 5.78 [0.902 to 11.7]; figure 2). Initial success in taking over transport had no bearing on subsequent successes in females (HDI_{mean} [CI] = −1.96 [−7.29 to 2.53]; electronic supplementary material, table S6).

Transport success was repeatable within individuals ($R = 0.377$, $p = 0.015$), a result that held when the analysis was restricted to only females and adjusted for trial type ($R = 0.313$, $p = 0.061$), meaning individuals who failed to transport in one trial type were more likely to also fail in a different trial type (figure 3).

(c) No sex differences in transport performance or pre-hatching behaviour

Latency to transport did not differ significantly between males (0.826 ± 1.230 days; $n = 24$) and females (1.467 ± 1.885 days; $n = 14$) that transported tadpoles (likelihood ratio test = 1.61, $p = 0.211$; electronic supplementary materials, table S7 and figure S2A), nor were there sex differences in transit time from tadpole pickup to drop-off ($\chi^2_{2,30} = 0.288$, $p = 0.592$; electronic supplementary material, figure S2B). Due to small numbers of transporting females in cue manipulation trials, we do not report on differences in transport behaviour across trial types.

All pre-hatching behaviours except for total time with eggs were significantly repeatable within individuals (number of pool visits: $R = 0.383$, $p = 0.001$; number of egg visits: $R = 0.178$, $p = 0.050$; total time in pool: $R = 0.251$, $p = 0.018$). No differences were observed between sexes or across treatments ($p > 0.05$; electronic supplementary material, figures S3 and S4); however, some pre-hatching behaviours were predictive of post-hatching transport behaviours. Specifically, females that made more visits to eggs on the day before hatching were marginally more likely to transport tadpoles ($\chi^2 = 2.811$, $p = 0.094$; electronic supplementary material, figure S5C). Among frogs that successfully transported tadpoles, males, but not females, that made more egg visitations on the day before hatching took less time to pick up hatched tadpoles ($\chi^2 = 3.232$, $p = 0.072$; electronic supplementary material, figure S6A). In both sexes, shorter tadpole transit times were associated with higher frequency of pre-hatching pool visits ($\chi^2 = 4.668$, $p = 0.031$; electronic supplementary material, figure S6B) and egg visits ($\chi^2 = 15.943$, $p < 0.0001$; electronic supplementary material, figure S6C), with the latter effect more pronounced in female transporters ($\chi^2 = 8.014$, $p = 0.005$).

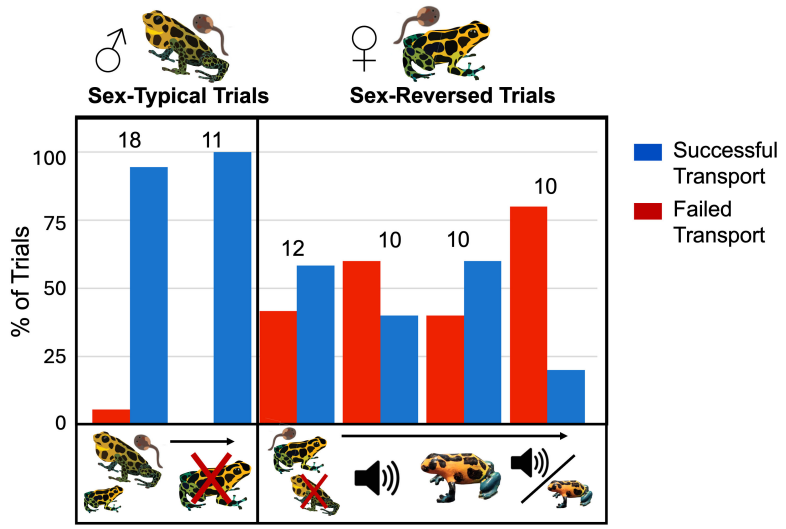

Figure 2. Variation in transport success across six trial types (from left to right): control (both males and females present); female removed; male removed; playback only (male removed, calls broadcast into tank); visual only (male removed, dummy male situated near egg clutch); and playback + visual (male removed, dummy male situated near egg clutch and cues broadcast into tank). Rates of transport success (blue) and failure (red) are depicted as a percentage of the total trials of that type, with number of trials denoted in text above the bars. In all sex-typical trials, transport was performed by the male, whether females were present or absent. In all sex-reversed trials, males were removed, and transport was performed by the female. In the one instance of transport failure observed in a sex-typical (control) trial, the tadpole was picked up but fell off prior to deposition. In all other instances of transport failure, transport was not initiated.

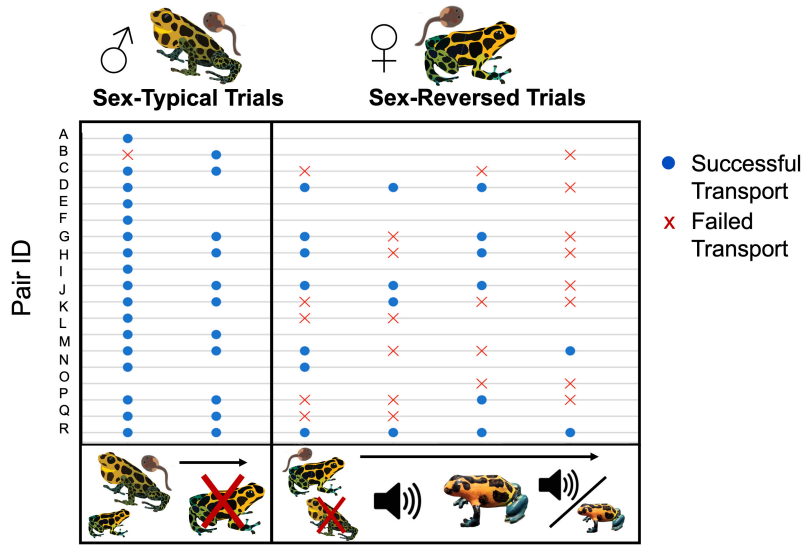

Figure 3. Individual variation in transport propensity across six trial types (from left to right): control (both males and females present); female removed; male removed; playback only (male removed, calls broadcast into tank); visual only (male removed, dummy male situated near egg clutch); and playback + visual (male removed, dummy male situated near egg clutch and calls broadcast into tank). Each row represents a distinct male–female pair, with male behaviour summarized in the first two columns (sex-typical trials) and female behaviour in the last four columns (sex-reversed trials). Blue circles denote successful transport, and red crosses denote failure of transport. Order of trials was randomized across pairs; however, not all individuals experienced all trial types.

(d) Hormone levels sustained across parenting cycle

Low plasma yields due to *R. imitator*'s small body size limited sample sizes for hormone comparisons. We found no significant differences in plasma cortisol across treatment groups ($F_{5,18}$ = 1.417, p = 0.265) but noted a non-significant trend of reduced cortisol levels in inflexible females compared to all other behavioural groups (figure 4; Tukey t = −1.73 to −2.58; $p > 0.05$).

(e) Individual variation in plasticity associated with gene expression signature

We examined differences in brain gene expression associated with differences in parental behaviour using principal components analysis (PCA). Samples separated along three primary principal components, which cumulatively explained 38% (PC1 = 17.9%, PC2 = 11.1%, PC3 = 9.0%) of variance in overall gene expression (figure 5). Differences among groups were marginally significant when considering separation along PC1 ($F_{5,24}$ = 2.116, p = 0.098), but not PC2 ($F_{5,24}$ = 0.6915, p = 0.635) or PC3

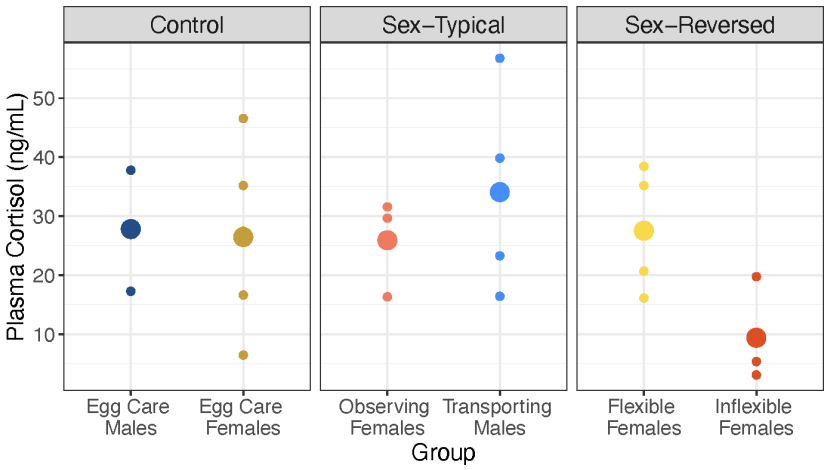

Figure 4. Concentrations of circulating cortisol (ng ml^{-1}) across behavioural groups. Transporting males and flexible females were sacrificed during tadpole transport within 1 day of hatching. Inflexible females were given 3 days post-hatching to transport tadpoles before sacrificing. Small points denote individual samples and large points denote group means.

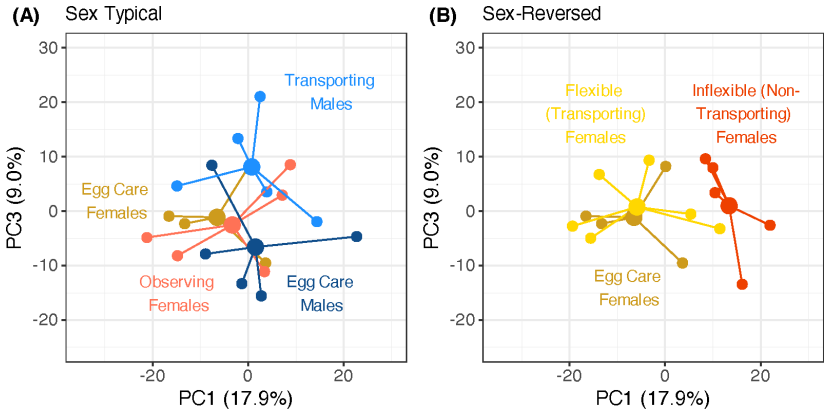

Figure 5. Principal components analysis of brain gene expression patterns among behavioural groups belonging to sex-typical trials (on the left) and sex-reversed trials (on the right). Same-sex egg care samples are plotted in both figures for reference.

($F_{5,24}$ = 1.75, p = 0.162). In sex-typical trials, all groups exhibited overlapping gene expression profiles, with no significant separation (PC1: $F_{5,24}$ = 0.437; p = 0.730; PC2: $F_{5,24}$ = 0.953; p = 0.440; PC3: $F_{5,24}$: 2.618, p = 0.089; figure 5A). In sex-reversed trials, inflexible (non-transporting) females separated significantly from caregiving parents along PC1 ($F_{5,24}$ =6.401; p = 0.013; egg care–non-transporting: Tukey = 19.933, p = 0.029; transporting–non-transporting: Tukey = 19.292, p = 0.020; figure 5B).

Relative to egg care parents of the same sex, transporting males exhibited more differences in gene expression (n = 74 differentially expressed genes; electronic supplementary material, table S8) than either observing (n = 54 differentially expressed genes; electronic supplementary material, table S9) or transporting females (n = 23 differentially expressed genes; electronic supplementary materials, figure S7 and table S10). The highest number of differentially expressed genes of any group was associated with females that failed to transport compared to both egg care (n = 178; electronic supplementary materials, figure S7 and table S11) and transporting females (n = 164; electronic supplementary material, table S12). There were 69 genes overlapping between these two contrasts (electronic supplementary materials, tables S11 and S12), among them enzymes involved in the hydrolysis of peptide and steroid hormones (e.g. Mephrin A subunit alpha, aldo-keto reductase family 1 member C4) and the synthesis and inhibition of neurotransmitters (e.g. gamma-aminobutyric acid receptor subunit rho-1, Pterin-4-alpha-carbinolamine dehydratase). Butyrophilin subfamily 3 member A3 and E3 ubiquitin/ISG15 ligase, two immune genes that were significantly downregulated in transporting females relative to egg care females (electronic supplementary material, table S11), were also downregulated in the contrast between transporting and non-transporting females (electronic supplementary material, table S12).

Within the transport stage, a female's social state (i.e. presence or absence of male partner) also accounted for differences in gene expression (n = 94 differentially expressed genes), notably in genes related to circulation (e.g. calcitonin gene-related peptide type 1 receptor isoform X1, histidine-rich glycoprotein-like) and neuronal function (e.g. glycogen phosphorylase (brain form), synaptotagmin-17 isoform X3, tectonin beta-propeller repeat-containing protein 2, pumilio homologue 2 isoform X2; electronic supplementary material, table S13).

Gene ontology (GO) term analysis revealed significant enrichment of immune response categories in transporting males (e.g. GO:0048525, negative regulation of viral process, GO:0050792, regulation of viral process; electronic supplementary material, table S14) and transporting females (e.g. GO:0002757, immune response-activating signalling pathway, GO:0002764, immune response-regulating signalling pathway; electronic supplementary material, table S15). Similarly, the major pathways enriched

in females observing male transport involved proliferation of T cells (e.g. GO:0046006, regulation of activated T cell proliferation, GO:0050798, activated T cell proliferation, GO:0043382) and other immune cells (e.g. GO:0045621, positive regulation of lymphocyte differentiation, GO:0070663, regulation of leucocyte proliferation, GO:0032604; electronic supplementary material, table S16).

Finally, genes differentially expressed between inflexible, non-transporting females and flexible, transporting females were significantly enriched in functions related to neural cell adhesion (e.g. GO:0060155, platelet dense granule organization, GO:0048041, focal adhesion assembly) and homeostasis (e.g. GO:2001137, positive regulation of endocytic recycling; GO:2001135, regulation of endocytic recycling; electronic supplementary material, table S18).

4. Discussion

Flexible parenting is widespread in nature and often differs between the sexes. Identifying the mechanisms responsible for the induction of care in the typically non-caregiving sex can offer critical insights into the processes that promote cross-sexual transfer and the evolutionary diversification of parental care [1,3]. Here, we studied the extrinsic and intrinsic factors affecting sex-reversed tadpole transport in the biparental poison frog, *R. imitator*. By manipulating social conditions experienced by parents of both sexes, we exposed sex and individual variation in the propensity to perform a male-typical parental behaviour. Collectively, our findings suggest that sex-reversed (female) tadpole transport is more likely in the absence of male signalling, that individual females vary in the propensity for tadpole transport, and that maintaining patterns of gene expression and hormones associated with a caring state may be a prerequisite to the takeover of transport behaviour.

(a) The sexes differ predictably in parental behaviour and propensity to perform a specific task

The first goal of this study was to characterize sex and individual variation in tadpole transport behaviour in a biparental frog for which sex-reversed (female) transport has been documented but not systematically studied. We predicted that if the necessity for female tadpole transport arises infrequently in nature, then both the propensity and performance of this task should be male-biased, whereas shared tasks should not show a sex bias. Consistent with previous accounts of *R. imitator* [25], we found that males and females contribute equally to pre-hatching egg care. However, we observed significant sex differences in transport, with males successfully transporting tadpoles in 96.6% of trials and females transporting in only 50% of trials when males were removed. Females were never observed transporting in the presence of males, although we have occasionally observed pairs transporting simultaneously.

That a strong sex bias in tadpole transport behaviour prevails while both parents are present implies that either males consistently pick up and transport tadpoles before females can or that females actively defer to their male partners. The former explanation seems unlikely, as we observed no sex differences in the latency to pick up tadpoles. In addition, *R. imitator* typically lay 2–4 eggs but transport only one tadpole at a time, such that the availability of hatched tadpoles is unlikely to limit female transport. We also could not attribute transport failures to tadpoles being lost in transit, as this was observed in only a single instance where the transporter was a male (figure 2). Rather, variation in transport success was explained by variation in transport *propensity*, with females less likely than males to initiate transport. Post-pickup, male and female transport behaviour (i.e. transit time from pickup to drop-off) was indistinguishable, suggesting that intrinsic sex differences affect transport initiation, but not transport performance. Intriguingly, individual variation in one behaviour prior to egg hatching (egg visitation rates) predicted female transport propensity in male removal trials. Although this effect was only marginally significant, it supports the idea that female decisions about transport may precede the window for transport.

(b) Partner cues influence probability of takeover by the non-typical caregiver

Results of our mate removal and cue manipulation experiments offer further insights into the extrinsic factors shaping sex-specific parental behaviour. Our study provides two lines of evidence supporting the prediction of Ringler *et al.* [28] that females monitor male acoustic signals to inform takeover decisions. We previously showed that pair-bonded female *R. imitator* discriminate and preferentially associate with advertisement calls of their partner [31]. Here, we show that male *R. imitator* modify calling behaviour in the absence of female partners and across care stages. While continuous calling from within one's territory likely functions in the maintenance of territorial boundaries during care (i.e. male–male communication [23,29,44]), results of our female removal trials imply an additional role in within-pair communication.

Clues as to the precise function for this calling behaviour come from our male removal trials, in which females with mates removed were provided audio recordings of their partner's calls. While the proportion of females successfully taking over tadpole transport was statistically indistinguishable between standard mate removal trials and trials using acoustic playback alone, the combination of realistic visual and acoustic stimuli during the transport window was associated with reduced female transport success. These patterns are consistent with the expectation that females are attuned to multimodal partner cues and use these to make care decisions [28]. In *R. imitator*, pair-bonded frogs typically remain in close visual contact throughout breeding periods, such that detached acoustic playback may yet signal an absent partner to a vigilant female. Similar patterns have been demonstrated in other biparental species, whereby one sex disproportionately 'monitors' efforts by the other to inform compensatory responses [45]. Theoretically, such social cues could be broadcast inadvertently and used in place of

offspring cues to minimize effort in direct evaluation by the flexible sex [46,47]; however, in the case of *R. imitator* males clearly modulate calling effort in response to partners, consistent with other lines of evidence that implicate acoustic communication directly in the coordination of biparental care [27]. The absence of any sex or treatment differences in egg visitation rates in our study further disqualifies the possibility that females use male cues as a substitute for offspring cues. Rather, it seems that females adhere to a simple decision rule of refraining from tadpole transport if males are present, and the acoustic and visual cues deployed in our experiments serve as placeholders (albeit imperfect ones) for an absent partner.

(c) Individual variation in transport propensity is repeatable

Another key result of our behavioural experiment was the demonstration of repeatable individual variation in parenting behaviours. Indeed, both pre-hatching behaviours and transport success were significantly repeatable within individuals, and females that failed to transport in the standard mate removal trial also failed to transport in a majority of cue manipulation trials (figure 3). The extent of individual variation in parenting behaviour in poison frogs and the factors that account for this variation are poorly understood. In our study, the propensity for sex-reversed tadpole transport did not reflect age, parental experience or morph (electronic supplementary material, table S1). Regardless of origin, individual variation in care can have important evolutionary implications. A recent study involving individual-based evolutionary simulations showed that transient, within-sex polymorphisms in care behaviour are almost always the first step in the evolution of sex role specialization [8]. Indeed, individual variation is theorized to be a critical substrate in the evolutionary diversification of parenting systems via cross-sexual transfer of parental behaviours [3]. While the conditions that expose polymorphisms in female transport ability are expected to arise relatively infrequently in *R. imitator* due to the high degree of male coordination that is necessary across stages of care [24,27], the existence of repeatable individual variation provides the possibility that changing environmental conditions could promote selection for females that provide care.

(d) The transition to active parenting is associated with subtle changes in gene expression

Consistent with previous hormonal and neurogenomic investigations of parental care in dendrobatids [12,32], we observed minimal sex differentiation in cortisol or brain gene expression. Remarkably, when comparing parents in the egg care stage to parents in the transport stage, neither sex showed large changes in cortisol concentrations or gene expression, whether in the sex-typical condition (male transporting, female observing) or sex-reversed condition (female transporting, male absent). Our results differ from those of Fischer & O'Connell [12], who in *D. tinctorius* observed significant increases in circulating cortisol and significantly altered expression of hundreds to thousands of transcripts in both sexes in association with the transition to active transport. Species differences may account for some of this variation; for example, because *R. imitator* are pair bonding, changes in hormone circulation and gene expression may arise early in the cycle to synchronize the physiological and neural states of males and females [48]. Indeed, large changes in neural induction in *R. imitator* when comparing non-parental frogs to parents in the transport stage [32] support the existence of an overall caring state that persists across care stages.

While subtle, variation in brain gene expression nevertheless revealed familiar mechanisms associated with parental care, including enrichment of immune response pathways in both observing and actively transporting parents relative to egg care parents. Changes in immune response pathways during care have been observed across taxa and are thought to confer immunoprotection to neurons during profound behavioural changes [48–51]. Interestingly, greater distinctions in neurogenomic state arose when comparing females in the presence (observing) versus absence (transporting and non-transporting) of male partners within a care stage than between care stages or behavioural states, most notably in genes related to circulation and neuronal function. This implies that mate removal triggers changes in female neural states regardless of subsequent behavioural decisions, an effect which may be mediated in part by male acoustic signalling.

(e) Absence of behavioural plasticity is associated with shifts in neurogenomic state

Contrary to our prediction that large changes in gene expression would be associated with the activation of tadpole transport, the most profound differences in our study consistently arose from contrasts involving females identified *a priori* as inflexible, on the basis of failing to takeover transport. While our behavioural analyses showed that transport propensity is repeatable, to ensure that females were not sampled prematurely (i.e. before having the opportunity to transport) sampling was postponed a day beyond the 80% transport window. Inflexible females showed non-significant reductions in circulating cortisol and significantly altered expression of genes involved in hormone and neurotransmitter regulation compared to egg-caring females. Additional differences included the upregulation of immune genes that were significantly downregulated in flexible females during transport as well as the differential enrichment of neural cell adhesion pathways, which could indicate remodelling of synaptic connections. Given that many expression differences attributed to non-transporting females were shared between contrasts with egg care and transporting females, we suggest that the neurogenomic state occupied by inflexible females represents a departure not only from a transport-capable state, but from a caring state altogether. There are at least two possible interpretations of these patterns: (1) inflexible females never achieve a caring state to begin with, and this is the cause of their failure to transport; and (2) inflexible females prematurely exit the caring state, potentially in response to an environmental trigger such as mate removal. Distinguishing between these alternatives will require further experimentation using designs with non-parental adult controls and sampling across multiple time points. However, if male removal does serve as the trigger for

inflexible females 'exiting' a caring state, it would suggest that the same environmental stimulus induces opposite responses in different females, consistent with the existence of a cryptic within-sex polymorphism.

5. Conclusion

This study found evidence for sex differences in the extrinsic motivators, but not the underlying neurogenomic mechanisms, of a sex-biased parental behaviour in a poison frog with clear division of labour, providing insights into how simple decision rules reinforced by communication within pairs can both maintain typical sex roles and promote flexibility. Specifically, we show that male calling behaviour can be used to track the progression of parenting cycles from egg care to tadpole transport and that these signals may function in suppressing parenting behaviours in females while males are present. Our data further suggest that the activation of specific parenting behaviours (here, tadpole transport) need not involve large changes in neurogenomic state; rather, genes expressed during 'priming' may account for the majority of what is needed for the active performance of the behaviour, whether by males or females. This finding reinforces our behavioural data, which show that male and female transporters do not differ in any observable performance measure (e.g. latency to initiate transport or duration of transport) but rather differ only in propensity to transport. Importantly, individuals lacking the capacity for sex-reversed behaviour show subtle variation in parental behaviours leading up to the window for transport and exhibit a distinct neurogenomic state during the transport window. Therefore, rather than being associated with dramatic changes in neurogenomic state in response to a social trigger, the mechanisms responsible for sex-reversed parental behaviour appear to lie in the ability to access and maintain activation of shared machinery even under sex-typical conditions.

Ethics. All animal care and experimental procedures were approved by the Animal Care and Use Committee of the University of Illinois at Champaign-Urbana (IACUC Protocol number 20147).

Data accessibility. All sequencing data have been deposited in NCBI GenBank under the BioProject ID PRJNA1345704. Raw sequence reads have been deposited in the Sequence Read Archive (SRA) under accession numbers SAMN52754457–SAMN52754487 along with the assembled transcriptome. All raw data and code for this project have been deposited in Dryad [52].

Supplementary material is available online [53].

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. J.B.M.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, visualization, writing—original draft, writing—review and editing; B.M.W.: data curation, formal analysis, methodology; S.E.W.: data curation, investigation, methodology, writing—review and editing; K.J.: investigation, project administration; M.E.P.: investigation; E.K.F.: conceptualization, funding acquisition, project administration, supervision, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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