EVIDENCE FOR EMPATHY IN PAIR BONDING POISON FROGS

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Abstract

Empathy is critical to individual and societal wellbeing in humans. More than a century ago, Charles Darwin hypothesized that the empathy-like phenotype is a phylogenetically widespread phenomenon. This idea remains contentious, partly due to few investigations among non-mammalian vertebrates. We provide evidence for Darwin's hypothesis by discovering key empathetic features, emotional contagion and ingroup bias, in pair bonding *Ranitomeya imitator* (Mimetic) poison frogs. We found that the corticosterone level of males appears to positively correlate with female partners across various conditions, including cohabitation and during experimental manipulation. This response is selective towards female partners relative to familiarized female non-partners and is irrespective of partnership longevity or lifetime reproductive output. However, hormonal state matching is not reflected behaviorally, indicating that behavioral state matching can be an unreliable proxy for emotional contagion. These results constitute the first evidence for emotional contagion in an amphibian. Together with findings in other taxa, they suggest empathy is evolutionarily widespread among social vertebrates.

Key words: empathy, emotional contagion, ingroup bias, pair bond, poison frog

Introduction

Empathy is the ability to be directly affected by and share the emotional state of another (Preston and de Waal, 2002), often triggering an adaptive behavioral response such as risk avoidance, social cohesion, conflict resolution, and social bonding (Batson, 2009; de Waal, 2008; Preston and de Waal, 2002). Empathy is considered a 'multi-level' phenomenon that extends from its most basic form, emotional contagion, or the ability to match the emotional state of another without conscious awareness (Keysers et al., 2022). This synchrony relies on a simple perception-action mechanism, where the perception of a "demonstrator's" emotional state triggers an automatic physiological representation of the same emotional state (i.e., "state matching") in the "observer" (de Waal and Preston, 2017; Preston and de Waal, 2002). More than a century ago, Charles Darwin provocatively argued that empathy-related behavior is not unique to humans, but is a phylogenetically widespread phenomenon, upon observing behavioral signs of sympathetic distress across a variety of species (Darwin, 1871/1982; Preston and de Waal, 2002). To date, the evolutionary origins and phylogenic scope of empathy remain contentious (Preston and de Waal, 2002; Schulkin, 2002), partially owing to the scarcity of evidence drawn from more distantly related vertebrates such as anamniotes.

Empathy may be especially apparent within pair bonds, a socio-sexual system emerging in humans and only 1-9% of other vertebrates (birds notwithstanding) (Fischer et al., 2019). Pair bonded relationships display among the highest levels of key empathetic predictors, including ingroup familiarity and selectivity, social closeness, and cooperation (Liévin-Bazin et al., 2018; Preston and de Waal, 2002). For example, pair bonded individuals rely almost exclusively on each other to execute highly coordinated ecological activities that are critical to inclusive fitness, including bi-parental care, joint territory defense, and reciprocal predator vigilance (Bales et al., 2021; Brandl and Bellwood, 2014; Fletcher et al., 2015; Reichard and Boesch, 2003). Since the fitness of pair bonded individuals is heavily reliant on each other, a mechanism for appropriately responding and attending to each other's needs is critical. However, outside of humans, empathy within pair bonded relationships has only been studied in voles and zebra finches (Burkett et al., 2016; Perez et al., 2015), leaving open the possibility of this phenomenon in other pair bonding vertebrates.

Here, we present evidence for emotional contagion in a pair bonding amphibian, the Mimetic poison frog (*Ranitomeya imitator*). *Rantomeya imitator* males and females form prolonged partnerships characterized by affiliative interactions, the mutual defense of a shared territory, and joint care for offspring (Brown et al., 2010, 2008). By subjecting male-female dyads to an "empathy assay" similar to one developed for rodents (Burkett et al., 2016), we tested the hypothesis that male observers would display emotional (fear) contagion and ingroup bias towards female partners (demonstrators) that were subjected to a stressor. Specifically, we predicted that males would state match stressed females hormonally and behaviorally despite never experiencing or observing the stressor

themselves, and that this response would be biased towards partners and increase with the longevity and lifetime reproductive output of partnerships.

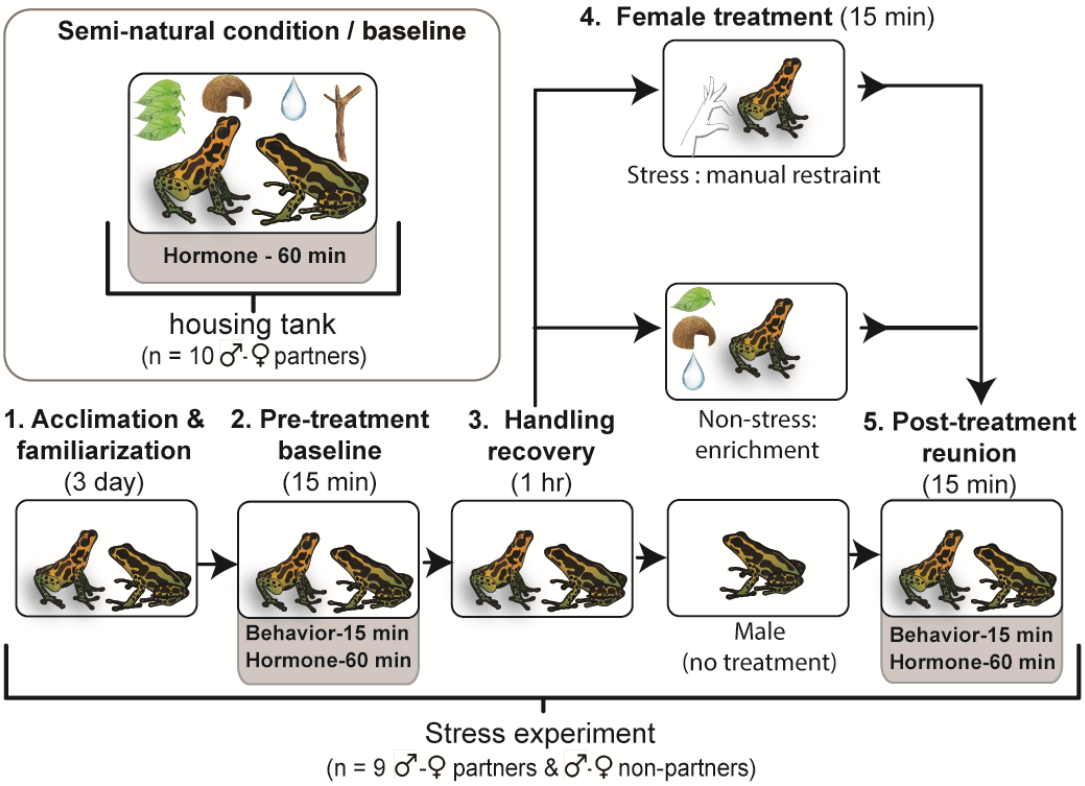

Figure 1. Experimental design for testing partner-selective emotional contagion in pair bonded male *R. imitator* poison frogs. Pair bonded males were assayed for state matching of female behavior and corticosterone hormone within housing terraria, and when females were experimentally subjected to a stress treatment that males did not observe themselves. In the stress experiment, the same males were assayed for state matching towards both female partners and familiarized female non-partners.

Methods

Establishment, longevity, and lifetime reproductive output of pair bonds

All procedures were approved by the Stanford University Animal Care and Use Committee (APLAC protocol numbers 32961 and 33880). *Ranitomeya imitator* used in this study were sexually mature adults reared in our breeding colony. Pair bonds were established by cohabitating male-female dyads in individual glass terraria (30.48 X 30.48 X 45.72 cm, Exoterra, Mansfield, MA) and allowing them to breed. Pair bond establishment was marked by the first successful reproductive bout consisting of tadpole deposition into a breeding pool. Once bonded, partnership longevity was calculated as the number of days from pair bond establishment to testing (132-1238 days across pairs). Tadpole deposition of each pair was monitored 3 times weekly. The number of tadpoles

deposited were recorded before being removed from housing tanks to facilitate ongoing reproduction. The lifetime reproductive output of pairs, calculated as the cumulative number of tadpoles deposited from partner establishment to testing (3-87 across pairs), was recorded up to the date of testing. Housing terraria were lined with sphagnum moss, live philodendrons, a climbing log, and film canisters for egg laying and tadpole deposition. Frogs were kept on a 12:12 h light cycle and fed wingless *Drosophila melanogaster* fruit flies dusted with vitamin supplements three times weekly.

Experimental design, and behavioral and hormonal sampling

Partner selective emotional contagion was examined using an “empathy assay” used for rodents (Burkett et al., 2016) and modified for amphibians. Pair bonded males (n = 9) and either their female partner or a female non-partner were removed from their housing terraria and co-housed within experimental behavioral observation terraria for 3 days prior to trials to acclimatize dyads to terraria and allow male-female non-partner dyads to become familiarized. The behavioral observation terraria (36.4 X 21 X 12.4 cm) were lined with moist sphagnum moss, contained two small, embedded canisters for shelter, and covered with a transparent lid that was punctured to allow for ventilation and video recording using a mounted GoPro®.

The day following acclimation, trials began and were conducted between 13-16:00. Male-female dyads were video recorded for pre-treatment behavior for 15 minutes. Immediately following, each animal was sampled for pre-treatment corticosterone levels by water-borne methods (Baugh et al., 2018; Baugh and Gray-Gaillard, 2021; Rodríguez et al., 2022). Each animal was placed in a separate sterile petri dish (8 cm diameter X 2 cm high) containing 20 mL milli-q water that was conditioned with essential ions for amphibians (Josh’s Frogs RO R/x, Osowwo, MI, USA) for 60 min. Then, both frogs were placed back into the behavioral arena for 1 hour to recover from potential handling stress. Following recovery, females were removed from the behavioral observation terraria and underwent either a 15 min hand restraint stress treatment or a 15 min non-stress treatment. The hand restraint treatment, which involves gently restraining a hind leg to inhibit movement, is standard for inducing a mild acute stress for studying stress hormones in amphibians (Coddington and Cree, 1995; Narayan et al., 2010; Narayan, 2013; Santymire et al., 2018). In the non-stress treatment, females were placed into terraria like the behavioral observational terraria, except enriched with leaves, a shelter log, and live philodendrons. These treatments were unobserved by males. Immediately following female treatment, females were reunited with males in the behavioral observation terraria and behavior and hormones were re-sampled as described above (**Fig. 1**).

All males underwent the experimental testing with their female partner and again with a female non-partner. The female non-partner was matched for social and reproductive status (all pair bonded and reproductively mature yet not active) and size

(length: ± 1 g, weight: ± 0.6 mm) to female partners as best possible. The order in which males underwent female partner/non-partner and female stress/non-stress treatments was assigned arbitrarily and was separated by at least 1 day. Immediately following trials, animals were placed back into their housing terraria. To compare individual corticosterone and male-female corticosterone co-variation between experimental and "semi-naturalistic" conditions, we also sampled corticosterone from pair bonded dyads that were naïve to experimental testing (n = 10) immediately following removal from their housing terraria using methods described above. Animals were not fed during or 12 hrs prior to experimentation to avoid response variation due to satiation state. To control for the potential influence of reproduction on corticosterone levels (Orchinik et al., 1991), only animals that were not actively reproducing at the time of testing (i.e., their housing terraria contained no eggs or tadpoles) were used. No reproductive behavior was observed during trials.

Behavioral quantification and statistical analysis

See Supplementary Methods S1 and Supplementary Table 1.

Corticosterone quantification

Water-borne hormone sampling and extraction was conducted following the methods of Rodriguez et al. (2022), Baugh et al. (2018), and Baugh and Gray-Billard (2020). Briefly, water from the water bath was immediately stored at -80°C for up to 2 weeks. Following, samples were thawed on ice, and water was collected with a 20 mL sterile syringe and pumped through a C18 cartridge (SPE, Sep-Pak C18 Plus, 360 mg Sorbent, 55–105 µm particle size, #WAT020515, Waters corp., Milford, MA) at a rate of ca. 10 mL/min. Cartridges were then eluted with 4 mL of 96% EtOH into 8 mL borosilicate vials. Two mL were used for corticosterone analysis, and the remaining 2 mL were stored at -80°C for re-running samples that did not initially pass quality control, or for future studies. Eluted corticosterone samples were then dried down with nitrogen gas at 37°C, resuspended with 250 µL of assay buffer (provided in ELISA kit, see below), and incubated overnight at 4°C.

Reconstituted samples were brought to room temperature and shaken at 500 rpm for 1 hr prior to laboratory assays. To estimate corticosterone concentration, we used a commercial enzymatic immunoassay (Enzo corticosterone ELISA kit, Catalog # ADI-901-097). Samples were plated in technical duplicate and assays were performed following the manufacturer's protocol. Plates were read at 405 nm, with correction between 570 and 590 nm, using a microplate reader (Synergy H1, BioTek Instruments, Winooski, VT, USA), and the concentration of corticosterone was calculated using the software Gen5 (version 3.05, BioTek Instruments, Winooski, VT, USA). The detection limit for the assay is 27 pg/mL, and samples that fell out of this range were removed from analysis. Samples

with the average intra-assay coefficient of variation (CV) above 20% were excluded from analysis. Cross-reactivity with deoxycorticosterone is 28.5%, and with other related steroid compounds is < 2% (see product manual for details).

Corticosterone statistical analysis

All statistical analyses and data visualizations were performed in R (V. 4.0.3; the R Foundation for Statistical Computing). Prior to analysis, corticosterone concentration was log-transformed to achieve normal distribution when necessary. First, we tested for differences in corticosterone concentrations between housing and the experimental pre-treatment (baseline) condition. For this, we performed a simple linear model including log-transformed corticosterone concentrations as the response variable, and the interaction between condition (housing and experimental pre-treatment baseline), dyad type (partner and non-partner) and sex as predictors. We further computed estimated marginal means (least-squares means) contrasts using the 'emmeans' function within the *emmeans* package (Lenth, 2022). P-values were adjusted using Tukey's method. Only pair bonded dyads were examined within the housing condition since animals were only housed with partners.

Following, differences in corticosterone concentration between experimental treatment conditions were examined separately for each dyad type and sex with a linear mixed-effects model (LMM) using the 'lmer' function within the *lme4* package (Bates et al., 2014). The model included log-transformed corticosterone concentration as the response variable, the interaction between treatment condition (non-stress and stress) and trial (pre- and post-treatment) as fixed effects, and frog identity as the random factor. Post-hoc analysis was conducted using estimated marginal means with Tukey's adjustment method.

Next, we tested corticosterone state matching of male-female dyads within and across conditions. We ran analyses separately for each condition (housing tank, experimental pre-treatment baseline, and post- stress/non-stress treatment) and dyad type (partner and non-partner). Since corticosterone concentration for the housing condition was normally distributed, we performed a simple linear correlation using Pearson's correlation coefficient. For the experimental conditions, we performed a LMM with male corticosterone log-concentration as the response variable, female corticosterone log-concentration and post-treatment condition (post-stress or post-non-stress) as fixed predictors, and frog identity as a random factor.

Finally, we tested whether male-female state matching predicted the longevity or lifetime reproductive output of partnerships. We ran a separate multiple regression for each condition (housing, experimental pre-treatment, post- stress, and post-non-stress treatments), using male corticosterone levels as the response, and female corticosterone levels, partnership endurance, and reproductive output as predictors.

Results

Males selectively state match their partners for corticosterone irrespective of the endurance or lifetime reproductive output of partnerships

Male corticosterone levels positively correlated with those of female partners within the housing baseline (Pearson's correlation test: t = 2.69, $r_{(8)}$ = 0.69, P = 0.02; **Fig. 2A**) and experimental pre-treatment baseline conditions (LMM: β = 0.5, t = 5.1, R^2 = 0.62, P < 0.001; **Fig. 2B**). There was a similar trend after controlling for the post-treatment conditions (stress/non-stress), although to a statistically non-significant extent (β = 0.34, t = 2.31, R^2 = 0.25, P = 0.08; **Fig. 2C**), likely due to small sample size (n = 5 and 6, respectively) and subsequent under-power within these treatments (see Supplementary Table 7 for details). By contrast, male corticosterone levels did not correlate with those of female non-partners across any condition (pre-treatment: LMM: β = 0.06, t = 0.21, R^2 = 0.003, P = 0.83; post stress/non-stress: LMM: β = -0.14, t = -0.55, R^2 = 0.34, P = 0.6; **Fig. 2D, E;** see Supplementary Table 7 for details). The extent of male-to-female partner corticosterone matching was not explained by the endurance or lifetime reproductive output of partnerships (P-values for all treatment conditions ≥ 0.05; Supplementary Table 9 and Supplementary Figure 3 for details).

Fear contagion assay does not provoke a fear response, nor do males behaviorally state match females

Subjecting females to the stress treatment caused no increase in corticosterone levels in female demonstrators or male observers across time points (planned least-squares means contrast: P ≥ 0.05 in all groups; Supplementary Tables 5, 6 and Supplementary Figure 1A, B, D for details). Furthermore, subjecting females to the stress treatment had no effect on the individual or coordinated fear behavior of female demonstrators or male observers of partner and non-partner dyads in any time point comparison (P ≥ 0.05 for all treatment comparisons; Supplementary Tables 2-4 and Supplementary Figures 1C, E and 2D-G for details). Together, this indicates that the assay treatment did not provoke a stress response as intended. Finally, males did not behaviorally state match females in any experimental condition, except for gaze and approach towards non-partner females in the non-stress condition (R^2 = 0.83, S = 20; P = 0.01; Supplementary Table 8 and Supplementary Figure 2A-C for details).

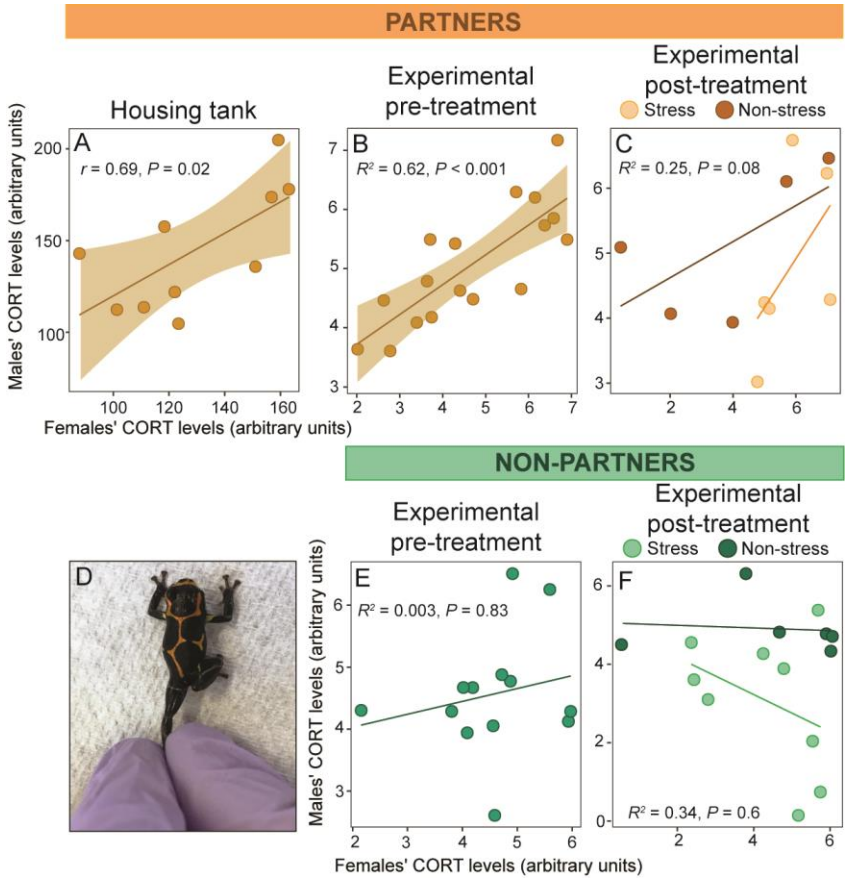

Figure 2. Partner-selective corticosterone matching in pair bonded *R. imitator* males. Males corticosterone (CORT) level matched (**A-C**) female partners, but not (**E-F**) familiar female non-partners, across semi-natural and experimental conditions. Regression lines are shown, with shaded regions denoting 95% confidence intervals for statistically significant results. (**D**) Example of gentle leg-restraint stress treatment.

Discussion

Our study suggests emotional contagion in a pair bonding amphibian, where males hormonally and selectively state match their female partners. These findings parallel those for mammals, birds, and zebrafish that live in familiar social groups (Fernandes Silva et al., 2019; Perez et al., 2015; Pérez-Manrique and Gomila, 2022). Here, we discuss the implications of this research considering the scientific controversy surrounding empathy across animals.

Emotional contagion in male poison frogs: evidence for deep evolutionary origins

We discovered that male pair bonded poison frogs display hormonal state matching exclusively towards female partners compared to familiar non-partner females. This is similar to other socially monogamous species, including humans and *T. guttata* song birds, where empathetic stress is more pronounced between partners compared to non-partners (Engert et al., 2014; Perez et al., 2015). However, since the stress treatment was insufficient for inducing a hormonal or behavioral response in female demonstrators, we cannot be completely certain male-to-female state matching resulted from males directly responding to female physiology or behavior — a criteria of emotional contagion. An alternative explanation is that male-female hormonal state matching resulted from

both sexes responding in a similar manner to an unknown external stimulus. This is unlikely however, as male-to-female hormonal state matching appears to be maintained after being separated into different experimental environments and is partner specific. We therefore suggest *R. imitator* males display emotional contagion selectively towards their female partners. Such socially selective emotional regulation was previously thought to rely on cortical brain regions and to therefore be unique to mammals (Decety, 2011). However, in addition to mammals, it has also been documented in amphibians (current study), fishes (Fernandes Silva et al., 2019) and birds (Perez et al., 2015). Together, these observations suggest that socially selective empathy is phylogenetically widespread among social vertebrates.

Inter-partner emotional contagion in a non-stressful context: ecology and adaptive function in pair bonded poison frogs

Understanding emotional contagion within an unaroused state (e.g., during rest or routine activity) is ecologically relevant yet poorly studied relative to stress or reward states. Modeling predicts that it is only adaptive and expected for an observer to experience emotional contagion towards others who are emotionally aroused (Nakahashi and Ohtsuki, 2015). Yet, studies in humans report evidence for sustained emotional contagion in non-stressful states, where individual diurnal cortisol patterns are consistently linked between romantic partners (Engert et al., 2018; Liu et al., 2013; Papp et al., 2013; Saxbe and Repetti, 2010). We found similar results in the current study, where partner-selective emotional contagion was observed across contexts that frogs experienced as non-stressful (i.e., during cohabitation and experimentally). Furthermore, this response was not reflected behaviorally. In an unaroused state, maintaining emotional resonance with but behavioral independence from group members might afford behavioral freedom in a context where coordination doesn't confer adaptive outcomes, while providing a means to "stand by" in preparation for contexts in which it does (e.g., the sudden need for bi-parental care). Finally, these results demonstrate that behavioral state matching can be an unreliable proxy for emotional contagion, reaffirming the need for measuring both physiological and behavioral components (Pérez-Manrique and Gomila, 2022).

Male-to-female emotional contagion is independent of the longevity or reproductive fitness of partnerships

In pair bonding species, empathy may be an affective mechanism through which cooperation occurs, enhancing lifetime reproductive output. In this process, partnership endurance might be a cause and/or a consequence of empathy. Indeed, several studies on pair bonding and bi-parental species have demonstrated partnership longevity increases parental cooperation, offspring output and survival, and ultimately lifetime reproductive fitness (Black, 2001; Griggio and Hoi, 2011). However, we found that inter-partner emotional contagion was not correlated with partnership longevity or lifetime reproductive output. Possibly, these relationships went undetected because they exist on a timescale that was not surveyed or were artifactually masked in a laboratory

environment. Additionally, inter-partner emotional contagion in the unaroused state might not reflect that during parental care. Alternatively, emotional contagion in poison frogs might have no impact on reproductive success or partnership endurance. Indeed, in humans, the duration of romantic relationships can have only a marginal association with intra-couple cortisol co-variation (Engert et al., 2018). Additional work is needed to resolve these alternative possibilities to inform the biological significance of inter-partner emotional contagion in *R. imitator*.

Conclusions

We found that pair bonded mimetic poison frog males display hormonal, but not behavioral, state matching selectively with their female partners, suggesting socially selective emotional contagion in amphibians. Along with similar findings in fish, birds, and mammals, this suggest that socially selective empathy is a widespread feature of social vertebrates.

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Competing interests

Lauren A. O'Connell is a reviewing editor for eLife.

Author contributions

Jessica Nowicki: Conceptualization, Methodology, Investigation, Data curation, Writing – original draft, visualization, Project administration, Funding acquisition. **Camilo Rodríguez:** Methodology, Software, Validation, Formal analysis, Data curation, Writing - review and editing, Visualization. **Julia Lee:** Investigation, Writing – review and editing. **Billie Goolsby:** Validation, Investigation, Data curation, Writing – review and editing. **Chen Yang:** Methods, Software, Formal analysis, Resources, Data curation, Writing – review and editing. **Thomas Cleland:** Methods, Software, Writing – review and editing. **Lauren O'Connell:** Methodology, Resources, Writing – review and editing, Supervision, Project administration, Funding acquisition.

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