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Original Article

Sexual selection does not drive hindwing tail elaboration in a moon moth, *Actias luna*

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The most emblematic animal traits are often attributed to sexual selection. While this pressure is an important force, elaborated traits that have been driven solely by natural selection are less enumerated. Here, we test an elaborate trait in moths—hindwing tails—that has been studied in an anti-predator context, but that remains unstudied for its role in mating. We gave female *Actias luna* (Saturniidae) moths a choice between two males of differing hindwing tail treatments. In our primary experiment, males with intact tails garnered more matings than males with tails removed. This difference appears to result from damage incurred by tail removal, however, as demonstrated with additional experiments. We created a tail/no-tail experimental set where we removed tails from both males, then reglued tails to one and applied glue only to the hindwings of the other. We found no significant difference in mating success between these males. To ensure that this result was not due to the glue itself, we offered females two intact males, with glue added to the wings of one. This set also had equal mating success. We therefore do not find evidence that tails play a role in sexual selection. These results, in combination with previous research on bat-moth battles using *A. luna*, indicate that the non-sexually dimorphic hindwing tail was likely driven by natural selection. We suggest that future research testing multiple selective forces is needed to reveal the prevalence of natural versus sexual selection as the primary force driving trait elaboration in diverse animal taxa.

Key words: **saturniidae, mate choice, alternative selective forces**

INTRODUCTION

Sexual selection is one of the primary drivers of some of the animal kingdom's most charismatic features (Andersson 1994). Elaborate traits—traits that are complex and conspicuous when compared to recent common ancestors—are commonly studied for their role in mate acquisition. Some notable examples of traits that attract female attention include male peacock (*Pavo*) tail trains that expand to reveal over 150 iridescent eyespots (Petrie et al. 1991), elongated caudal fin "swords" in male swordtail fish (Basolo 1990) and the structurally convoluted call of male Túngara frogs (*Physalaemus*) (Rand and Ryan 1981). The handicap principle (Zahavi 1975) hypothesizes that sexually selected traits provide an honest signal of the bearer's vigor, driven by an inherent cost of the signal. This concept has been met with some criticism (Grafen 1990; Penn and Számadó 2020), as it can be difficult to detect the cost of a trait (Somjee 2021). For instance, the peacock's tail does not seem to pose a measurable locomotor deficit (Askew 2014; Thavarajah et al. 2016). Studies into other elaborate traits have revealed costs, including increased energetic budgets with increasing sword length (Basolo and Alcaraz 2003) and heightened predator attraction to more complex frog calls (Page and Ryan 2008).

Some elaborate traits are driven by both sexual and natural selection. Female poison frogs (Maan and Cummings 2009; Dreher et al. 2017) and chemically defended *Heliconius* butterflies (Finkbeiner et al. 2014), for example, prefer more brightly colored males, providing evidence that natural and sexual selection together drive conspicuous aposematic male coloration. Moreover, a trait that evolved initially in response to one of these pressures may be co-opted and enhanced in response to the other. Many moths use ultrasonic sound production to attract mates, with males and females of some species performing duets that likely function to identify conspecifics (Conner 1999; Nakano et al. 2008, 2009). Lineages that use ultrasound production for mating also commonly use it in an anti-predator context, to warn bats of bad taste and to jam bat sonar (Nakano et al. 2014; Barber et al. 2022). More evolutionary analyses are needed to determine which evolutionary force was the primary driver of the origination of this trait.

An elaborate trait can also arise solely by natural selection—some birds and ungulates flash conspicuous color patches as pursuit-deterrent signals (Caro 1995; Stang and McRae 2009) and multiple unrelated caterpillars (i.e., a non-reproductive animal)

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have horns behind their head that reduce wasp attacks (Kandori et al. 2022). Aside from larval examples, however, it can be difficult to determine whether a trait has evolved in the absence of sexual selection and often with deeper investigation, a reproductive function emerges. For instance, while pursuit-deterrent traits seem to be directed at predators, they might also provide quality information to potential mates (Byers et al. 2010). A clear understanding of whether a trait has evolved as an anti-predator trait without the influence of sexual or social selection requires thorough understanding of the study system and multiple lines of evidence. One intriguing example is the hindwing tails of swallowtail butterflies (*Papilio*) that appear to deflect bird strikes (Chotard et al. 2022). Morphometric analysis of male and female hindwing tails in swallowtails show no sexual dimorphism, which could indicate a lack of sexual selection (Koutrouditsou and Nudds 2021), but mating experiments are needed. Parsing the different evolutionary pressures that drive the formation of elaborated traits requires holistic understanding of an animal's biology and ecology.

Saturniid moths are one such well-studied system. Within this speciose group (~3400 described species (Kitching et al. 2018)), an elaborate anti-predator trait has evolved multiple times—long hindwing tails that can be more than double the length of the moth's wingspan (Barber et al. 2015). In each independent evolutionary origin, these tails are twisted and their tips cupped, resulting in structures that spin behind the moth as it flies. Staged battles between insectivorous bats and tailed *Actias luna* and *Argema mimosae* moths demonstrate that tails draw bat attack away from the vital body core. Tails increase the moth's escape success compared to individuals whose tails have been experimentally removed, as well as non-tailed members of the same tribe (Saturniini) (Barber et al. 2015; Rubin et al. 2018). Kinematic analysis of moth flight during bat-moth battles indicates that these survival differences are not due to differing flight abilities, but instead are likely due to echoic reflections off the tail tips that create an alternative target for the bat to strike (Rubin et al. 2018).

Male saturniids do much of the flying to seek out mates (Janzen 1984; Lamarre et al. 2015). Due to their short lifespan (~7 days), saturniids do not forage and females usually begin pheromonally calling from a perch close to their pupal site within 24 h of eclosion (Janzen 1984; Scoble 1992). A female can draw in multiple conspecific males with the pheromonal plumes that she releases from glands at the tip of her abdomen (Brown 1972). She often mates with the first male that arrives and begins laying eggs the next night, without coupling with any other males (mondandry) (Janzen 1984; Morton 2009). Moreover, unlike moths in many other families, female saturniids demonstrate no neural antennal responses to their own species pheromone, making it unlikely that they select mates based on male pheromones (Holdcraft et al. 2016). It is possible that females make a visual assessment of potential mates, especially on full moon nights when the nocturnal light levels are elevated, but a deeper understanding of the visual perception of saturniid moths would clarify this possibility (Sondhi et al. 2020; van der Kooi et al. 2021).

We tested whether the elaborate, anti-bat hindwing tails of *A. luna* also play a role in reproduction. We used tailed *A. luna* moths as our model to query conspecific females as to their perception of the tail. Morphologically, male and female *A. luna* appear (to the human eye) quite similar with both sexes possessing an equally long tail. While sexual dimorphism can be an indicator of a sexually selected trait, it is not a requirement for the trait to influence mate selection, as it can signify differing natural histories between the sexes, a lack of cost associated with the trait, or an instance of unresolved intersexual gene conflict (Slatkin 1984; Pennell and Morrow 2013; West-Eberhard 2014). To determine whether sexual selection is driving tails in the same direction as nocturnal predation pressure, we staged mating experiments where each female *A. luna* had a choice between two males of different hindwing tail treatments. Based on saturniid natural history and previous work in this system, we hypothesized that the presence or absence of a tail would not affect mate selection. By testing sexual selection, and leveraging previously amassed knowledge about natural selection pressures on tails, we can gain deep understanding of its evolutionary route. *Actias luna* may stand as an important case study for understanding the relative selective forces that can drive trait elaboration.

METHODS

Behavioral paradigm

To determine whether hindwing tails are under sexual selection, we staged mating choice tests in mesh-sided tents (Big Agnes Tiger Wall 3 Carbon Tent: 2.2 × 1.5 × 1.1 m, Eureka Suma Tent 2P: 2.2 × 1.4 × 1.1 m, BioQuip Collapsible Cage: 0.6 × 0.6 × 0.9 m) placed outdoors in Nashville, TN and Gainesville, FL during three months (April–June), over three years (2020–2022), for a total of 78 trials. All moths were either purchased from Carolina Biological Supply as pupae, or were reared in the Kawahara Lab. We exclusively used moths that were no older than 1–2 days post-eclosion in our experiment. To ensure that recently eclosed females did not emit pheromone and no moths mated before trials, we kept pupae in a room with constant light (Millar et al. 2016). On experimental nights, we placed two unmated male *A. luna* adults of different treatments and one unmated female adult together in a tent (Figure 1a,b). Male treatments are defined as follows and were presented in the following dyads (Figure 1c):

Tails vs. no tails

To determine whether males with tails secure more matings than males without tails, we offered females intact (tails intact) and ablated (tails removed at the base of the hindwing) males ($n = 25$).

Damage control

To determine whether differences in the above trials were driven by damage from cutting off the hindwing tails (in ablated treatment), we offered females sham control males (tails removed and reglued to the hindwing), and ablated control males (tails removed and glue applied to the cut location—to control for the glue added to sham control moths). Glue was applied to the underside of the wings in both treatments ($n = 27$).

Glue control

To ensure that the smell of the glue was not masking the effect of tails in the *damage control* trials, we offered females intact males (tails intact), and glue control males (tails intact and glue applied to the underside of the base of the hindwing; $n = 25$).

We randomly assigned males to receive one of the two treatments in each dyad and randomly assigned the dyad to a female. To allow enough time for possible mating, we placed moths in their experimental tent at dusk and left them until dawn, at which point we recorded which individuals had mated (Figure 1b). *Actias luna* moths stay in copula for the entire duration of the night once they

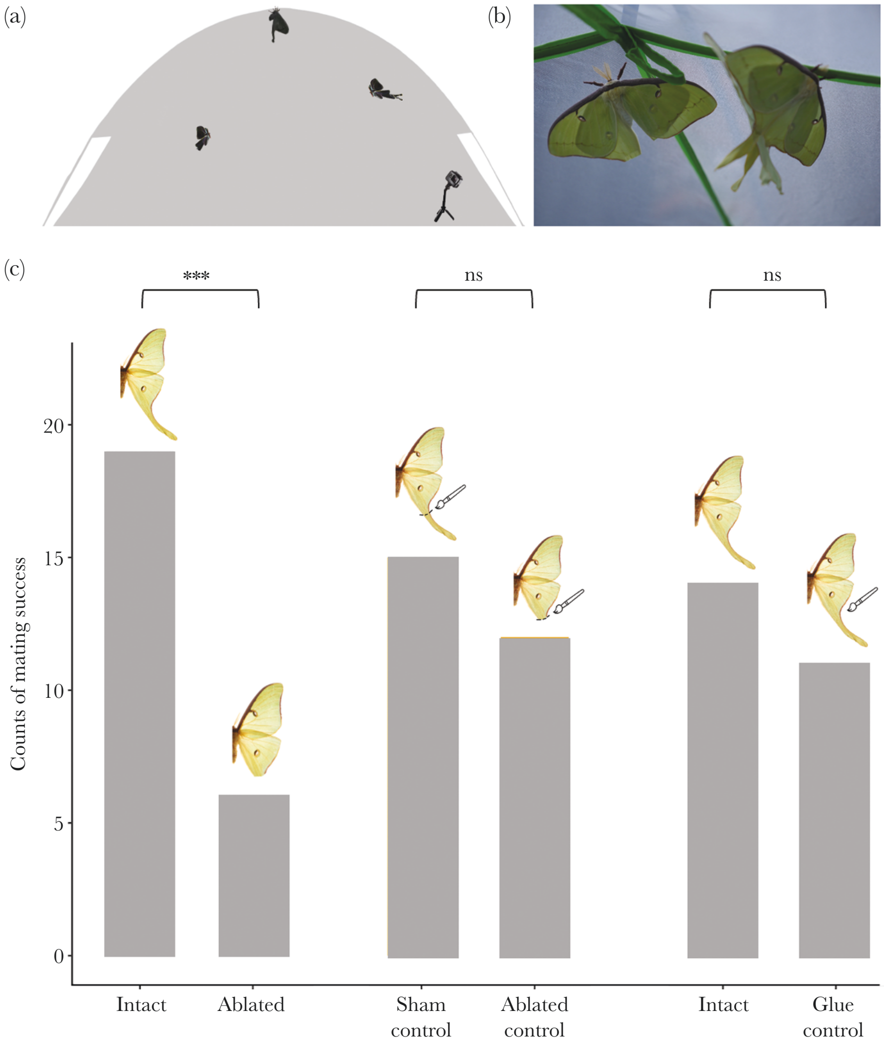

Figure 1.
Hindwing tails of *Actias luna* moths do not drive mating success. (a) We conducted experimental mating trials in mesh tents, each populated with one female and two males of differing hindwing treatments. (b) We left moths for the entirety of the night and in the morning noted which male had succeeded in mating with the female. (c) To test if hindwing tails are involved in mating success, we conducted trials with three different experimental sets of hindwing tail treatments (represented by A. luna images in the three comparisons above). While males with unmodified tails (intact) garnered more matings compared to those with tails removed (ablated), control trials reveal that this is due to the damage incurred by cutting the tails. The difference in mating success between a tailed and non-tailed male disappears when comparing males with tails removed and then reglued (sham control) and males with tails removed and glue applied to the base of the hindwing (ablated control). Glue control trials comparing mating success of males with unmodified tails (intact) and males with glue added to intact tails (glue control) indicate that the glue itself did not influence trial outcome in the sham vs. ablated control experiment. Each bar represents the total number of matings that a given treatment acquired (individual males and females were only given the opportunity to mate once). Mating success was only compared within experimental sets (intact vs. ablated: total $n = 25$ trials; sham control vs. ablated control: total $n = 27$ trials, intact vs. glue control: total $n = 25$ trials). The triple stars indicate a statistically significant difference ($P < 0.05$), while "ns" indicates no significant difference ($P > 0.05$).

have paired, and we therefore consider our approach to be a reliable metric for mating success. When possible, we filmed the interactions from within the tent, using an infrared (IR) light (Raytec) and an IR-converted GoPro Hero4 (Backbone) camera set to timelapse mode on a small tripod. These moths are unlikely to see in the IR portion of the spectrum (van der Kooi et al. 2021) and thus we do not believe the IR light influenced the outcome of the trial. We checked each tent for mating ~3 h after dark, and again after sunrise. In the event that a female was not pheromonally calling by ~3 h after dark, which can be discerned by the eversion of her genitals, we replaced her with a new female and allowed the trial to continue throughout the night. At the 3-h check, we recorded the temperature and humidity with a digital monitor, as well as the relative amount of nocturnal light using a Sky Quality Meter ((SQM), Unihedron; units: magnitudes per square arcsecond). This device cannot relay information about the spectrum or relative intensity of different wavelengths of nocturnal light, but rather gives an integrated measurement of night sky brightness. We used the nightly SQM output as a proxy of the available light that moths would have to inform mating decisions and only compare light levels within this study. To create a size metric for individual males, we took size-calibrated photos of each male moth and extracted the surface area of his right fore- and hindwings using imageJ (Schneider et al. 2012).

For interactions that were captured by video, we used VLC media player to view and extract time stamps for behaviors of interest. We noted when a female first presented a receptive posture—pinning her wings back and everting her genitals—and how much of the total time each male spent close to her, which we defined as in view of the camera (entire roof of the tent), as well as how many times each male physically contacted the female before any mating occurred, which we term "bopping." Because videos were captured in timelapse, we did not measure the absolute time between when the female first began presenting and when mating occurred, but rather noted the sequence of events among individuals.

Statistical analysis

We built a series of generalized linear models in R studio using the "lme4" package (Bates et al. 2015), fitted with a binomial distribution to compare mating success across treatments. We ran separate sets of models for each individual dyad, as each was a functionally distinct experiment. To determine the effect of potential confounding factors, we built a series of binomial models with treatment always included as a fixed effect, in addition to one of the following: male surface area (using right forewing and hindwing surface area as a proxy), environmental nocturnal light level (SQM), temperature, humidity, and cage category (large, small). We scaled numerical variables (male surface area, SQM, temperature, humidity) to make them more readily comparable across different units. We performed model checks using the "DHARMa" package (Hartig 2018) and tested for multicollinearity in models that contained multiple predictor variables using the car package (Fox and Weisberg 2019; although none of these more complex models were included in the final dataset). To compare model fit for the three model sets (one set for each dyad), we used the Bayesian Information Criterion (BIC), which penalizes model scores for additional parameters while avoiding inflated Type I errors due to successive pairwise testing (Taper and Ponciano 2016) (see Supplementary Material and Rubin and Kawahara (2023) for model checks, BIC tables, and model outputs). We then ran a separate binomial model for each experimental set, using size difference between the males as the sole fixed effect and mating success of one of the treatments as the dependent variable, which estimates the intercept when males are the same size (see Vega-Trejo et al. 2014). Finally, to compare the outcomes of all trials, we built an intercept model that included all treatments, with experimental set identifier included as a random factor. Recent simulation studies indicate that a grouping factor (random effect) with fewer than five levels can still accurately estimate fixed effects values (Gomes 2022). All code and outputs can be found in Rubin and Kawahara (2023).

RESULTS

We found no evidence that females prefer males with tails (Figure 1c). Males with tails (intact) received significantly more matings than males with tails removed (ablated) (n = 25; intact mean = 0.76 ± SE: 0.09, ablated = 0.24 ± 0.10, $P = 0.002$) in our first set of trials. We found, however, that damage control trials with males whose tails were removed and replaced with glue (sham control) and males whose tails were removed and glue added to the base of the hindwing (ablated control) did not exhibit the same difference (n = 27; sham control = 0.56 ± 0.10, ablated control = 0.44 ± 0.10; P = 0.42). A set of glue control trials revealed that this lack of difference was not due to females' aversion or attraction to the presence of glue (n = 25; intact = 0.56 ± 0.10, glue control = 0.44 ± 0.10; $P = 0.40$) (Figure 1c). To extract these parameter estimates, we compared BIC scores of the models to find the best fitting model for each experimental set. We found that for all three experiments, either the model that included treatment only as fixed effect performed the best, or the models that included treatment only or treatment and male size performed similarly (within 2 BIC of each other). Parameter estimates from these two top models were functionally the same and thus, given the small sample size, we proceed with interpretation of the simpler (treatment only) model. Temperature (range: 61–93 °F), humidity (range: 19–94%), and light level (range: 13.31–21.47) were never significant covariates. We verified that only the results of the intact vs. ablated trial statistically differed from the expected 50–50 mating success using a cross-trial comparison intercept model (tail experiment P-value = 0.01, damage experiment P-value = 0.56, glue experiment P-value = 0.55). Our size only models, which estimate whether a size difference between the males affects the mating success of a male treatment, revealed that for all experimental sets, size difference was not a significant factor (tail experiment P-value: 0.52, damage experiment P-value: 0.236, glue experiment P-value: 0.227. All code and model outputs are provided in Supplementary Material and (Rubin and Kawahara 2023).

Of the 77 pairings, we were able to video record 18 mating events. This small sample size was due to a limited number of available cameras at the time (1–2), cameras being used to film tents where mating did not occur, or the female pheromonally calling from a part of the tent that was off-camera. In nearly all trials, females flew to the top of the tent and called from this elevated location (Figure 1a). Males in different treatments generally spent a similar amount of time near the female ("near" = in camera view). In only 4/18 videos (22.2%) did a male spend less than half of his time near the female and this did not seem to impact trial outcome. That is, in just half of these cases, the male that spent more time near the female succeeded in mating with her. In 11 video recordings, the male that successfully mated bopped the female more than the male that failed. This value did not differ significantly from random chance, based on an exact binomial test ($P = 0.48$). On average, males bopped females 13.4 times (standard error: 2.7) before mating. The maximum number of times that a single male bopped a female was 73. In trials that did not result in mating (excluded from these analyses because there was no mating success), males bopped females, but females did not present a receptive posture.

DISCUSSION

We tested the effects of sexual selection on an elaborate hindwing trait in a saturniid moth and found no evidence that this pressure drives hindwing trait elaboration. While hindwing tails provide a measurable anti-predator benefit against echolocating bats (Barber et al. 2015; Rubin et al. 2018), they do not appear to provide a mating benefit. We did find a difference in mating success between tailed and non-tailed moths, but this difference was likely driven by the damage created by the ablation procedure, rather than by female choice. In tethered laboratory experiments where the moth is under acute predatory attack, there is no significant kinematic difference between intact and ablated or sham control moths (Rubin et al. 2018). It may be that in the context of a few-second-long bat attack, moths with damaged wings can perform as well in flight as intact moths to evade predators. However, in a longer-duration, lower-intensity context, such as finding and acquiring mates over the course of an entire night, a damaged moth may be at a disadvantage due to overall lower flight performance. From our data, we cannot determine whether wing damage hampers flight, or whether it saps energetic resources that lead to an overall reduction in flight time, or reduced mating drive. We note that in all video recordings, damaged and intact moths spent approximately the same amount of time near the female (i.e., within frame of the camera) and both were fully capable of flying to the top of the cage where the female was often resting.

We also note an intriguing "bopping" behavior observed in multiple males, in which a male tapped the female as it flew by her (Video S1). After several bops, the female often assumed a receptive posture, pinning back her wings to better reveal her everted genitalia. This receptive response has been documented in another saturniid moth, *Hyalophora cecropia*, in response to male "fluttering" (Sasaki et al. 1983). Although we had low power, we did not find a significant effect of bopping on mating success. This bopping behavior and its limited influence may be due to cage constraints. For example, it may be that males were accidentally hitting the female in their attempts to disperse or to locate the source of the pheromone plume ("fanning") (Loudon and Koehl 2000). It is also possible that the limited space allowed a mating opportunity for males who would otherwise not have been able to mate with the female, as the female was primed to be receptive by the bopping of the other male. Wild or larger-cage studies may reveal more about the role of this behavior in the mating biology of moths.

Male bopping could aid in species identification if the behavior promotes a transfer of chemical information between sexes. Expression of pheromone binding proteins on female antennae is often quite low, however, and females do not demonstrate behavioral or physiological responses to pheromone challenges (Steinbrecht et al. 1995; Callahan et al. 2000). Our glue control trials did not reveal any statistical difference in mating success between an unmodified male and a male with glue added to his wings, indicating that the addition of this substance was unlikely to influence mate selection. It is possible that females did not respond to glue because the chemical composition of the glue was too divergent from naturally occurring biological compounds that would have stimulated her olfactory receptors. Alternatively, this result may simply belie a lack of chemosensory discrimination by female *A. luna*.

In addition to their lack of chemosensory discrimination, we also found no evidence that females were using vision to assess potential mates. The light environment (as measured by an integrated SQM reading) did not have explanatory power in our study, nor did females seem to be using a visual cue—tails or no tails—to distinguish amongst males, as described above. Saturniid moths have the capacity to detect light wavelengths between 340 and 560 nm, roughly ultraviolet to green (van der Kooi et al. 2021). We did not compare the receptive behavior towards conspecific males with behavior towards heterospecific males in this study, and thus we cannot know whether females are using certain reflectance spectra for species identification, although this assessment would presumably not be affected by the presence or absence of tails. To date, most studies of moth nocturnal vision have focused on foraging in hawkmoths (Sphingidae) (Kelber and Roth 2006). More work is needed to study saturniid vision and how it impacts non-foraging behavior.

Sexual selection is often credited with molding some of the world's most striking animal traits due to the tight genetic link between the bearer of the trait and the selective agent on that trait (Lande 1981). We did not find evidence that sexual selection has driven the luna moth's elaborate hindwing tails. Evidence from prior predator-prey studies leads us to conclude that bat predation is likely a major driver of hindwing tail evolution in saturniid moths. Tails improve moth escape success from predatory attack, with increasingly long tails creating an increasingly successful deflection effect against echolocating enemies (Rubin et al. 2018). These seemingly conspicuous appendages do not seem to come with a tradeoff from diurnal predation, however (Rubin et al. 2023). Without an evolutionary cost, lineages that evolve this trait should experience pressure almost exclusively towards elongation. In accordance with this, comparative phylogenetic analyses of saturniids do not support tail length reduction in any clade across the family (Barber et al. 2015; Rubin et al. 2018). However, flight constraints likely limit tail elongation past a certain point (Park et al. 2010; Aiello et al. 2021; Hamilton et al. 2021) and further research into saturniid kinematics may reveal such a limit. In sum, the various pressures and constraints on this elaborate trait appear to fall within the bounds of natural selection. Understanding a system in this holistic manner is critical for tracing its evolutionary route and may help reveal broad patterns about the types of selective pressures that have most commonly led to trait elaboration.

SUPPLEMENTARY MATERIAL

Supplementary material can be found at http://www.beheco.oxfordjournals.org/

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DATA AVAILABILITY

Analyses reported in this article can be reproduced using the data and code provided by Rubin and Kawahara (2023).

AUTHOR CONTRIBUTIONS

Juliette Rubin: Conceptualization-Equal, Data curation-Equal, Formal analysis-Equal, Funding acquisition-Equal, Investigation-Equal, Methodology-Equal, Project administration-Equal, Validation-Equal, Visualization-Equal, Writing – original draft-Equal. Akito Kawahara: Conceptualization-Equal, Funding acquisition-Equal, Project administration-Equal, Resources-Equal, Supervision-Equal, Writing – review & editing-Equal

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