

Predator-induced shape plasticity in *D. pulex*

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Abstract

All animals and plants respond to changes in the environment during their life cycle. This flexibility is known as phenotypic plasticity and allows organisms to cope with variable environments. A common source of environmental variation is predation risk, which describes the likelihood of being attacked and killed by a predator. Some species can respond to the level of predation risk by producing morphological defences against predation. A classic example is the production of pedestals and head spikes in the water flea, *Daphnia pulex*, which defend against predation from *Chaoborus* midge larvae. Previous studies of these defences have focussed on changes in pedestal size and the number of spikes along a gradient of predation risk. Although these studies have provided a model for continuous plasticity, they do not capture the whole-organism shape response to predation risk. In contrast, studies in fish and amphibians focus on shape as a complex, multi-faceted trait made up of different variables. In this study, we analyse how multiple aspects of shape change in *D. pulex* along a gradient of predation risk from *C. flavicans*. These changes are dominated by the inducible morphological defence, but there are also changes in the size and shape of the head and the body. We detected change in specific modules of the body plan and a level of integration among modules. These results are indicative of a complex, multi-faceted response to predation and provide insight into how predation risk drives variation in shape and size at the level of the whole organism.

INTRODUCTION

A major challenge facing all organisms is to adapt to environments that vary within their lifespan. A route to responding to and surviving such variation is phenotypic plasticity, the ability of individual genotypes to change phenotype when exposed to different environments during their life-cycle. One pervasive, natural source of environmental variation is the risk of mortality from predation, known as predation risk. Predation risk induces a suite of changes in the behaviour, life history and morphology of many plants and animals. Of particular interest are morphological responses to predation risk which range from the production of spines to changes in the shape of a portion of the body or the entire body plan of an organism.

Many studies of predator-induced shape change have focussed on linear assessments of shape, which measure the distance between two points. Key examples include changes in defensive dorsal spine length, body depth that affects vulnerability to gape-limited predators and morphological features associated with behavioural swimming escape responses. However, this type of analysis only captures a subset of the overall shape variation.

Measurement of overall shape is a multivariate analysis (i.e. it involves multiple different variables). A well-established method for assessing multivariate plasticity in shape is geometric morphometrics, which uses anatomical coordinates as shape variables to measure relative differences in shape. This approach has

been used extensively to measure predator-induced changes in shape for a wide range of organisms, such as fish, amphibians and snails.

Geometric morphometrics not only allows the measurement of shape overall, but also the extent of modularity and integration between different aspects of shape (Klingenberg, 2014). Modularity refers to the level of covariation between different traits within morphological structures, or modules, relative to the level of covariation between these structures. Integration refers to the level of co-variation between different traits throughout a morphological structure or even a whole organism (Klingenberg, 2014). Therefore, modularity exists if the level of integration within modules is strong compared to the integration between modules (Klingenberg, 2009).

In addition to considering multivariate shape, there has been a shift from the standard "two-environment" approach for assessing phenotypic plasticity and estimating reaction norms (Roff 1999) to analysing changes along a gradient. More recently, it has become standard to evaluate plasticity in multiple traits and along environmental gradients of more than two environments.

Despite such work, shape has rarely been assessed as a plastic trait in water fleas (*Daphnia* species), an iconic organism for the study of size-selective, predator-induced phenotypic change. Instead, research has largely focused on assessing the production of inducible morphological defences, such as the head spikes of *Daphnia pulex*, called 'neckteeth', which develop in response to predator cues (kairomones) released from their midge larvae predators. Although there has been some research into the dorsal pattern of induced morphology in *D. pulex*, the question of how overall shape changes in response to predation risk remains unanswered.

Other examples of predator-induced changes in *Daphnia* suggest that overall shape may change in response to predation risk. Considerable variation exists in many features of the *Daphnia* spp. body plan including body width, alignment, shoulder shape and carapace strength. Furthermore, changes in body size are well-documented in the context of size selective predation theory and empirical assessment of life history responses to predation risk. Together with associated research on fish, this suggests that overall shape might increase survival and therefore provide important fitness benefits to plasticity.

In this study, we evaluate shape plasticity along a gradient of increasing predation risk in three genotypes of *D. pulex* which differ in their sensitivity to predator cues. We apply morphometric landmark-based analysis to photographs of *Daphnia* taken by, in which *D. pulex* were exposed to six levels of predation risk from their midge larvae predator, *Chaoborus flavicans*. We combine geometric morphometrics with phenotypic trajectory analysis to formally evaluate the multivariate change in shape and estimate measures of both modularity and integration to evaluate if there are coherent units of the body plan that respond to predation risk, and whether these units change independently or together.

In advance of the analysis, we predict narrower bodies and bigger heads will form part of the predator-induced response in *D. pulex*. Narrower bodies enhance the predator escape response in fish and amphibians and we expect that bigger heads are more likely to interfere with predation linked to the neckteeth defence. Given these two predictions, we might also predict modularity and / or integration of these responses. From a modularity perspective, we predict that the head region and lower body are separated in their response to predation due to the nature of the induced morphological change in the head region. This means that the neckteeth defence and associated shape changes of the head would be localised to that part of the animal and relatively independent of changes to the body. Furthermore, under the modularity hypothesis, we might also predict that changes in the dorsal region, where the neckteeth form, respond independently to the ventral portion of the daphnid. As for the integration hypothesis, we predict some level of integration between the head and lower body regions driven by a negative developmental correlation, where the head gets larger and the body gets narrower and longer.

MATERIALS AND METHODS

To evaluate these hypotheses, we first analysed the phenotypic trajectories of three *D. pulex* genotypes along

a gradient of predation risk, focusing on the magnitude, direction and shape of the change in multivariate trait space. Second, we performed modularity and integration tests to evaluate the head-body and dorsal-ventral hypotheses mentioned above. In the following, we introduce the study system, data and design before details on the above process.

Study system

In this study, we focus on one of the most iconic predator-induced defences in water fleas (*Daphnia* species), the ‘neckteeth’ of *D. pulex* (Fig. 1). The neckteeth defence comprises a swollen area on the back of the head (neck-pedestal) and spikey bits which grow on top. The defence grows in response to predator cues (kairomones) released by *Chaoborus* midge larvae. The onset of kairomone sensitivity begins in the late embryonic stage, when the neck-pedestal begins to grow, and the spikey bits develop later in the early juvenile stages. The maintenance of the defence requires consistent exposure to predator cues and usually lasts until the third instar, after which the *Daphnia* are large enough to escape size-selective predation. Also, it is well known that the induction of the defence increases with the level of predation risk. Although the exact mechanism is unclear, it has been shown that the neckteeth defence increases prey survival by up to 50%.

Study design

We used data originally collected in Dennis *et al.* 2011. This included photographs of second and third instar *D. pulex* exposed to six different concentrations of *C. flavicans* kairomone, including a control (0, 0.1, 0.25, 0.5, 0.75 and 1 $\mu\text{l ml}^{-1}$). Animals were photographed at the second and third instar because this is when the defence is usually expressed the most. For each treatment, third-generation mothers of at least their third brood had been exposed to the relevant cue concentration and offspring of the subsequent brood were transferred to glass jars which contained 50ml of hard artificial pond water, food (2×10^5 cells ml^{-1} of algae) and the appropriate concentration of predator cue. Animals were transferred to a new jar containing fresh media and cue daily, until they reached maturity. Photographs of the animals were taken at the second and third instars.

Three genotypes were analysed for shape plasticity from this dataset. Two (clones Chardonnay and Cletus) showed a characteristically low level of induced morphology and one (clone Carlos) a characteristically high level. Animals from second and third instars were pooled so the data formed a factorial design of six predation risk levels x three genotypes. There were 5-10 replicate photos (i.e. replicate individuals) at each level of predation risk for each clone (totalling 518 photos).

Digitisation

We digitised images using geometric morphometrics to create two-dimensional anatomical co-ordinates (reference points, called ‘landmarks’) that characterized body shape. First, lateral-view photographs were uniformly orientated (head at the top) and prepared for landmarking in Microsoft Paint. This involved drawing a vertical line from the eye to the base of the tail (a standard measure of daphnid length) and then two perpendicular lines, one at the midpoint of the original line and another intersecting the rostrum. These lines provided a clear method to capture the approximate location of the dorsal and ventral midpoints, as well as the region on the back of the neck where defences were induced, consistently between different images (Fig. 2A). This process identified a total of six landmarks for each photo including (1) the centre of the eye (near the top of the head), (2) the neckteeth defence (or the corresponding area in the controls), (3) the base of the tail, (4) the dorsal (back) midpoint, (5) the ventral (belly) midpoint and (6) the rostrum (snout). These landmarks were motivated by a method developed for linear dimensions in two species of *Daphnia*, *D. dentifera* and *D. mendotae* and were selected to capture key aspects of the defence, head and body shape.

Images were then digitised by applying landmarks using the *geomorph* package v4.0.5 in R v4.2.1. Before these images were analysed in R, they were standardised using generalised Procrustes superimposition from the *geomorph* package v4.0.5. This process aligned the images to account for differences in the size, position and orientation of the original specimen so that shape could be compared in a meaningful way. The resulting

Procrustes shape coordinates (Fig. 2D) were used in the subsequent analyses.

The amount of human (intra-observer) error in selecting landmarks was measured using two sets of identical photographs. The same landmarks were applied to 30 photographs of either the defended or undefended morph, and the standard error of each landmark was measured from the Procrustes shape coordinates.

Data analysis

To test the statistical significance of variation in shape across different factors, including size (a commonly used factor in morphometric analyses, measured as the mean position of all the landmarks for an individual specimen), instar, genotype and predation risk, we performed Procrustes ANOVA using the `procD.lm()` function from the *geomorph* package v4.0.5 . The Procrustes ANOVA used a permutation procedure of 10,000 iterations to assess the importance of variation in shape across the different factors for our set of Procrustes-aligned coordinates.

To further understand the relationship between different factors, specifically genotype and predation risk, we performed trajectory analysis using the `trajectory.analysis()` function in *RRPP* package v1.3.1 for R . The phenotypic trajectory analysis measured morphological variation between treatments in terms of its magnitude (distance moved in shape space), direction (angle of the change in shape space) and shape (relative position of the change in shape space). The mean phenotypic trajectories were visualised using principal component analysis and were connected in order of increasing predation risk. Thin-plate spline deformation grids were used to describe the principal component axes by indicating the departure from the mean shape of the sample to the lower and upper bounds of the sample (see .

We evaluated modularity and integration of morphological (co)variation using the covariance ratio and partial least-squares (PLS) analysis .The CR is a ratio of the overall covariation between modules relative to the overall covariation within modules. The significance of the CR is tested by comparison to a distribution of values obtained by randomly assigning landmarks into subsets. A significant result, which indicates modularity, is found when the observed CR is small relative to this distribution.

When used with landmark data, PLS analysis is referred to as singular warps analysis . The analysis calculates normalized composite scores (linear combinations), one from the X-variables and one from the Y-variables, that have the greatest mutual linear predictive power. Similar to the test for modularity, the observed PLS value is compared to a distribution of values obtained by randomly permuting the individuals (rows) in one set relative to those in the other. A significant result, which indicates integration, is found when the observed PLS correlation is large relative to this distribution.

We applied the CR and singular warps analyses with 999 iterations to test for two non-mutually exclusive potential patterns of modularity and integration between 1) the head (snout, head top, neck) and lower body (belly, tail base, back) regions, and 2) the dorsal (head top, neck, back) and ventral (snout, belly, tail base) regions. It is important to note that these tests do not represent two ends of a continuum. Both modularity and integration can co-occur and it is entirely possible to find modules (by rejecting a null model of no covariation within modules) and detect integration between these modules (by rejecting a null model of no covariation between modules). We performed these tests across clones and predation risk levels.

Ethics statement

We confirm that the methods employed in this study were reviewed and approved by the institutional review committee and all animals from the original experiment were cared for in accordance with institutional and national guidelines.

RESULTS

Observer error

The amount of human (intra-observer) error in selecting landmarks was very low for both the defended and undefended morphs (defended mean SE = 0.74×10^{-3} [unitless], undefended mean SE = 0.72×10^{-3}

[unitless]). This suggests that any differences in shape measured in the following analyses are the result of the treatments applied, rather than any observer error.

Predation risk alters shape

Analysis of *Daphnia* shape using Procrustes ANOVA revealed that shape varied across all factors, including size, instar, clone and predation risk (all $p < 0.001$). The response to the level of predation risk did not vary by instar (predation x instar interaction; $F = 1.39$, $df = 5$, $p = 0.13$), but it did vary by genotype (predation x genotype interaction; $F = 4.92$, $df = 10$, $p < 0.001$). The phenotypic trajectory analysis showed that this interaction was not based around how much the clones responded to predation cues (path distances were equivalent, all pairwise differences $p > 0.05$), but around differences in the direction of these changes in multivariate trait space (the angle of the change in shape space) and the shape of these changes (the relative position of the changes in shape space, all pairwise differences $p < 0.01$) along the six cue concentration gradient (Fig. 3-5). Specifically, the direction of change in clone ‘Cletus’ differed from both clone ‘Chardonnay’ and ‘Carlos’ (both $p < 0.05$) indicating that the landmarks that changed in ‘Cletus’ were different than in the other two clones and we can see the evidence for differences in the shape of the trajectories in Fig. 5, where each clone follows a slightly different path along the cue concentration gradient.

Neckteeth, head height and belly-bulge

The principal component axes underlying the trajectory analysis indicated that 55% of variation in shape was captured by the first PC, rising to 71% with PC2 and 83% with PC3. A visualisation of the trajectories in 2D PC space, along with detail on what shapes were associated with the PC-axes, revealed several key insights (Fig. 3-5). Based on the deformation grids outside the PC-axes, we were able to define PC1 as neck-change (i.e. the inducible defence), PC2 as head height and PC3 as belly-bulge.

Fig. 3 revealed that the most significant change in shape occurred along PC1, the inducible defence. The trajectories of all three clones moved in parallel to PC-axis 1, which showed that the neck region was larger when there was more cue. Also, there were clear differences among the genotypes linked to head height (PC2 axis). We suggest that this is largely representative of clone differences, as Fig. 3 and 4 indicated relatively large differences between genotypes compared to the effect of the cue concentration.

Head and body shape is modular, but there is integration across all body regions

We assessed two hypotheses using modularity and integration tests. First, we asked whether there was evidence for modularity and integration between the head (snout – head top – neck) and body regions (belly – tail base – back). Second, we asked whether there was evidence for modularity and integration between the back of the head (head top – neck – back) and the front of the body (snout – belly – tail base).

We found evidence for modularity between the head and body (Fig. 6A). The analysis of modularity showed that the observed CR was significantly different to the distribution of variables randomly assigned to modules ($CR = 1$, $CI = 0.00$, $p < 0.05$). Therefore, this suggested that there was a high level of coordination between shape variables within the head and within body regions compared to between them. Our integration test between the head and body modules suggested that they also respond to predation risk in an integrated manner (r-PLS: 0.825, $p = 0.001$). Fig. 6B showed that, moving from the origin out, as the neck region enlarged, the back moved higher, the tail was drawn towards the front of the body and the snout shifted backwards.

We found no evidence for modularity ($CR = 1.29$, $CI = 0.00$, $p = 0.54$; Fig. 6C) in the back and front regions. However, our integration test suggested that these two groupings of landmarks do respond to predation risk in an integrated manner (r-PLS: 0.917; $p = 0.001$). Fig. 6D showed similar results to Fig. 6B; moving from the origin out, as the back was expanded by the inducible defence, the back moved higher, the tail was pulled towards the front of the body and the snout was pulled towards the back.

DISCUSSION

Our understanding of how organisms respond to predation risk has traditionally focused on a small number of specific traits in only a few environments. Recent advances have moved the standard of plasticity research to a multi-trait approach including morphology, life history and behaviour along environmental gradients. This has been complemented by a growing appreciation that morphometric analyses applied to organism shape can provide added value to analyses of phenotypic plasticity.

Here, building on this growing use of morphometrics in plasticity research, we evaluated ‘shape’ plasticity among three genotypes of *D. pulex* exposed to a gradient of six levels of predation risk. Our objective was to use morphometric shape as a ‘summary’ trait affected by responses to predation risk in life history and morphology to evaluate several hypotheses about the impact of predation risk on whole organism plasticity. Our motivation was linked to size selective predation theory and work on the response of fish to predation risk where size and shape are both linked to survival . We found that the *D. pulex* response to predation risk involves both modular and integrated changes, with changes in the inducible neckteeth defence linked to changes in head and body shape. This suggests that there is a complex response to predation, including strong developmental correlations in how *Daphnia* body plans are organised.

Our first set of results from the trajectory analysis showed how morphology changed in response to a gradient of predation risk. In accordance with previous studies of the inducible defence and our initial hypothesis, we found that head width (neck-change) increases along the gradient of predation risk . Also, we found that head height varied among clones and body width (belly-bulge) was a non-linear feature of change. This last result is contrary to our hypothesis derived from the fish literature; we predicted that body width would decrease along a rising gradient of predation risk linked to swimming/evasion, but in reality, body width varies regardless of the level of predation risk.

Our second set of results from the modularity and integration tests showed evidence for modularity between the head and the body, as expected, but also integration across these two body regions. This suggests that there is a high level of coordination within the head and the body, but there is also an integrated ‘trade-off’ among aspects of the body plan under predation risk. Specifically, as we predicted, as the neck region enlarges, the back moves higher, the tail is drawn towards the front of the body and the snout shifts backwards. Contrary to our other hypothesis, we found no evidence for modularity in the back and front regions. However these two groupings of landmarks are integrated in their response to predation risk, which is likely to be derived from the modular and integrated response of the head and lower body.

It is well-documented that the inducible defence of *D. pulex* increases in response to higher levels of predation risk . This morphological defence has long been hypothesized to form part of a whole-organism response to predation that includes changes in size and life-history traits linked to the allocation of energy to growth, defence morphology and reproduction .

In comparison, there has been some work on body width, which is another key aspect of shape that we analysed, but it is unclear what role this plays in the response to predation risk. Previous studies have used linear morphometrics to show a relatively small increase in body width in defended compared to undefended morphs of *D. pulex* and *D. magna* which suggests that body width may play a minor role in the response to predation. In support of this, it has been shown that body width is a better predictor for prey size range in *Chaoborus* than body length . *Chaoborus* usually swallows prey that cannot be deformed only if its diameter is not wider than the larva’s head capsule diameter . The increase in body depth might additionally make prey handling for the larvae more difficult. Alternatively, the increased body width in defended compared to undefended morphs may result from the increase in strength, and possibly thickness, of the carapace . Similar to the changes in body width, variation in head height, something revealed in our analysis, has not been well-documented, but may be linked to hydrodynamics .

In this study, there was both a modular and whole-organism, integrated response to predation (*sensu* . This is entirely possible, as modularity and integration are not two ends of a continuum, but represent two mutually compatible destinations of selection. It is commonly thought that the evolution of modularity and integration is linked to trait functionality. In the case of modularity, there can be selection for ‘variational

adaptation’, where traits that often respond together to environmental pressures, such as predation risk, are integrated into one module, and traits that rarely need to be changed at the same time are packed into another module. This may explain why changes in the head of *D. pulex*, which form the main response to predation risk, are relatively independent of changes in the body.

Possible functions of the observed integration could be for carapace stability or an improved escape response. Alternatively, integration could increase fitness by co-ordinating the response of multiple traits to spatial and/or temporal variation in the environment. Regardless, our results deliver strong support for the existence of developmental constraints (correlations) that are worth investigating to understand the nature of predator induced phenotypic plasticity.

Shape variation in response to predation risk has not previously been studied in *D. pulex*. In this study, we exposed *D. pulex* to six levels of predation risk and evaluated shape plasticity using geometric morphometrics and phenotypic trajectory analysis. We now have a better understanding that *D. pulex* shape is a multivariate response to predation risk, that there is genetic variation in this response and that the responses can be both modular and integrated, with associated adaptive and non-adaptive (constraint) hypotheses in need of further evaluation. Thus, there are two clear ‘next-steps’. The first is to establish the adaptive benefit/costs of shape change and variation. The second is the molecular ecology of the developmental constraints. The availability of genomic tools, the clonal nature of *Daphnia*, well-established experimental protocols and recent high throughput image analysis are an outstanding platform for future research.

REFERENCES

Table 1. Observer error for each landmark in the defended and undefended morphs.

Landmark	Intra-observer error	Intra-observer error
	Defended, standard error (unitless)	Undefended, standard error (unitless)
1 – eye	0.74×10^{-3}	0.70×10^{-3}
2 – neckteeth defence (or corresponding area)	0.61×10^{-3}	0.64×10^{-3}
3 – base of the tail	0.81×10^{-3}	0.69×10^{-3}
4 – dorsal midpoint	0.59×10^{-3}	0.61×10^{-3}
5 – ventral midpoint	0.77×10^{-3}	0.76×10^{-3}
6 – rostrum	0.92×10^{-3}	0.92×10^{-3}
Mean	0.74×10^{-3}	0.72×10^{-3}
Maximum	0.92×10^{-3}	0.92×10^{-3}
Minimum	0.59×10^{-3}	0.61×10^{-3}

FIGURE LEGENDS

Figure 1. (A) Defended and (B) undefended morphs of *D. pulex*. Animals are third instar juveniles exposed to either $1\mu\text{l ml}^{-1}$ or $0\mu\text{l ml}^{-1}$ (control) of *C. flavicans* predator cue. The enlarged image shows a close-up of the neckteeth defence.

Figure 2. Image digitisation and Procrustes superimposition. (A) First, images were prepared for landmarking in Microsoft Paint. This process identified a total of six landmarks for each photo (open circles) including (1) the centre of the eye (head top), (2) the neckteeth defence (or the corresponding area in the controls), (3) the base of the tail, (4) the dorsal (back) midpoint, (5) the ventral (belly) midpoint and (6) the rostrum (snout). (B) Second, the landmarks (closed circles) were applied in R to create digitised images that captured key aspects of shape (links). (C) Third, Procrustes superimposition was performed to align digitised images in terms of size, position and orientation. The digitised images in grey differ from the image in black in terms of size, position and orientation. (D) The final set of digitised images.

Figure 3. The first set of three *D. pulex* phenotypic trajectories along a gradient of predation risk. The principal components summarise changes in neck shape (PC1) and head height (PC2), which are visualised by the deformation grids along the axes. Individual data points for each specimen are shown by the small points, whereas the large points correspond to the mean phenotype for each treatment. The colour indicates the level of risk (0, 0.1, 0.25, 0.5, 0.75 and 1 $\mu\text{l ml}^{-1}$) and the lines connect each treatment in order of increasing predation risk. The shape of the points refers to the specific clone used in the experiment (circles – Carlos, squares – Cletus, triangles – Chardonnay).

Figure 4. The second set of three *D. pulex* phenotypic trajectories along a gradient of predation risk. The principal components summarise changes in belly bulge (PC3) and head height (PC2). See the description of figure 3 for the legend.

Figure 5. The third set of three *D. pulex* phenotypic trajectories along a gradient of predation risk. The principal components summarise changes in neck shape (PC1) and belly bulge (PC3). See the description of figure 3 for the legend.

Figure 6. Modularity and integration tests. (A) Histogram of CR coefficients from simulations of random partitions of the head and body, with the observed CR coefficient indicated by the arrow. (B) PLS scores for the head and body, with the line of best fit and deformation grids which visualise the shape identities and how they change along the axes. (C) The same as (A), but for the front and back. (D) The same as (B), but for the front and back. The specific landmarks involved in the tests are represented by the diagrams above the plots. If there was modularity, the observed CR coefficient would be significantly less than one, and if there was no integration, the line of best fit would look like either a horizontal or vertical line.

AUTHOR CONTRIBUTIONS

Sam Paplauskas : Conceptualisation (supporting), investigation (equal), data curation (lead), formal analysis (equal), funding acquisition (lead), investigation (equal), validation (lead), visualisation (lead), writing – original draft preparation (lead), writing – review and editing (equal). **Oscar Morton** : Conceptualisation (supporting), investigation (equal). **Mollie Hunt** : Conceptualisation (supporting), investigation (equal). **Ashleigh Courage** : Conceptualisation (supporting), investigation (equal). **Stephanie Swanney** : Conceptualisation (supporting), preliminary (equal). **Stuart R. Dennis** : Writing – review and editing (supporting). **Dörthe Becker** : Writing – review and editing (supporting). **Stuart K. J. R. Auld** : Writing – review and editing (supporting). **Andrew P. Beckerman** : Supervision (lead), conceptualisation (lead), formal analysis (equal), investigation (equal), methodology (lead), visualisation (supporting), writing – review and editing (equal)

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DISCLOSURE STATEMENT

The authors have no conflict of interest to declare.

DATA ACCESSIBILITY STATEMENT

The data that support the findings of this study are openly available in Dryad at <https://doi.org/10.5061/dryad.zkh1893fx>.





