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Interactive effects between donor and recipient species mediate fitness costs of heterospecific pollen receipt in a co-flowering community

Gerardo Arceo-Gómez[1] · **Rainee L. Kaczorowski**[2] · **Cheril Patel**[1] · **Tia-Lynn Ashman**[2]

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Abstract

Evaluation of pollen transfer in wild plant communities revealing heterospecific pollen receipt is common, yet experimental hand pollinations have revealed high among-species variation in the magnitude of its effect on recipient fitness. The causes of this among-species variation are unknown, however, prompting the investigation of underlying factors. Here, we conducted a hand-pollination experiment with ten co-flowering species to determine whether the effects of heterospecific pollen receipt are mediated by the pollen donor or recipient species alone, or whether the effects are determined by the interaction between them. We further assessed species traits potentially mediating interactive effects in heterospecific pollen receipt by evaluating the relationship between heterospecific pollen effect size and three different predictors reflecting a unique combination of pollen donor and recipient characteristics. Our results show, for the first time, that the magnitude of the heterospecific pollen receipt effect is determined by the specific combination of donor and recipient species (i.e., interactive effects). However, we were unable to uncover the specific combination of traits mediating these effects. Overall, our study provides strong evidence that an understanding of heterospecific pollen receipt effects based on recipient or donor characteristics alone may be insufficient. This study is an important step toward an understanding of consequences of heterospecific pollen receipt in co-flowering communities.

Keywords Co-flowering · Pollen transfer · Pollination · Pollen size · Stigma area

Introduction

Plants typically co-flower in multispecies communities where the amount of flowering overlap (e.g., Caradonna et al. 2014; Arceo-Gómez et al. 2018b) and pollinator sharing can be high (e.g., Olesen and Jordano 2002; Bascompte et al. 2003; Bascompte and Jordano 2007; Olesen et al. 2007). As a result, pollinators frequently transfer pollen between flowers of different plant species, i.e., heterospecific pollen transfer (hereafter, HPT; Waser 1978; Morales and Traveset 2008; Ashman and Arceo-Gómez 2013; Fang and Huang 2013; Tur et al. 2016; Johnson and Ashman 2018). Increasing evidence also suggests that HPT can have severe effects on the reproductive success of pollen recipients (reviewed in Morales and Traveset 2008); with marked reductions in seed production (e.g., on average 20% in response to 50:50 mix of pollen; Ashman and Arceo-Gómez 2013). Thus, HPT has the potential to act as a strong selective force driving floral evolution (Morales and Traveset 2008; Arceo-Gómez et al. 2015) and mediating co-flowering community assembly (Ashman and Arceo-Gómez 2013). Studies on the effects of heterospecific pollen receipt (hereafter, HPR), however, have also revealed high among-species variation in the magnitude of the effect (Ashman and Arceo-Gómez 2013). While some species can suffer severe reductions in seed production (e.g., Matsumoto et al. 2010), others are unaffected by HPR (e.g., Kwak and Jennersten 1991; reviewed in Morales and Traveset 2008, Ashman and Arceo-Gómez 2013), and the underlying causes of this variation are largely unknown (but see

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✉ Gerardo Arceo-Gómez
gomezg@etsu.edu

✉ Tia-Lynn Ashman
tia1@pitt.edu

[1] Department of Biological Sciences, East Tennessee State University, Johnson City, TN 37614, USA

[2] Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA 15260, USA

Arceo-Gómez and Ashman 2016; Wipf et al. 2016; Arceo-Gómez et al. 2018a). Thus, to advance our understanding of the ecological and evolutionary consequences of HPT, it is critical that studies move beyond the simple assessment of significance in the effect of HPR (i.e., effect vs. no effect) and evaluate the factors that underlie among-species variation in the magnitude of the effects.

It has been proposed that the magnitude of the effect of HPR is determined by traits of the recipient associated with vulnerability to heterospecific pollen (hereafter, HP) or traits of the donor that increase the potential of HP to interfere with conspecific ovule fertilization (Ashman and Arceo-Gómez 2013). This suggests that HPR effects are the outcome of specific traits associated with either pollen donor or recipient species. Less explored, however, is the possibility that the fitness costs of HPR are mediated by the specific combination of pollen donor and recipient characteristics. For instance, it has been hypothesized that HP grain size can be an important determinant of the effect of HPR as it will affect the donor's ability to attach to, and physically block, recipient stigmas (Ashman and Arceo-Gómez 2013). However, pollen grain size alone may be a poor indicator of HP donor's ability to block stigmas and interfere with conspecific pollen germination as the same HP grain can be considered 'large' or 'small' relative to the size of the recipient's stigma. Thus, we propose that the role that HP grain size plays in mediating HPR effects needs to be evaluated relative to the size of the recipient's stigma (i.e., interactive effects), which is often correlated with conspecific pollen grain size (Cruden and Miller-Ward 1981; Heslop-Harrison 1981). In this sense, we predict that the effect will be greater as the recipient stigma–heterospecific pollen size ratio decreases (i.e., as stigma and pollen approach the same size), as fewer pollen grains would be needed to completely block the recipient's stigma and impede conspecific pollen germination and pollen tube growth. For instance, in a recent study, Arceo-Gómez et al. (2018a) show that few (1–5) *Zea mays* pollen grains were sufficient to decrease *Mimulus guttatus* pollen tube growth when deposited on *M. guttatus* stigmas. Interestingly, *Z. mays* pollen (> 100 µm in diameter; Pleasants et al. 2001) is more than three times the size of *M. guttatus* pollen (> 30 µm) and covers approximately 7.5% of the *M. guttatus* stigma surface area suggesting that the large HP grain relative to *M. guttatus* stigma size could be one cause for its strong detrimental effects (Arceo-Gómez et al. 2018a). It is important to note that pollen grains that are the same size or larger than the stigma are not expected to be found on stigmas as these typically show weaker attachment and tend to fall off the stigma.

Another trait that is an emergent property of the HP donor and recipient pair is the degree of flowering overlap between them, which can also be a strong indicator of the costs associated with HPR. For instance, we can expect effects of HPR to increase with decreasing flowering overlap between pollen donor and recipient species, as divergence in flowering time may be an evolutionary response to high costs associated with HPT (e.g., Waser 1978; also see Campbell and Motten 1985; Morales and Traveset 2008; Ashman and Arceo-Gómez 2013). For instance, a greenhouse study of three HP donors on *Mimulus guttatus* showed that *Helianthus exilis* caused the largest reduction in seed production compared to two other HP donors, and *H. exilis* also had the smallest amount of flowering overlap with *M. guttatus* (Arceo-Gómez and Ashman 2011; Arceo-Gómez et al. 2018b). Furthermore, in an experimental study, Waser (1978) showed that HP transfer was likely the main cause for divergence in flowering time between *Ipomopsis aggregata* and *Delphinium nelsoni*.

Finally, the phylogenetic distance between pollen donor and recipient species can also be an important indicator of the magnitude of HPR effects (e.g., Arceo-Gómez and Ashman 2016). Closely related species typically share similar stigma morphology and pollen-stigma recognition systems (Martin 1970; Harder et al. 1993; Hiscock and Allen 2008) that may make pollen germination and pollen tube growth hard to prevent when pollen comes from closely related heterospecific species (Ashman and Arceo-Gómez 2013, 2016). This lack of rejection at the stigma level can lead to fertilization and ovule usurpation by HP (e.g., hybridization) and/or high rates of seed abortion (e.g., Arceo-Gómez and Ashman 2011).

Despite the clear potential for interactive effects between pollen donor and recipients to mediate, and thus predict the magnitude of HPR effects, no study has simultaneously evaluated the role of HP donor, HP recipient and their interaction in driving the fitness effects of HPR. Accordingly, in this study, we ask the following questions: (1) Are the effects of HPR on seed production determined by the HP donor, HP recipient, or by the interaction between donor and recipient species? (2) Does HPR effects increase with decreasing stigma–pollen size difference, decreasing flowering overlap and/or decreasing phylogenetic distance between recipient and pollen donor species pairs?

Materials and methods

Study system

We examined the costs of HPT among insect-pollinated plant species that coexist in serpentine seep communities at the McLaughlin Natural Reserve in California (38°51′29.45″N 122°24′33.49″W). Serpentine seeps are species-rich communities (Koski et al. 2015; Arceo-Gómez et al. 2018b) dominated by small herbaceous perennials and annuals (Freestone and Inouye 2006; Koski et al. 2015). Plants have a peak seasonal flowering season (April–June) due to their high dependency on water availability (seasonal rainfall), resulting in a high degree of flowering overlap among species (Arceo-Gómez et al. 2018b). Previous studies have showed substantial pollinator sharing among plant species (e.g., Koski et al. 2015) in these communities which results in a high degree of heterospecific pollen transfer (e.g., Arceo-Gómez et al. 2016; Ashman, Arceo-Gómez and Kaczorowski, unpublished data). From these communities, we selected six recipient and four donor species (Table 1). All pollen donor and recipient species co-occur in at least one serpentine seep at our sites (Arceo-Gómez et al. 2018b) and share a variety of insect flower visitors (Koski et al. 2015; Ashman, Arceo-Gómez and Kaczorowski, unpublished data). There was a tenfold difference in diameter of pollen grains from selected HP donor species and a 100-fold difference in stigma area of selected recipient species (Table 1). Experimental plants were grown from seeds or seedlings (*Stachys albens*) collected from the field in 2015, under homogeneous conditions (day temp: 73–75 °F, night temp: 63–65 °F, 12–14-h days) in the greenhouse at the University of Pittsburgh, and given fertilizer, water and pest control as needed. All pollen donors and recipients flowered simultaneously in the greenhouse, except for *S. albens* which flowered a few months earlier.

Experimental design

To evaluate whether the effects of HPR are mediated by HP donor, recipient or their interaction, we conducted hand pollinations of all HP recipient species with (1) a mixture of conspecific and single HP donor species (one for each of the four donors) and (2) a control (pure conspecific pollen). There were 5–40 recipient plants of each species, and these were haphazardly distributed across three greenhouse benches. Pollen donor plants (all plants from the four heterospecific donor species, as well as conspecific plants to be used in pollen mixes) were kept separate from recipients on a single bench.

Hand pollinations

Mixed pollinations were created in an additive fashion to create a 20% HP treatment that mimics average receipt in nature (Ashman and Arceo-Gómez 2013). Mixes were created using knowledge of the number of pollen grains per anther (e.g., Arceo-Gómez and Ashman 2011). All pollen was collected fresh for mixes (and used within 48 h) or preserved in a − 20 °C freezer (*S. albens*) until used (*e.g.*, Arceo-Gómez and Ashman 2011). In mixed pollinations, pollen from multiple individuals was combined for use in hand pollinations. Pollen was applied by contacting fresh stigmas 1–2 times with a toothpick (e.g., Arceo-Gómez and Ashman 2011) so as not to oversaturate the stigma. Flowers on a recipient were assigned a treatment randomly.

Styles of each hand-pollinated flower were collected after flowers had wilted or 24 h after pollination if styles were not persistent (i.e., TRLX) and stored in 70% ethanol. Styles were softened with 1 M KOH and mounted on slides (e.g., Arceo-Gómez et al. 2015, 2016), and the number of conspecific and heterospecific pollen grains on the stigma counted under a compound microscope (Leica DM3000) at 40 × magnification. These HP and conspecific load sizes were used as covariates in analyses (see below). Fruits from

Table 1 Heterospecific pollen donor and recipient species used in hand-pollination experiments. Stigma area is given for recipient species, and pollen diameter (mean ± SE; $N = 3–10$) is shown for all species studied. Species codes used for each plant are also given. The alternative name is an accepted pseudonym for the species

Plant species name	Species code	Alternative name	Plant family	Experimental role	Stigma shape	Stigma area (mm^2)	Pollen diameter (μm)
Anagallis arvensis	ANAR	.	Primulaceae	Pollen recipient	Cylinder	0.061	26.80 ± 0.64
Centaurium trichanthum	CETR	*Zeltnera trichantha*	Gentianaceae	Pollen recipient	Double sphere	0.396	38.90 ± 1.09
Mimulus guttatus	MIGU	*Erythranthe guttata*	Phyrmaceae	Pollen recipient	Flat lobes	4.186	34.87 ± 0.32
Mimulus layneae	MILA	*Diplacus layneae*	Phyrmaceae	Pollen recipient	Flat lobes	2.754	37.98 ± 1.24
Mimulus nudatus	MINU	*Erythranthe nudata*	Phyrmaceae	Pollen recipient	Flat lobes	2.636	33.14 ± 0.97
Trichostema laxum	TRLX	.	Lamiaceae	Pollen recipient	Double cone	0.267	54.75 ± 0.89
Lotus micranthus	LOMI	*Acmispon parviflorus*	Fabaceae	Pollen donor	.	.	24.86 ± 3.06
Plagiobothrys stipitatus	PLST	.	Boraginaceae	Pollen donor	.	.	3.75 ± 0.48
Stachys albens	STAL	.	Lamiaceae	Pollen donor	.	.	35.09 ± 0.86
Streptanthus breweri	STBR	.	Brassicaceae	Pollen donor	.	.	15.25 ± 0.41

each pollinated flower were collected at maturity, and the number of seeds counted with the aid of a dissecting microscope (Zeiss Stemi SV6 with Fostec light source or Wild Heerbrugg M5 with Leica Lux 1000 light source). For each recipient plant, we also collected one non-pollinated flower in 70% ethanol and later enumerated number of ovules/flower to account for variation in ovule numbers in response to seed set (see below).

Recipient and donor trait data

To estimate stigma area for each recipient species, we took a photograph of a stigma at 35 × magnification (unless size required a lower magnification) using the integrated camera on a dissecting scope (Leica EZ4 W). We measured stigmatic area from photographs using ImageJ 1.51 k software (Rasband 1997). The area was measured directly for flat stigmatic surfaces and estimated for spheres, cylinders or conical surfaces from their length and/or diameter based on shape (spheres: $4\pi r^2$, cylinders: $(2\pi rh)+(2\pi r^2)$, cones: $\pi r(r+(\sqrt{(h^2+r^2)})$. To estimate pollen size for each species, we acetolyzed anthers (Dafni 1992) and photographed pollen grains ($N = 3$–10 per species) at 400 × magnification (unless size required a lower magnification) with a camera-mounted compound microscope (Olympus CX41 with DP12 camera). From each photograph, we quantified the diameter (or the longest dimension) of each pollen grain using ImageJ 1.51 k software (Rasband 1997). Stigma–pollen size ratio was calculated as recipient stigma area divided by the heterospecific pollen diameter. Thus, as the ratio approaches one, the size of the recipient stigma equals heterospecific pollen size.

To create an index of co-flowering overlap for every pair of HP donor-recipient species, we used data on the number of open flowers for each species within 9–13 1 × 3 m plots distributed across each of 5 seeps for the duration of the 2016 flowering season (for details see Arceo-Gómez et al. 2018b). We estimated Schoener's index of niche overlap (Schoener 1970) as applied to flowering at each seep:

$$\mathrm{SI} = 1 - {}^{1}\!/_{2}\, \Sigma_k \left| p_{ik} - p_{jk} \right|,$$

where p_{ik} and p_{jk} are the proportion of flowering by species i and j, respectively, occurring on day k (Forrest et al. 2010). The SI ranges from 0 (no overlap in flowering) to 1 (complete flowering overlap; Forrest et al. 2010). We averaged across sites where both species were present (2–5 sites).

We estimated phylogenetic distance between each HP donor and recipient species pair from a phylogeny of the donors and recipients using 'Phylomatic' (Webb and Donoghue 2005). For this, we used the most recent megatree in Phylomatic (R20100701.new) as the base tree and the final tree was re-ultrametricized with branch lengths scaled to time using the BLADJ function in 'Phylocom' (Webb et al. 2008). We then estimated branch length between HP donors and recipients using the function 'phydist' in 'Phylocom' (Webb et al. 2008).

Data analyses

We estimated the plant-level effect of HPR for each HP treatment as follows: (number of seeds of HP treatment − number seeds of control treatment)/number of ovules. This index reflects the decrease in seed production as a result of HPR relative to pure conspecific pollen while accounting for variation in ovule number across plants and species. We used a mixed model to evaluate whether the magnitude of the 'HPR effect' varied among HP recipient species, HP donor species, or with their interaction. We used individual plant ID as a random variable and conspecific pollen load and the exact proportion of heterospecific pollen in the HP mix applied as covariates. Residuals were normally distributed (Kolmogorov–Smirnov, $P > 0.05$).

To evaluate whether HPR effect size varied with stigma–pollen size ratio, co-flowering overlap and/or phylogenetic distance, we estimated an effect size for every HP donor–recipient combination. For this, we calculated standardized Hedges' d ([mean seed set of mixed pollination − mean seed set of conspecific pollination]/pooled SD) as a measure of effect size (Hedges and Olkin 1985). We then used multiple regression to evaluate the effects of the predictors on Hedges' d, while also accounting for differences in mean HP proportion applied to stigmas for each species. Residuals were normally distributed (Kolmogorov–Smirnov, $P > 0.05$). We used a stepwise regression (proc phreg; SAS 2010) to eliminate variables with low explanatory power and obtain the model with the strongest support. The average proportion of HP deposited on stigmas was removed from the final model (Wald $\chi^2 = 0.3$, $P = 0.5$) as a result of this analysis. All statistical analyses were conducted in SAS (SAS 2010).

Results

Heterospecific pollen effect size (Hedges' d) varied between − 1.1 for the TRLX-LOMI pair and 0.39 for the MINU-STBR pair (Fig. 1). The magnitude of the plant-level HPR effect varied significantly among HP recipients ($F_{5,\,303} = 6.76$, $P = 0.001$) but not among HP donors ($F_{3,\,303} = 1.12$, $P = 0.3$). Variation in the magnitude of the HPR effect was also significantly influenced by the interaction between HP donor and recipient species ($F_{15,\,303} = 1.85$, $P = 0.02$; also see Fig. 1). Conspecific pollen load size and the exact proportion of HP deposited on stigmas were significant covariates in the model ($F_{1,\,303} = 4.5$, $P = 0.03$; $F_{1,\,303} = 30.97$, $P = 0.001$, for conspecific and heterospecific

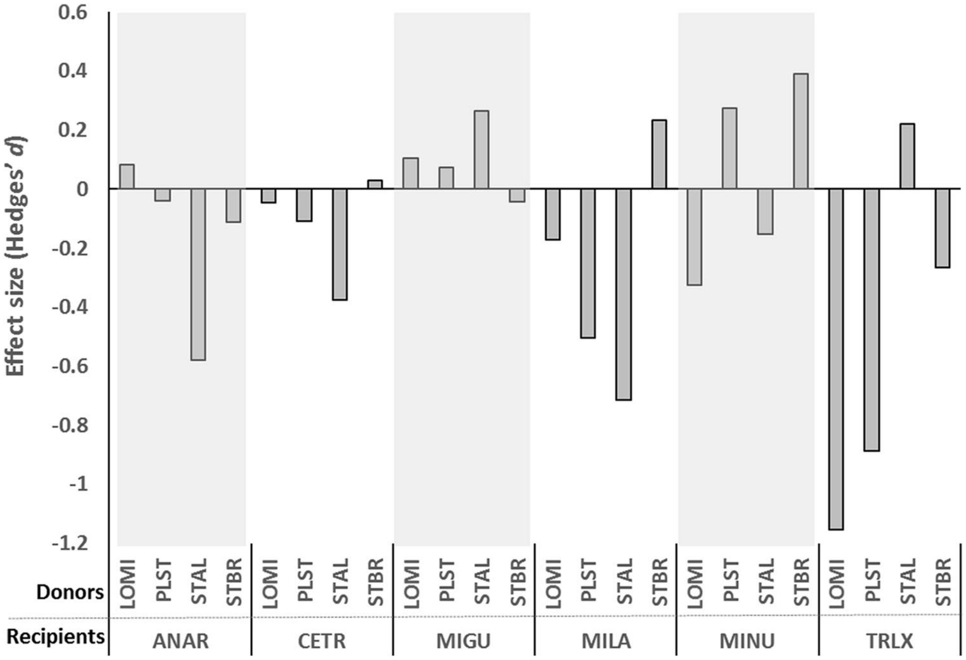

Fig. 1 Hedges' *d* effect size [(mean seed set of mixed pollination – mean seed set of conspecific pollination)/pooled SD] for each heterospecific pollen donor and recipient pair combination. The magnitude of the effect size on seed set ranged from negative (negative values) to positive (positive values). Full species names associated with each code are given in Table 1

loads, respectively). Surprisingly, however, none of the recipient–donor trait indices nor phylogenetic distances significantly explained variation in HPR effect size (Hedges' *d*; $F=0.5$, $P=0.6$).

Discussion

Here, we show, for the first time, that the fitness effects of HPR are not mediated by donors or recipient species alone but rather by the interactive effects between them, as indicated by the significant donor by recipient interaction (also see Fig. 1). For instance, in our study, the effect of the HP donor PLST ranged from the second strongest negative effect (−0.8 in TRLX) to slightly positive (0.2 in MINU) depending on the identity of the recipient species (Fig. 1). Along these same lines, the recipient MILA experienced the third strongest detrimental effect when pollinated with STAL (effect size −0.7) but was unaffected by STBR (effect size 0.2; Fig. 1). These interactive effects are also apparent in studies of pollen allelopathy (i.e., chemical inhibition of conspecific pollen germination and tube growth), where large variation in the magnitude of the HP effect has been observed when the same HP donor has been tested across different recipient species (e.g., Murphy and Aarssen 1989; Murphy 2000). This suggests that pollen recipients in addition to donors play a role in mediating HP costs even when there are known traits (i.e., allelopathy) mediating the detrimental effects on seed production. It is important to note, however, that large differences among recipient species in terms of their vulnerability to HP effects were also observed (Fig. 1). Thus, even though the specific donor by recipient combination seems to be the major determinant of HP effects, some recipient species may still be overall more vulnerable to these effects than others.

In this study, we were unable to uncover the underlying HP donor–recipient trait combinations mediating HPR effects. It is possible, however, that HP donor–recipient trait combinations other than those studied here, i.e., stigma–pollen size ratio, flowering overlap or phylogenetic distance between HP donor and recipient, may be more important in mediating HP effects. For instance, it is interesting that the effect of LOMI as HP donor was substantially different between two closely related recipients (Fig. 1). LOMI effects ranged from strongly negative in *Mimulus nudatus* (effect size: −0.3) to neutral/positive in *M. guttatus* (HP effect size 0.1; Fig. 1). A possible contributing factor is that these two recipient species have different mating systems. While *M. nudatus* is predominantly a selfing species (Ritland and Leblanc 2004), *M. guttatus* is considered mostly outcrossing (Willis 1993). Outcrossing species may be more resistant to the effects of HPR (Ashman and Arceo-Gómez 2013) because they are commonly exposed to great diversity in pollen from different sources on their stigma (Brandvain and Haig 2005; Mazer et al. 2010). This can lead to stronger competitive ability compared to self-pollinating species, which typically experience homogeneous pollen loads (Ashman and Arceo-Gómez 2013). On the other hand, the recipient *Trichostema laxum*, which is considered a highly outcrossing species (Spira 1980), had the largest HPR effect size (−1.1) when pollinated with LOMI, but it had a neutral/positive effect (0.2) when pollinated with STAL (Fig. 1).

These combined results suggest that the recipient's mating system may act in combination with other HP donor traits in mediating HPR effects, but this prediction still needs to be tested.

It is also important to acknowledge that we tested our predictions across six recipients and four HP donors (24 HP donor–recipient combinations), and even though, to our knowledge, this is the largest study of this nature to date, our ability to detect a significant effect may still be limited by insufficient variation in the traits studied and/or limited sample size. Thus, it is possible that a wider range of variation in these traits across donor and recipient species pairs could have yielded significant results. Thus, future studies should evaluate the role of HP donor–recipient interactive effects in mediating HP costs using a larger set of HP donor and recipient species. Such predictive knowledge would be particularly important for the conservation of native plants as alien species can readily integrate into plant pollinator (e.g., Lopezaraiza-Mikel et al. 2007) and pollen transfer networks (Emer et al. 2015; Johnson and Ashman 2018) and have been shown to be particularly harmful via HPT (Arceo-Gómez and Ashman 2016). To our knowledge, this is the first study to evaluate the importance of interactive effects between HP donor and recipient species in mediating the costs of HPT, and thus, we emphasize the need for more studies to fully understand their importance and the underlying traits involved. Such knowledge would be valuable in advancing our predictive understanding of the ecological and evolutionary consequences of HP transfer in natural communities.

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Author contribution statement TLA and GAG conceived and designed the experiments. RLK and GAG performed the experiments. CP and RLK processed samples and collected data. GAG and CP analyzed the data. GAG and RLK wrote the manuscript; all authors provided editorial advice.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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