Optimal inaccuracy: estimating male fitness in the movement-assisted dichogamous

species *Clerodendrum infortunatum*

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Abstract

In hermaphroditic species, sexual interference can drive the evolution of dichogamy,

where sporophylls (reproductive parts) are separated in time. However, the separation of

sporophylls can lead to pollination inaccuracy, especially in movement-assisted dichogamy,

where sporophylls alter their position over time. Is pollination inaccuracy minimised by the

second sporophyll taking the exact position of the first? Are the sporophylls optimally

positioned and stable in their respective active phases? We address these questions in

Clerodendrum infortunatum, a protandrous, movement-assisted dichogamous species. We

made predictions from optimality arguments, and tested these by measuring sporophyll

angles over time, by experimentally manipulating flowers, and by estimating correlates of

the resultant fitness, taking into account pollen export, pollination inaccuracy and the

resultant total pollen delivered. Contrary to expectation, anthers do not have a fixed position

in the male phase, and pollination inaccuracy is high. Further, when pollen load is highest,

anthers are paradoxically not positioned at the pollen export peak. Also, pollen export and

pollination accuracy peaks do not align. This seeming maladaptiveness of anther positioning

nevertheless results in highest overall male fitness, measured as the total pollen delivered

over the entire male phase. Instead of a simple positional exchange of sporophylls, stamens

display a more complicated dynamic strategy which appears close to optimal even though

naive measures of pollination inaccuracy are high. Such a strategy of maximising overall

male fitness, integrating over the dynamics of stamen trajectory, could well be a general

characteristic of protandrous movement-assisted dichogamy.

Introduction

The diversity of flowering plants is breathtaking. The vast number of species are accompanied by remarkable variation in their life history strategies. One such strategy is the sexual system of flowering plants [1], which ranges from monoecy (male and female flowers occur on the same individual) to dioecy (male and female flowers occur on different individuals), with several variations, including androdioecy, andromonoecy, and many more. The most common sexual strategy in flowering plants is hermaphroditism (male and female sexual organs occur in the same flower, and the plant bears only this type of flower), which occurs in nearly 72% of all angiosperms [2].

Why should hermaphroditism be so common in flowering plants? The theory of plant resource allocation in economic terms [3,4] suggests that this is the most efficient strategy as it minimises resource investment. This is because a common investment in attractive traits, including petals and nectar, promotes both male and female reproductive success at the same time. However, this advantage comes with an associated cost – that of self-pollen deposition. This cost is borne by both self-compatible and self-incompatible species. In self-compatible species, self-pollen deposition increases the possibility of inbreeding depression, where offspring vigour decreases down the generations [5,6]. In addition, primarily male reproductive success might decline because of sexual interference [7–9]. Sexual interference leads to wastage of pollen because deposited self-pollen on stigmas cannot be deposited on stigmas of other individuals and are therefore removed from the cross-pollen pool. On the other hand, in self-incompatible species, sexual interference is the only cost to bear.

Flowers have evolved different strategies to minimise these costs. The details vary from species to species, but the general theme is either spatial or temporal separation of sporophylls (stamen and pistil) in the flower [7,10]. In herkogamy, both sporophylls are active

(reproductively mature) at the same time, but separated in space. By contrast, in dichogamy,

sporophylls become active at different times. In this way, dichogamous flowers have separate

male and female phases. In the special case of movement-assisted dichogamy, the sporophyll

that matures earliest is positioned in the presentation zone (where it exports or receives pollen,

as the case may be) in the first phase. In the second phase, it moves away and the second

sporophyll becomes active and takes that position.

Any spatial separation of sporophylls has an associated problem of pollination

inaccuracy [7,11]. For successful cross-pollination, the pollen export and receipt positions must

be similar among flowers in a population. This is because, in vector-pollinated plants, pollen

transfer is maximum when the part of the pollinator's body that touches (and therefore picks

up) pollen from the anthers is the same part of the body that touches (and therefore deposits)

pollen to the stigma. Because particular species of pollinators tend to show stereotypical

foraging postures at flowers, this requirement is most closely met when anthers and stigmas in

different flowers are at exactly the same position. For this reason, any spatial separation

between anther and stigma necessarily increases pollination inaccuracy even as it serves the

purpose of decreasing self-pollen deposition.

Pollination accuracy or inaccuracy, then, clearly affects optimal presentation of the

sporophylls. A reasonable starting point would be that the optimal position of a mature

sporophyll is where the sporophylls has the highest probability of touching the relevant body

part of the pollinators; this leads to the highest pollen export and deposition. It follows from

this that mature sporophylls in natural populations should occupy this 'optimal presentation

zone'. Though early natural history studies [12–14] took this for granted, the assumption is rarely

tested explicitly [11].

Different floral adaptations lead to varied consequences. The permanent spatial

separation between sporophylls of herkogamous species is expected to lead to high pollination

inaccuracy [8]. In dichogamous species, by contrast, the first sporophyll makes way for the

second, and it is potentially possible for both sporophylls to occupy the same position, albeit

separated in time. Does this mean that pollination inaccuracy is lower in dichogamous species

than in herkogamous species? Unfortunately, few studies have attempted to answer this

question [15]. Species that show movement-assisted dichogamy present a further complication

as their sporophylls are in motion. In this case, the theory of optimal sporophyll placement

depends on an additional assumption that after entering the optimal presentation zone, mature

sporophylls remain stationary. However, this is not always the case [16]. If sporophylls are not

stationary in the presentation zone, then pollen export and deposition will vary dynamically as

the sporophylls move. This means that fitness of traits needs to be estimated across the

lifetime (and therefore position) of sporophylls to understand the overall outcome.

Therefore, a detailed study on adaptive fitness of movement-assisted dichogamy

should address the following aspects in sequential order. First, whether male and female

sporophylls take on the same position during maturity. Second, whether they both take on

'optimal' positions. To assess that one needs to compare indices of fitness between the

observed phenotype with other variants [11,15,17]. These variants can come from among the

natural variation within populations, but when that variation is small (possibly due to strong

selection), artificial variants must be created. Morphological trait manipulations are routinely

used by floral biologists to create artificial variants [18–20]. Even, within the range of natural

variation, one may need to create artificial variants in order to study dynamic traits (e.g.,

sporophyll position in movement-assisted dichogamy). To estimate a fitness index of a

sporophyll at a specific position, one needs to create an artificial variant, which is fixed at

that position over time.

A population may depart from optimality (called adaptive inaccuracy) in one or both

of two ways: the mean of the population lies away from the optimum (called bias), and

phenotypic variation is high such that many individuals are far from the optimum (called

imprecision) [21]. In a perfect world, with all members of the population perched on the

adaptive peak, both bias and imprecision are zero. However, maladaptation in the population

can reveal itself through nonzero bias, or nonzero imprecision, or both. Depending on the

situation, selection may be directional, or stabilizing, or both.

To investigate the adaptive fitness of movement-assisted dichogamy in detail, we

studied the complete (no overlap between sexual phases) dichogamous species *Clerodendrum*

infortunatum (henceforth *Clerodendrum)* [16]. Though *Clerodendrum* is a self-compatible

species, it mainly breeds by xenogamy and geitonogamy [22,23]. Thus, for reproduction,

Clerodendrum in natural population heavily depends on pollinators. We predicted that

pollination inaccuracy of the natural population is low. We also tested the prediction that in

flowers, anthers and stigma are positioned optimally to maximise their fitness. In manipulated

flowers, we fixed anthers and stigma at various positions within and beyond their natural

ranges, and estimated pollen export and deposition rate at each position. We predicted that the

resultant curves would be unimodal, where the highest peaks indicate the optimal presentation

zone for each of the sporophylls. We expected the peaks for anthers (pollen export) and

stigmas (pollen deposition) to overlap strongly. We then investigated whether anther and

stigma positions in the natural population overlap with the expected male or female optimal

presentation zones as estimated through the morphological manipulation experiments. We

estimated cumulative male fitness over the entire male phase. We also estimated bias and

imprecision as a measure of the degree of maladaptation in the natural population.

Methods

Observations and experiments

The Anamalai Hills, dominated by mid-elevation tropical wet evergreen rainforest [24],

are situated in the southern Western Ghats in India. The Valparai plateau (700m-1600m asl)

lies within these hills. Though the Valparai landscape is dominated with tea and coffee

plantations, many privately owned rainforest fragments (ranging from 0.3 to 650 ha in size)

still persist. These forest fragments harbour a significant diversity of rain-forest flora and

fauna [25,26]. Two such forest fragments were selected for this study, namely Iyerpadi Riverine

(10°21'12" N, 76°59'54" E, Area 1.76 ha) and Varattuparai Colony (10°21'22" N, 76°56'30" E,

Area 33.4 ha). Within these forest fragments, we studied the shrub *Clerodendrum*

infortunatum Gaertn. [Lamiales: Lamiaceae] between December 2014 and May 2015.

Clerodendrum produces dichogamous and protandrous flowers, where the male phase

precedes the female phase. Flowers are mainly pollinated by butterflies [22,23].

To describe the spatiotemporal dynamics of sporophylls, we monitored flowers

between 18 February 2015 and 26 April 2015. Thirty two flowers from 18 plants were tagged

with coloured thread, so that they could be monitored throughout their lifetime of two or more

days [16]. We monitored flowers every two hours from the morning (6 AM) to evening (6 PM),

with a total of seven visits per day. The next visit was on the morning of the next day. During

each visit, a lateral photograph of the flower was taken. Stamen and style angles were

measured later from the photographs using ImageJ software (Fiji distribution) [27]. In order to

measure sporophyll positions in a consistent manner, we defined the geometry of a flower as

follows. Taking the horizontal floral axis as the starting point, the basal direction of the plane

was defined as being at an angle of 0° and, measuring counter-clockwise, the distal direction

as 180° (Fig. 1). Angles from this plane to the anthers and stigma were measured from the

point where the floral axis was intersected by an imaginary vertical line along the plane where

corolla tube opens up.

Fig. 1. A. A natural *Clerodendrum* flower at male phase. B. A schematic diagram of a morphologically manipulated flower, where anthers were set at a fixed position near 135°. All angles [30°, 90°, 135°, 160°, 215°, 270°, 350°] at which sporophylls were set in different treatments are also shown.

The fitness consequences of adopting different phenotypes (sporophylls at different angles) were measured by experimentally manipulating floral structures. Because sporophylls are in continuous motion, it is impossible to find sporophylls at a fixed position in natural flowers. Also, sporophylls take on a narrow range of positions in their active phases, and the consequences of adopting a position outside this range cannot be assessed in natural populations. Flowers were therefore manipulated to set the active sporophyll at a specific angle, and the other sporophyll at its normal inactive position (roughly 270°) (Fig. 1). These experiments were carried out between 28 April 2015 and 24 May 2015. All morphological manipulations were done on the male stage flower (target flower) so that nectar content was roughly similar and could not influence visitation differentially. *Clerodendrum* flowers are white, and so white double-sided adhesive tape was used to attach the sporophylls (after removing the existing sporophylls) to the flower at a specific angle. The tape was without any detectable odour, which might alter visitor behaviour. On a small piece of tape, stamens or style were attached and then that piece was attached to another small piece at 90° (Fig. 1). This second piece was attached to the flower. Stamens and styles used in this experiment were collected from a separately bagged inflorescence to make sure that the used stigmas did not contain any pre-deposited pollen, and the pollen loads of the used anthers were intact. Styles were collected from female-phase flowers and stamens were collected from male-phase flowers in the donor inflorescences.

In this manner, active sporophylls were experimentally set at the following angles –

30°, 90°, 135°, 160°, 215°, 270° and 350°. These angles were chosen in order to cover

virtually all possible approach angles around the flower. Treatments in which sporophylls

were affixed towards the distal end of the flower ($< 90°$ and $> 270°$) were unlikely to receive

pollen from pollinators, but provide an estimate of pollen deposition from other sources (e.g.,

from the shaking of branches). To check for possible adverse effects of manipulation, we

compared sporophylls manipulated to be in the presentation zone with unmanipulated

sporophylls (see Results section). Pollen of unmanipulated flowers was quickly depleted

(personal observations), and hence flowers with manipulated stamens were kept exposed for 6

hours (0800 to 1400 hrs). By contrast, pollen deposition rates were low, so flowers with

manipulated stigmas were kept exposed for 24 hours (0800 of day 1 to 0800 of day 2). The

exposure durations were about 3/4th and 3/5th of the natural male and female phase durations

respectively [16]. We have assumed that pollen export and deposition rate is zero at 0° and 359°

(the distal end of the flower), since in nature, the pedicel blocks the pollinator approach path

at those angles. We also assumed that pollen export and pollen deposition rates over the

duration of the experiments (6hr and 24hr respectively) were constant for a particular

sporophyll position. After the exposure period, sporophylls were collected in vials containing

70% ethanol: all four anthers from a flower in a single vial and the stigma in a separate vial.

This storage method has a caveat – pollen grains loosely attached to the stigma surface might

get washed off. However, the remaining pollen on the stigma is likely to provide a reasonable

estimate of the number of germinating pollen since germinating pollen adheres tightly to the

stigma surface because of pollen-pistil interaction [28,29].

Pollen were counted in the lab, from 28 April to 21 June 2016. Stigmas were excised

and placed on microscope slides; they were first washed in 95% ethanol, and then stained

with Safranin O [30–32]. After a drop of glycerine was added, the stigma was squashed under a

coverslip, and pollen were counted under a compound microscope (100x magnification).

Because *Clerodendrum* anthers have a large number of pollen grains, it was not possible to

conduct a total count of the leftover pollen on anthers, and so a suitable sampling method

needed to be devised. We used a slightly modified version of the protocol described by

Shivanna [33]. Anthers were placed in a 1 mL vial with 70% ethanol solution. A drop of

detergent was added to keep the pollen suspended. The anther was crushed with a glass rod to

release pollen grains. The suspension was mixed thoroughly by repeatedly drawing it into and

expelling it from a syringe without the needle. While gently shaking the vial we took a 0.01

mL volume of solution with a micropipette. The solution was spread on a haemocytometer

glass slide, covered with a coverslip. The total number of pollen grains in the solution was

estimated by averaging the number of pollen grains across the ten replicate subsamples,

dividing the average by the volume of the subsample (0.01 mL) and then multiplying it by the

total suspension volume (1 mL).

Analyses

All angular data were summarised using circular statistics. Because anther and stigma

angles in active phases (i.e., male and female phases respectively) were in a small range

(between 150° and 180°), simple linear differences were calculated where needed. We arrived

at an operational definition for determining male and female phases by defining an angle of

transition between these two floral phases. This transition angle was set as 180° (based on

sporophyll movement [16]). When the average anther angle was less than (greater than) the

critical anther angle, the flower was considered to be in the male phase (female phase). The

critical angle is also the transition angle, so when the average anther angle is less than 180°,

this implies that the style angle is greater than 180°. In this way, although we have defined

phases based only on average anther angle, the definition captures the position and movement

of the stigma as well. At anthesis (hour 0), a large proportion of the anthers remains

undehisced, and stamens remain partly coiled (personal observations). For this reason we

consider the initial anther stage to begin at hour 2, when pollen from the dehisced anthers

become available for export.

Positional mismatch, which can lead to pollination inaccuracy, is estimated as the

absolute difference in measurements between the mean anther angle, and the mean stigma

angle of the natural population in their respective active phases. As the average anther angle

changed with time in the male phase (Supplementary Fig. 1), positional mismatch was

estimated at different time points in the male phase.

Using the experimental constructs, which manipulated sporophyll position, we

estimated pollen removal (from anthers) and pollen deposition (to stigmas), and with this,

modelled male fitness in relation to sporophyll angle. A phenomenological modelling

approach was used, where the best fit model was used to describe the relation, and parameter

values were either estimated from the data (see Results section) or collected from the

literature.

The number of exported pollen cannot by itself be used as a measure of male fitness.

Pollen export by vectors is very inefficient process, where most of the exported pollen does

not deposit on conspecific stigmas [34]. A better measure of male fitness is the estimated number

of exported pollen that is eventually deposited on conspecific stigmas. This depends on a

combination of pollen load, export rate, and the proportion of exported pollen that is

deposited per unit time (deposition rate). Following from the theory of pollination accuracy,

we assumed that pollen removed by pollinators from an anther at a particular position (angle)

is deposited on stigmas at that same position. In all, the male fitness of a flower with anthers

at a specific angle is a combination of the pollen export rate, pollen deposition rate and

available pollen load on anther at that angle. For the purposes of this study, we assumed that

all pollen deposited on a flower comes entirely from another single flower. If this assumption

is violated, the pollination efficiency parameter would change; we explore the possibility in

the Results section.

Pollen export rate was modelled using results from the morphological manipulation

experiment. For each manipulated treatment, we subtracted pollen counts of the anthers

(retained pollen) from the average initial pollen load of *Clerodendrum*, which is 6082 [22,23]. The

proportion of pollen exported from specific anther positions was calculated per 0.36 hr, which

is the time taken by anthers to move 1° (Supplementary Fig. 1). Pollen export rate is not

constant across the male phase, but rather changes with anther position. Export rate is initially

low, and as anthers move towards the floral axis, the rate increases and then decreases. Pollen

export rate E was modelled as a function of anther angle (x) in the following manner (Eq. 1).

Parameter n is the anther angle (on a linear scale) where pollen export rate would become 0.

Parameter m ($m > 0$) is the visitation rate. With the increase in number of visits pollen export

rate increases.

$$E = mx\left(1 - \frac{x}{n}\right) \qquad\qquad (1)$$

The number of pollen deposited at any specific angle was divided by the estimated

number of pollen exported from that angle to estimate the proportion of exported pollen that

was deposited on a stigma at that specific angle. The proportion of exported pollen that was

deposited per 0.36 hr (the deposition rate curve) (A) was modelled as a function of anther

angle (x) using a bell-curve function with a single peak (Eq. 2). Parameter a ($a > 0$) represents

the height of the curve, which is the indicator of the efficiency of the pollinator. Parameter b is

the angle at which pollen deposition is highest. This angle corresponds to the approach angle

of the pollinators. Parameter c ($c > 0$) is the strength of pollination accuracy selection. With

increasing strength of pollination accuracy selection, the pollen deposition rate curve becomes

narrower (Supplementary Fig. 2).

$$A = ae^{-c(x-b)^2} \qquad\qquad (2)$$

Pollen delivery rate depends on the pollen export rate and exported pollen's rate of

deposition onto conspecific stigmas. The deposition rate depends on pollination accuracy.

Therefore, the pollen export rate function (Eq. 1) was multiplied by the pollen deposition rate

function (Eq. 2) to estimate pollen delivery rate (Eq. 3) with respect to anther angle. This

equation estimates the proportion of exported pollen delivered to conspecific stigmas per unit

time (0.36 hr) at a specific anther angle.

$$D_r = EA \qquad\qquad (3)$$

The available pollen load (P) on anthers changes with time as stamens move.

Available pollen depends on the initial pollen load ($P(v) = 6082$, see above) and pollen export

rate (E, Eq. 1). The change in pollen load on anthers with time can be described by an

extension of the exponential population growth equation, where the rate is negative and

depends on anther position. The anther angle (*x*) was used as a proxy for time, as it changes

with time in a linear fashion for most of the duration of the male phase (Supplementary Fig.

1). Thus, anther pollen load is modelled here as a function of anther angle rather than as a

function of time. Change in pollen load (*dP*) with change in angle (*dx*) depends on export rate

j and pollen load *P*. The rate parameter *j* was replaced by the Eq. 1. By integrating both side

(definite integration, where *v* is the initial anther position and *s* is the anther position, where

pollen load needs to be calculated) we derive an equation (Eq. 4) that describes pollen load at

different anther positions (*P(s)*).

$$\frac{dP}{dx} = jP$$

$$\frac{dP}{dx} = mx(1 - \frac{x}{n})P$$

$$\frac{dP}{P} = (mx - \frac{mx^2}{n})dx$$

$$\int_v^s \frac{dP}{P} = \int_v^s (mx - \frac{mx^2}{n})dx$$

$$lnP(s) - lnP(v) = \left\{ \left(\frac{ms^2}{2} - \frac{ms^3}{3n}\right) - \left(\frac{mv^2}{2} - \frac{mv^3}{3n}\right) \right\}$$

$$lnP(s) = lnP(v) + \left\{ \left(\frac{ms^2}{2} - \frac{ms^3}{3n}\right) - \left(\frac{mv^2}{2} - \frac{mv^3}{3n}\right) \right\}$$

$$P(s) = P(v)e^{\left\{ \left(\frac{ms^2}{2} - \frac{ms^3}{3n}\right) - \left(\frac{mv^2}{2} - \frac{mv^3}{3n}\right) \right\}}$$

296   Because the rate is negative (pollen load decreases with time as anthers move) the exponent in

297   Eq. 4 is negative.

$$P(s) = P(v)e^{-\left\{ \left(\frac{ms^2}{2} - \frac{ms^3}{3n}\right) - \left(\frac{mv^2}{2} - \frac{mv^3}{3n}\right) \right\}} \tag{4}$$

298   The proportion of pollen delivered to conspecific stigmas per 0.36 hr at different

299   anther positions is described by Eq. 5. This is the male fitness curve, and is the product of

300   available pollen load (Eq. 4) and the pollen delivery rate (Eq. 3).

$$D_p = P(s)D_r \tag{5}$$

As active anthers are not stationary, overall male fitness (Eq. 6, total pollen delivered

to conspecific stigmas in the entire male phase) can be estimated only by summing fitness

over the lifetime of the anther (i.e., over the multiple positions it takes). This was done by

integrating the resulting male fitness function between the limits v (anther starting angle,

when anthers reach maturity) until u (180°– the transition angle, when sexual interferences

take place [16]). We have assumed that sexual interference is the key force driving the evolution

of dichogamy in *Clerodendrum*. A model has shown that sexual interference alone can lead to

the evolution of dichogamy [35]. Also, as flowering is asynchronous in *Clerodendrum* and

geitonogamy takes place [22,23], dichogamy can not help flowers much in reducing selfing.

However, it can minimise the sexual interference.

$$T_p = \int_v^u D_p dx \tag{6}$$

After the male fitness curve is estimated, it becomes possible to estimate the bias and

imprecision in the degree to which natural phenotypes match the optimum (the peak of the

fitness curve; found by numerical exploration). Bias is defined as the deviation between the

fitness of mean of the population and the theoretical maximum fitness. Imprecision is the

population variance of fitness, which is the scatter of individuals around the population mean.

Adaptive inaccuracy is estimated [21,36,37] as the sum of imprecision and the square of bias, with

the unit being the square of total pollen delivered.

All analyses were carried out in R, version 3.5.1 [38]; with the following additional

packages: nlme [39], boot [40,41] and circular [42,43]. Error bars in figures are 95% nonparametric

bootstrap confidence limits. This method circumvents restrictive assumptions about the data

distribution [44]. We draw inferences from the data based on the estimated effects and their

uncertainty as measured by their confidence intervals. In addition to the estimation approach

[45–47], we also presented results of null hypothesis significance tests in which the p-values

assess our ability to discern experimental effects statistically.

Results

In the male phase, the anthers are tightly clustered around 150° (mean vector angle, α

= 150.85°; [95% CI = 145.92°, 156.04°]; mean vector length, r = 0.98; N = 24). Similarly, in

the female phase, stigmas are closely arranged around 167° (mean vector angle, α = 167.17°,

[95% CI = 163.82°, 170.74°]; mean vector length, r = 0.99; N = 24). Although the difference

between the mean orientation of male and female sporophylls is only 17°, the low within-

group variation means that the difference is statistically discernable (Fig. 2; $p < 0.001$;

Watson-Williams test for homogeneity of means).

Fig. 2. Arrows show circular summary statistics of the angles of anthers at hr 2 in their active

phase and the angles of stigmas in their active phase. The direction indicates the mean, and

the length signifies the concentration. Radial lines flanking the group mean vectors mark the

95% confidence intervals.

While the stigma remains stable over its entire active phase the anthers move

continuously [16]. For the most part of the male phase, the anther angle changes approximately

linearly with time (Supplementary Fig. 1). To be specific, anthers take roughly 0.36 hr to

move 1°. The difference in movements between stigma and anther means that the positional

mismatch between the sporophylls changes with time. At eight hr from anthesis, positional

mismatch is minimal (Supplementary Fig. 3). Subsequently, it increases dramatically

towards the end of the male phase.

We compared pollen retention between control (male phase natural flower) and

treatment (160° angle floral constructs of anthers) flowers to estimate the effectiveness of

manipulated male flowers. Similarly, we compared pollen deposition between control

(female phase natural flower) and treatment (160° angle floral constructs of stigma) to

estimate the effectiveness of manipulated female flowers. There is a relatively small (and not

statistically discernible) difference between retained pollen number in control and treatment

($M_{control}$ = 1203, 95% CI = [768.03, 1730.97], $M_{treatment}$ = 1353.85, 95% CI = [944.62,

1812.23], Welch two sample t-test, t = -0.43124, df = 19.728, p = 0.671, $N_{control}$ = 10, $N_{treatment}$

= 13). Similarly, there is a small (in absolute terms, and not statistically discernible)

difference between deposited pollen number in control and treatment, though there is

considerable variation ($M_{control}$ = 2.25, 95% CI = [1.12, 3.5], $M_{treatment}$ = 4.2, 95% CI = [1.5,

7.5], Welch two sample t-test, t = -1.1314, df = 11.794, p = 0.2804, $N_{control}$ = 8, $N_{treatment}$ = 10).

Fig. 3. A. Pollen retained at anthers set at specific angles in morphologically manipulated

Clerodendrum flowers. B. Deposited pollen at stigmas set at specific angles in

morphologically manipulated flowers. C. Proportion of pollen exported per 0.36 hr from

anthers set at specific angles. The fitted curve representing pollen export rate function (Eq. 1)

is plotted. D. Proportion of exported pollen deposited per 0.36 hr at stigmas set at specific

angles. The fitted curve representing pollen deposition rate function (Eq. 2) is plotted. Error

bars are 95% nonparametric confidence intervals.

As expected, pollen retention for anthers affixed towards the basal end of the flower

is high (Fig. 3A), and when Eq. 1, (with parameters $m = 0.0004300188$; $n = 381.4351$) is

fitted to the proportion of pollen exported per 0.36 hr, the curve is rather flat at the peak

indicating a relatively broad range of positions over which pollen are removed (Fig. 3C). By

contrast, the pollen deposition curve (Eq. 2, $a = 0.00001725803$; $b = 170.0948$; $c =$

0.0008193516) shows a sharp peak with respect to position (Fig. 3D).

When overlaying the pollen export and pollen deposition functions (Fig. 4A), we see

that their peaks (190.7° and 170.1° respectively) are separated. The product of these two

functions, which describes pollen delivery rate, peaks at 170.8°, virtually identical to the

pollen deposition peak (Fig. 4B) because the pollen export function is relatively flat in this

region (Fig. 4A). The mean anther angle of the population (150.9°) at the beginning of the

male phase (hr 2) turns out to be far away from the delivery peak (Fig. 4B). Available pollen

load is highest at hr 2 (150.9°), after which it decreases rapidly (Eq. 4, Fig. 4B). When

anthers come to the delivery peak, their pollen has been substantially depleted (Fig. 4B).

From the starting position (150.9°) as anthers move, the number of pollen delivered from

different positions declines steadily (Eq. 5, Fig. 4C).

Fig. 4. A. Pollen export rate and pollen deposition rate focused on a narrower range of

angles. The resultant delivery rate is the product of the two equations. Maxima of each

function are indicated. B. Pollen load (Eq. 4) and delivery rate functions in relation to anther

angle, with the maxima of each function. C. The resultant delivered pollen at different anther

angle (Eq. 5).

385   The different starting positions of anthers lead to different numbers of total pollen

delivered (Eq. 6) to a single stigma from a single set of conspecific anthers (i.e., on a single

flower) in the entire male phase (Fig. 5). The highest total pollen delivery would happen if

anthers started at 146.3°. Surprisingly, starting at the position of the maximum delivery rate

(170.8°) leads to the lowest total pollen delivered. The total pollen delivered in case of the

natural starting position (150.9°) of anthers is close to the maximum peak. The overall

adaptive inaccuracy of the natural population is 0.0004341271 pollen^2, with imprecision

(0.0004330152 pollen^2) making a much larger contribution than bias (0.000001111884

pollen^2).

Fig. 5. Total pollen delivered (Eq. 6) to a single stigma from a single set of conspecific

anthers (single flower) in the entire male phase for different starting positions of anthers.

Cubic polynomial function ($y = -0.00000047066x^3 + 0.0001597981x^2 - 0.01653496x +$

0.5393821) is fitted to the data, where y is the index of total pollen delivered in the entire

male phase and x is the starting position of anthers. The points that indicate the maximum

total pollen delivered, total pollen delivered in natural population and total pollen delivered if

anthers would start at delivery peak are shown.

With the increase in visitation rate, both the total number of delivered pollen and

optimum start angle increases (Fig. 6A). With increasing pollinator efficiency, the total

number of delivered pollen increases (Fig. 6B). However, the position of optimum remains

the same. By contrast, with increasing strength of pollination accuracy selection, the optimal

peak moves closer towards the pollen delivery peak, but the number of total pollen delivered

decreases (Fig. 6C).

Fig. 6. A. The relation among visitation rate (parameter *m*) and number of total pollen

delivered (Eq. 6) and optimum starting position of anthers. B. The relation between

pollinator efficiency (parameter *a*) and the number of total pollen delivered (Eq. 6). C.

Anther starting positions where maximum pollen delivery (Eq. 6) occurs at different strength

of pollination accuracy selection (parameter *c*). Cubic polynomial functions are fitted to the

data.

Discussion

Contrary to expectation, *Clerodendrum* sporophylls do not take on identical positions

at the beginning of their respective active phases (Fig. 2). The mean stigma angle (167.1°) in

the natural population is close to the pollen deposition peak (170.1°). However, the mean

anther angle (150.9°) at the beginning of the male phase is away from the pollen export peak

(190.7°). In addition, while the stigma is stationary in its active phase [16], the anthers move at

the rate of 1° per 0.36 hr (Supplementary Fig. 1). As a result of that pollination accuracy

changes over time (Supplementary Fig. 3).

Anther movement during the active male phase results in a wide pollen export angle

(Fig. 4A). In the counterfactual situation, if anthers were stationary, pollen would be

exported from a restricted position and this would result in a narrow pollen export angle.

Pollen export angle has a crucial role to play in pollination. In general, if pollination

accuracy is maintained, we would expect that a narrow export angle would result in more

efficient pollen export than would a wide export angle. The observed wide export angle leads

to a lower deposition rate because pollen is exported from a broader zone, which includes the

accuracy peak but is not exclusively restricted to it.

If a narrow export angle is advantageous, then why is the pollen export angle of

Clerodendrum so wide (Fig. 4A)? Another way of asking this is why are anthers not stationary

during the male phase? Further, why are anthers positioned at a non-optimal angle at the

beginning of the male phase, when pollen load is high (Fig. 4B)? If variation in stigma angle

is high in the population, then a wide pollen export angle might ensure pollen deposition at

various positions. Moreover, there is an observed trade-off between pollen export and

pollination accuracy (i.e., the deposition rate of exported pollen) (Fig. 4A) possibly because of

the presence of pollen thieves in the visitor assemblage [22,48]. During the male phase, the

deposition rate of exported pollen gradually increases (Fig. 4A), while the amount of pollen

available to be exported decreases (Fig. 4B). A resolution to this conundrum could come from

the recognition that only a few pollen grains are sufficient for pollination [49,50], implying that

there would be no point in flooding optimally placed stigmas with additional pollen. In

general, optimal resource use would involve increasing quantity where quality is low and

reducing quantity where quality is high. This may be what *Clerodendrum* flowers do to

maximise male fitness. This strategy could result in the fertilisation of the greatest number of

stigmas in the population, including those away from the optimal stigma position.

Contrary to the expectation that the natural population of anthers should be at the top

of the delivery curve, at the beginning of the male phase anthers are far from the delivery

peak (Fig. 4B). However realised male fitness is actually related to the number of delivered

pollen, which in turn depends on not only on the delivery rate but also on the available pollen

load (Fig. 4B). Additionally, estimating overall male fitness requires integrating pollen

delivered over the entire male phase. How might overall fitness be related to the starting

position of the anthers? One might naively expect that anthers should start out at the position

associated with peak pollen delivery because pollen load is highest at the start of the male

phase. Instead, theoretically starting at this position would lead to low pollen delivery,

because of sexual interference [7]. This means that pollen load should be high at angles where

delivery rate is low, and as pollen load decreases, anther angle should change such that

delivery rate increases. Multiplying these curves (Fig. 4C) and integrating over the duration of

the male phase (Fig. 5) shows that, counter-intuitively, anthers should start away from the

position of peak delivery in order to maximise total pollen delivery. This is precisely what is

seen in the natural population, in which pollen can thus be delivered to flowers with a wide

range of stigma angles (Fig. 5). Although this argument is based on empirical evidence from

Clerodendrum, it is likely to hold true for all movement-assisted dichogamous species. This

also implies that the long-standing notion that the style and stamen take identical positions in

their active phases does not necessarily hold in movement-assisted dichogamy. Firstly, the

stamens are not stationary. Secondly, even if one focusses on the initial position of stamen,

this, too, deviates from the position of the style. Instead, the story is more complicated, and

anthers use a sophisticated and dynamic strategy to maximise male fitness.

Fig. 7. Number of pollen delivered at different anther angles at different pollination accuracy

selection strength (parameter *c*).

Pollination accuracy is a strong selection force in natural populations because it

determines pollen deposition on conspecific stigmas [11]. It is a form of stabilising selection and

is expected to be high, especially in complete dichogamous species [15,20,51]. However, the

pollinator environment is rarely constant for a species on an evolutionary timescale. In fact, it

varies considerably in space and time even within an ecological timescale. In such a situation,

fluctuating selection [52] is the likely outcome. A generalist species that is visited by multiple

pollinators whose relative abundance fluctuates over time and space is likely to experience

different strengths of pollination accuracy selection. One reason is that accuracy selection

strength could be pollinator-specific. For example, hawk moth pollinators might induce high

accuracy selection strength, because they have a stereotypical and restricted approach angle to

flowers: they always aligns themselves vertically in front of the flower and insert their

proboscis from a distance. By contrast, the approach angle of butterflies is more variable, as

they typically land on the inflorescence first and then insert their proboscis: this means that,

depending on the landing position, the approach angle could be wide. In addition, the

pollinating surface area of butterflies is large as their wings can carry pollen. On the contrary,

in hawk moths, only the base of the proboscis (close to the head) can carry pollen. Due to

these behavioural differences, pollination accuracy selection strength can differ from species

to species, especially if they belong to different pollinator guilds. *Clerodendrum* is a generalist

plant, visited by diverse groups of visitors, such as bees, butterflies, and hawk moths [22,23].

Therefore it is important to investigate the effect of different strengths of pollination accuracy

selection on anther movement dynamics. With increasing strength of pollination accuracy

selection (Supplementary Fig. 2), the optimal anther starting position should come closer to

the pollen delivery peak (Fig. 6C) as number of pollen delivered at initial anther angles

decreases (Fig. 7).

Different pollinators are also likely to vary in their pollination efficiency. However,

the model presented here suggests that this factor will not fundamentally change the

optimum starting position of anthers; instead, it would alter the number of pollen delivered in

the entire male phase (Fig. 6B). A further factor is visitation rate, which fluctuates greatly

within and across populations. An increase in visitation rate would increase the total pollen

delivered, and as well as shift the maximum pollen delivery peak towards the pollen export

peak (Fig. 6A) as pollen gets quickly depleted at higher visitation rate (Fig. 8).

Fig. 8. At different visitation rate (parameter *m*) change in pollen load as anthers move

during the active phase.

For *Clerodendrum*, in which anthers move in their active phase, we have

quantitatively shown that the starting position of anthers is close to the theoretical optimum.

However, one might raise a more fundamental question about why anthers in their active

phase are mobile at all. After all, a strategy of stable anther placement at the position that

maximises delivery rate would generate the highest fitness. This question remains

unresolved.

Overall, we conclude that *Clerodendrum* anthers are not in a stable position during

their active stage: instead they move, and as a consequence pollination accuracy also changes

over the male phase. Pollen export and pollination accuracy appear to trade off and their

peaks do not align. Anthers start away from the position where pollen delivery rate is

maximum. We demonstrate that overall male fitness, which depends on the total pollen

delivered over the entire male phase, would in fact be lowest if anthers start at this position.

Instead, starting away from the delivery peak means that pollen load is high where delivery

is low, and pollen load is low where delivery rate is high. We argue that, because of sexual

interference, this strategy in fact leads to the best outcome. In this way dynamic anthers

maximise male fitness in a rather complicated way. Although our model was built to explain

movement-assisted dichogamy specifically in *Clerodendrum*, a similar approach could be

used to model movement-assisted dichogamy in general. In conclusion, by testing a variety

of implicit assumptions that have been made about floral adaptations, our study reveals how

ecological aspects of pollination can impact the intricacies of floral adaptation and evolution.

References

1. Barrett, S. C. H. Understanding plant reproductive diversity. *Philos. Trans. R. Soc. B Biol. Sci.* **365**, 99–109 (2010).

2. de Jong, T. & Klinkhamer, P. *Evolutionary ecology of plant reproductive strategies*. (Cambridge University Press, Cambridge, 2005).

3. Bazzaz, F. A. & Grace, J. *Plant resource allocation*. (Academic Press, San Diego, 1997).

4. Campbell, D. R. Experimental tests of sex-allocation theory in plants. *Trends Ecol. Evol.* **15**, 227–232 (2000).

5. Charlesworth, D. & Charlesworth, B. Inbreeding depression and its evolutionary consequences. *Annu. Rev. Ecol. Syst.* **18**, 237–268 (1987).

6. Husband, B. C. & Schemske, D. W. Evolution of the magnitude and timing of inbreeding depression in plants. *Evolution* **50**, 54 (1996).

7. Lloyd, D. G. & Webb, C. J. The avoidance of interference between the presentation of pollen and stigmas in angiosperms I. Dichogamy. *N. Z. J. Bot.* **24**, 135–162 (1986).

8. Webb, C. J. & Lloyd, D. G. The avoidance of interference between the presentation of pollen and stigmas in angiosperms II. Herkogamy. *N. Z. J. Bot.* **24**, 163–178 (1986).

9. Cruzan, M. B. & Barrett, S. C. H. Postpollination discrimination between self and outcross pollen covaries with the mating system of a self-compatible flowering plant. *Am. J. Bot.* **103**, 568–576 (2016).

10. Bertin, R. I. & Newman, C. M. Dichogamy in angiosperms. *Bot. Rev.* **59**, 112–152 (1993).

11. Armbruster, W. S., Pélabon, C., Hansen, T. F. & Bolstad, G. H. Macroevolutionary patterns of pollination accuracy: a comparison of three genera. *New Phytol.* **183**, 600–617 (2009).

12. Gray, A. *Structural botany*. vol. 1 (American Book Company, New York, 1879).

13. Knuth, P. & Müller, H. *Handbook of flower pollination: based upon Hermann Müller's work 'The fertilisation of flowers by insects'*. vol. 1 (Clarendon press, Oxford, 1908).

14. Vogel, S. Ecophysiology of zoophilic pollination. in *Physiological plant ecology III: Responses to the chemical and biological environment* (eds. Lange, O. L., Nobel, P., Osmond, C. & Ziegler, H.) 559–624 (Springer-Verlag, Berlin, 1983).

15. Armbruster, W. S., Hansen, T. F., Pelabon, C., Perez-Barrales, R. & Maad, J. The adaptive accuracy of flowers: measurement and microevolutionary patterns. *Ann. Bot.* **103**, 1529–1545 (2009).

16. Mukhopadhyay, A. & Quader, S. Fine-tuned spatiotemporal dynamics of sporophylls in movement-assisted dichogamy: a study on *Clerodendrum infortunatum*. bioRxiv doi: https://doi.org/10.1101/2020.05.30.124818 (2020).

17. Armbruster, W. S. Estimating and Testing the Shapes of Adaptive Surfaces: The Morphology and Pollination of Dalechampia Blossoms. *Am. Nat.* **135**, 14–31 (1990).

18. Nilsson, L. A. The evolution of flowers with deep corolla tubes. *Nature* **334**, 147–149 (1988).

19. Johnson, S. D. & Steiner, K. E. Long-tongued fly pollination and evolution of floral spur length in the *disa draconis* complex (orchidaceae). *Evolution* **51**, 45–53 (1997).

20. Cresswell, J. E. Manipulation of female architecture in flowers reveals a narrow optimum for pollen deposition. *Ecology* **81**, 3244–3249 (2000).

21. Armbruster, W. Floral integration, modularity, and precision: distinguishing complex adaptations from genetic constraints. in *Phenotypic integration: studying the ecology and evolution of complex phenotypes* (eds. Pigliucci, M. & Preston, K.) 23–49 (Oxford University Press, Oxford, 2004).

22. Kumar, B. D., Deepika, D. S. & Raju, A. J. S. Reproductive biology of the hill glory bower *Clerodendrum infortunatum* L. (Lamiaceae). *Ann. Bot.* **7**, 43–56 (2017).

23. Reddy, T. B. & Reddi, C. S. Butterfly pollination of *Clerodendrum infortunatum* (Verbenaceae). *J. Bombay Nat. Hist. Soc.* **92**, 166–173 (1995).

24. Pascal, J. P. *Wet evergreen forests of the Western Ghats of India: ecology, structure, floristic composition and succession*. (Institut Français de Pondichéry, Pondicherry, 1988).

25. Umapathy, G. & Kumar, A. The occurence of arboreal mammals in the rain forest fragments in the Anamalai Hills, south India. *Biol. Conserv.* **92**, 311–319 (2000).

26. Sridhar, H., Raman, T. R. S. & Mudappa, D. Mammal persistence and abundance in tropical rainforest remnants in the southern Western Ghats, India. *Curr. Sci.* **94**, 748–757 (2008).

27. Schindelin, J. *et al.* Fiji: an open-source platform for biological-image analysis. *Nat. Methods* **9**, 676–682 (2012).

28. Vazquez, D. P. & Simberloff, D. Indirect effects of an introduced ungulate on pollination and plant reproduction. *Ecol. Monogr.* **74**, 281–308 (2004).

29. Ortega, E., Dicenta, F. & Egea, J. Rain effect on pollen–stigma adhesion and fertilization in almond. *Sci. Hortic.* **112**, 345–348 (2007).

30. Green, F. J. *Sigma-Aldrich handbook of stains, dyes, and indicators*. (Aldrich Chemical Co, Milwaukee, 1990).

31. Wood, G., Gabriel, A. & Lawson, J. Palynological techniques—processing and microscopy. *Palynol. Princ. Appl.* **1**, 29–50 (1996).

32. Jones, G. D. Pollen analyses for pollination research, unacetolyzed pollen. *J. Pollinat. Ecol.* **9**, 96–107 (2012).

33. Shivanna, K. R. & Tandon, R. *Reproductive ecology of flowering plants: a manual*. (Springer, New Delhi, 2014).

34. Johnson, S. D., Neal, P. R. & Harder, L. D. Pollen fates and the limits on male reproductive success in an orchid population. *Biol. J. Linn. Soc.* **86**, 175–190 (2005).

35. Sargent, R. D., Mandegar, M. A. & Otto, S. P. A model of the evolution of dichogamy incorporating sex-ratio selection, anther-stigma interference, and inbreeding depression. *Evolution* **60**, 934–944 (2006).

36. Orzack, S. H. & Sober, E. How (not) to test an optimality model. *Trends Ecol. Evol.* **9**, 265–267 (1994).

37. Orzack, S. H. & Sober, E. Optimality models and the test of adaptationism. *Am. Nat.* **143**, 361–380 (1994).

38. R Core Team. *R: A language and environment for statistical computing.* (R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/, 2018).

39. Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D. & R Core Team. *nlme: Linear and nonlinear mixed effects models. R package version 3.1-137. <URL: https://CRAN.R-project.org/package=nlme>*. (2018).

40. Davison, A. C. & Hinkley, D. V. *Bootstrap methods and their application*. vol. 1 (Cambridge university press, Cambridge, 1997).

41. Canty, A. & Ripley, B. *boot: Bootstrap R (S-Plus) Functions. R package version 1.3-20.* (2017).

42. Pewsey, A., Neuhäuser, M. & Ruxton, G. D. *Circular statistics in R*. (Oxford University Press, Oxford, 2013).

43. Agostinelli, C. & Lund, U. *R package 'circular': Circular Statistics (version 0.4-93). URL https://r-forge.r-project.org/projects/circular/*. (2017).

44. Chernick, M. R. & LaBudde, R. A. *An introduction to bootstrap methods with applications to R*. (John Wiley & Sons, Inc, Hoboken, 2014).

45. Nakagawa, S. & Cuthill, I. C. Effect size, confidence interval and statistical significance: a practical guide for biologists. *Biol. Rev.* **82**, 591–605 (2007).

46. Wasserstein, R. L. & Lazar, N. A. The ASA's statement on p-values: context, process, and purpose. *Am. Stat.* **70**, 129–133 (2016).

47. Wasserstein, R. L., Schirm, A. L. & Lazar, N. A. Moving to a World Beyond "p < 0.05". *Am. Stat.* **73**, 1–19 (2019).

48. Hargreaves, A. L., Harder, L. D. & Johnson, S. D. Consumptive emasculation: the ecological and evolutionary consequences of pollen theft. *Biol. Rev.* **84**, 259–276 (2009).

49. Shore, J. S. & Barrett, S. C. The effect of pollination intensity and incompatible pollen on seed set in *Turnera ulmifolia* (Turneraceae). *Can. J. Bot.* **62**, 1298–1303 (1984).

50. Quesada, M., Fuchs, E. J. & Lobo, J. A. Pollen load size, reproductive success, and progeny kinship of naturally pollinated flowers of the tropical dry forest tree *Pachira quinata* (Bombacaceae). *Am. J. Bot.* **88**, 2113–2118 (2001).

51. Opedal, Ø. H., Bolstad, G. H., Hansen, T. F., Armbruster, W. S. & Pélabon, C. The
    evolvability of herkogamy: quantifying the evolutionary potential of a composite trait.
    Evolution **71**, 1572–1586 (2017).

52. Eckert, C. G., Ozimec, B., Herlihy, C. R., Griffin, C. A. & Routley, M. B. Floral
    morphology mediates temporal variation in the mating system of a self-compatible plant.
    Ecology **90**, 1540–1548 (2009).