The buck in the milkweed: evidence of male–male interference among pollinaria on pollinators

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Summary

- Direct physical confrontation among conspecifics for access to mates is a form of sexual selection well known among animals, but not thought to take place in plants. Consequently, no structures are known that can be considered as weapons that evolved under such confrontation. Pollinaria of milkweeds may physically compete for access to attachment points on the pollinators' body, and occasionally pollinaria may link onto pre-existing pollinaria on a pollinator resulting in concatenation.
- We hypothesized that concatenation may result in interference between proximal and distal pollinaria, and that features of nonconcatenating pollinaria might be attributed to prevention of concatenation. We tested this by analyzing pollen donation efficiency, experimental manipulation of the phenotype and the phylogenetic patterns of co-occurrence of traits.
- It is shown that concatenation was able to diminish the reproductive performance of proximal pollinaria, that horns on pollinaria prevented concatenation, and that horn acquisition was correlated with a loss of concatenation. The experimental removal of horns in species that did not concatenate caused reversion to concatenation.
- The present work could be the first evidence of male physical struggles and of the acquisition of weapons related to these struggles, that are analogous to those known in animals.

Introduction

Owing to their immobility and lack of sensory perception, plants are thought not to compete physically for male reproductive opportunities (Lloyd & Webb, 1977; Willson, 1979, 1990, 1994; Arnold, 1994; Stanton, 1994; Grant, 1995; Murphy, 1998; Skogsmyr & Lankinen, 2002; Delph & Ashman, 2006; Moore & Pannell, 2011), with several pollen traits having been suggested to enhance the chances of pollen in fertilizing ovules, such as its early and abundant production, germination ability, pollen tube growth rate and pollen grain size (Andersson, 1994; Skogsmyr & Lankinen, 2002). These forms of sexual selection through male competition may be better described as scrambles amongst pollen grains to be the first to reach stigmas and fertilize the ovules therein. Interference competition by an apparently direct chemical interaction among pollen grains on stigmas has been reported previously for the wild radish (Marshall *et al.*, 1996). However, to our knowledge no study has yet found either direct physical interaction or interference competition among individuals of a species during pollination – that is, before pollen is deposited on stigmas. In addition, we have not come across any morphological product of evolution being attributed to this kind of competition in plants.

Most biologists agree that males fighting in competition for females has no scope in plants (Andersson, 1994). In fact, it has been consistently believed that direct physical interaction and a consequent evolutionary acquisition of defensive structures of the sort known in animals – such as weapons including horns that can be wielded in physical confrontations – does not take place in plants (Lloyd & Webb, 1977; Queller, 1987; Stanton, 1994; Grant, 1995; Murphy, 1998; Skogsmyr & Lankinen, 2002; Delph & Ashman, 2006; Moore & Pannell, 2011).

In milkweeds (Apocynaceae, subfamily Asclepiadoideae), large amounts of pollen typically aggregate in two pollinia which are connected by accessory sterile structures, making a complex pollinarium (Harder & Johnson, 2008). These sterile structures of the pollinarium consist of one median sulcate structure (the corpusculum), and two arm-like structures (the caudicles). Physical interaction between pollinaria takes place when a pollinarium already picked by a pollinator is inserted into a recipient flower by the same pollinator. In some species, insertion of a pollinium may result in the permanent mechanical linking, in a chain-like fashion, of a pollinarium of the recipient to the caudicle of the donor pollinarium. The pollinarium picked up first occupies a proximal position with respect to the pollinator, with the next pollinarium added occupying a distal and outermost position (Figs 1, 2, and Supporting Information Video S1). (Concatenation is the process of a pollinarium linking to a pre-existing one.) Although the adaptive value of pollinarium chaining in milkweeds remains unknown (Coombs *et al.*, 2012), it has been suggested as a means to increase the amount of pollen removed by pollinators and the chances of subsequent attachment of new pollinaria (Coombs *et al.*, 2012). This, however, would only be

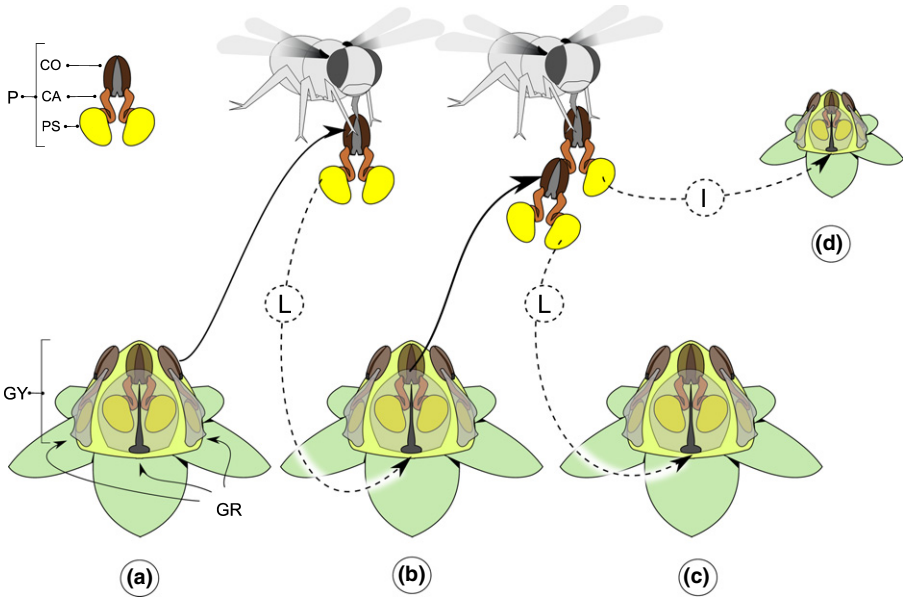

Fig. 1 Diagram of the pollination process in a milkweed where the pollinaria concatenate. Pollinarium parts are shown in the top left. (a–d) Successive visits to flowers by one insect. After having introduced the glossa in a guide rail of the flower (a), a pollinarium becomes attached to the pollinator's glossa. The pollinator then moves on to (b), where one pollinium of the pollinarium that was picked up first is delivered into the guide rail and a second pollinarium is picked up, which it clips to the same caudicle of the first pollinarium that has just delivered the pollinium. If the pollinator moves to (c), the proximal pollinarium with respect to the pollinator can only deliver its remaining second pollinium with interference (I) from the distal pollinarium, whereas if the pollinator moves to (d), the distal pollinarium may deliver either of its two pollinia free from interference (L). CA, caudicles; CO, corpusculum; GR, guide rails; GY, gynostegium; P, pollinarium; PO, pollinium.

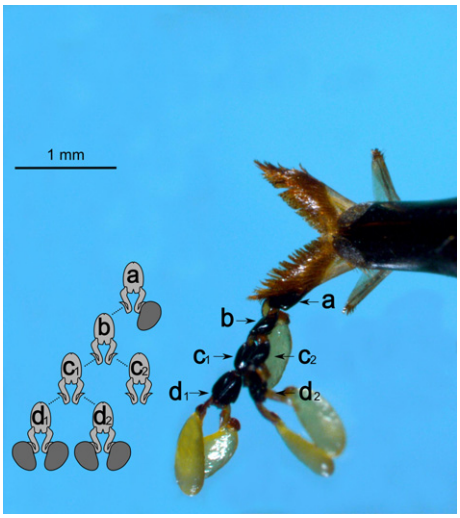

Fig. 2 Dorsal view of the mouth parts of *Pepsis* sp. showing one pollinaria chain of *Morrenia odorata* on the glossa formed by successive and dichotomous concatenations from one initial pollinarium (a). Letters tag the corpuscles; left, schematic tree.

plausible if the proximal and distal pollinaria come from the same individual plant. If, on the other hand, pollinaria chains are multiparental, which can often be the case, the selective effect of concatenation would be meaningless to functional coordination among pollinaria.

Concatenation may result in differences in reproductive performance between proximally and distally positioned pollinaria: first, a proximal pollinarium can be impaired in delivering one of its two pollinia due to spatial interference by the outermost pollinarium; second, a distal pollinarium might benefit from a proximal pollinarium providing a privileged outermost position where it can cling onto pollinators (Figs 1, 2).

If the possibility of building multiparental chains is high, then it should be advantageous for a pollinarium to avoid other pollinaria becoming linked to it. In fact, the chances of building multiparental chains are expected to be high when there are few flowers per plant and few attachment sites on the body of pollinators. Multiparental chains are also favored when self-pollination is avoided through long periods of pollinarium reconfiguration; that is, gradual reorientation by bending or twisting of the pollinarium after withdrawal from the anther, to attain the correct position for insertion into a stigma (Peter & Johnson, 2006). Species of the milkweed genus *Oxypetalum* often bear horn-like structures on pollinaria attachment sites to which no biological meaning has been attributed, but which are suggestive of a means to avoid concatenation. Related to this, it is also known that the ability to concatenate is species specific and varies within the Oxypetalinae clade (Vieira & Shepherd, 1999; Wiemer *et al.*, 2011).

In the present study, the hypothesis of sexual selection mediated through physical male interference was approached by addressing the two aspects of reproductive differences among the interacting pollinaria mentioned above, that is, the interference of distal pollinaria on proximal ones, and the occurrence of advantageous traits to maintain an outermost position. The effects of interference were addressed in four concatenating milkweed species by analyzing the consequences of pollinarium

position on a chain with respect to its pollen delivery performance. For these four species we tested whether proximal pollinaria – that is, those picked up first – experienced impaired delivery of their own second pollinium, or whether delivery occurred more often for pollinaria at the distal end of a chain. Impairment was measured as reduced second pollinium delivery efficiency, and the function of traits supposedly advantageous to maintain an outermost position was tested in two ways: first, by analyzing a milkweed phylogeny, including species that do and do not concatenate, to try to determine if caudicle horns are associated with the avoidance of concatenations; and second, by manipulating the pollinarium phenotype to directly test the influence of the caudicle horns on concatenation.

We specifically addressed the following predictions:

- The reproductive performance of a pollinarium depends on its position in a chain.
- The evolutionary acquisition of horn-like extensions on the attachment sites of pollinaria is associated with the prevention of concatenation.
- The evolutionary or experimental loss of horn-like extensions is associated with the occurrence of concatenation.

Materials and Methods

Study system

In Asclepiadoideae flowers bear five pollinaria that are located on a widened stylar cone, the gynostegium (Fig. 1; Endress, 1994). The corpusculum and caudicle enable both the coupling of the pollinarium to the pollen vector and the subsequent delivery of the pollinia into other flowers, with the corpusculum being functionally comparable to a nautical clamp cleat due to the way it firmly fastens to thread-like structures such as the bristle of an insect pollinator or the caudicles of a pollinarium. In addition, a pollinarium transported by a pollinator may come into contact with a pollinarium of a recipient flower during the process of pollinium insertion. Initially, a pollinarium is removed from a flower when pollinators withdraw a body appendage that has slipped into a guide rail that subtends one clasping corpusculum (Queller, 1983). The pollinia are inserted one at a time into different guide rails and get sliced off the pollinarium by sharp edges at the end of the rail (Liede, 1996; Wiemer *et al.*, 2011). During this process of insertion of a pollinium into a guide rail, the corpusculum of the corresponding pollinarium of the recipient flower may clip onto the caudicle of the pollinarium picked up first, thus building up a chain. Up to two pollinaria may become attached to each pollinarium, and several pollinaria may be concatenated dichotomously in branched tree-like complexes (Fig. 2).

Pollen donation efficiency

In order to measure the pollen donation efficiency of species which make pollinaria chains on the pollinators, we studied four milkweed species during October to December 2009 (Table S1). One species (*Philibertia gilliesii*) attached pollinaria on the legs, with the remaining three (*Morrenia odorata, M. brachystephana* and *Tweedia brunonis*) fixing them on the glossa of pollinators. By assuming that missing pollinia represent successful insertions (Theiss *et al.*, 2007; Wiemer *et al.*, 2011), the inspection of the pollinaria transported by milkweed pollinators allowed us to estimate the frequency of second donations made by proximal compared to distal pollinaria. This assumption that missing pollinia is a good estimation of successful insertion is based on the fact that pollinaria are very sturdy and do not split unless cut with sharp edges, and also that there is not an 'over-delivery' of pollinia as should be expected if there were a pollinia loss in addition to insertion (Theiss *et al.*, 2007; Wiemer *et al.*, 2011). The second donation efficiency was estimated by inspecting, under a dissecting microscope, 551 pollinaria from 295 visitors to the four concatenating milkweed species (the number of pollinaria for each milkweed species is given in Fig. 3). Then, using a χ^2 homogeneity test for each species separately, the null hypothesis was tested that delivery of the second pollen load is equally probable for distal and proximal pollinaria; that is, the proximal pollinarium was not impaired by the attachment of a distal pollinarium (see Notes S1).

Association between ability to concatenate and presence of horns

We tested if the presence of horns was associated with the prevention of concatenation. Ancestral reconstructions of the pollinarium trait 'presence of caudicle horns' and 'prevention of concatenation' were carried out on the Oxypetalinae phylogeny by the most parsimonious reconstruction method (MPR optimization) utilizing Mesquite (Maddison & Maddison, 2004). This analysis was performed on the existing *Oxypetalum* phylogeny based on four molecular markers (*trn*C-*ycf*6, *trn*H-*psb*A, *trn*D-*trn*T and ITS) and one morphological matrix containing a total of 32 characters (Farinaccio, 2007). Inclusion of other

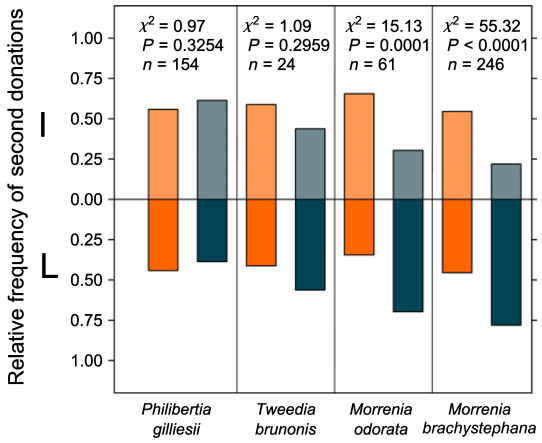

Fig. 3 Pollen donation efficiency in four concatenating milkweed species: efficiency of donation of the second pollinium after the first pollinium has been donated without (L) and with (I) interference. This interference occurs when a pollinarium becomes concatenated during the first donation by the pollinarium that was picked up first. The relative frequencies expected by a null hypothesis of no interference (orange) and observed (dark blue) are shown (for more details see text and Supporting Information Notes S1).

Oxypetalinae genera in the analysis was based on data from a subfamily-wide reconstruction obtained with sequences of *trn*L intron and *trn*L-F intergenic spacer of South American taxa (Rapini *et al.*, 2007). Information on the prevention of concatenation is available for 15 species in this clade (Vieira & Shepherd, 1999; Wiemer *et al.*, 2011; and present work) and thus the resulting tree was trimmed so that it included just these species of interest for this analysis. We tested if the evolution of horns on the caudicles was associated with prevention of concatenation in this sub-sample of species. To this end, we tested for independence with Pagel's 'correlation test' using Mesquite, setting up 1000 simulation replicates to calculate the significance (Pagel, 1994; Maddison & Maddison, 2004).

Functionality of the pollinarium horn

In order to test if horns prevented concatenation, we experimentally removed horns from the pollinaria and compared their ability to concatenate against nondissected pollinaria. Flowers of *Oxypetalum solanoides* were used, a species whose pollinaria bear horns and which does not concatenate (Table S1). The pollinaria were manually removed from the flowers using an eyelash mounted on a toothpick, which emulated an insect bristle. Then the horns were excised with a scalpel under the dissecting microscope, and the pollinaria held clasped at the tip of the eyelash were brought into contact with a guide rail in order to deposit one pollinium. For manual pollinium insertion attempts with nondissected ($n = 20$) and dissected ($n = 20$) pollinaria, we recorded whether chains were formed during insertion (total number of attempts $n = 40$) using a Sony CCD-Iris recorder (Tokyo, Japan) mounted on a Leica M420 stereomicroscope (Wetzlar, Germany). To evaluate whether the frequency of attempts resulting in concatenations was independent of the presence of horns, homogeneity was tested with a χ^2 test, computing *P*-values by a Monte Carlo simulation with the 'chisq.test' function using R v2.15.0 and setting up 10 000 simulation runs (R Core Team, 2012).

Results

Pollen donation efficiency

For two of the four studied milkweed species (*Morrenia brachystephana* and *M. odorata*), the number of second donations by pollinaria that had concatenated a pollinarium during the first donation was significantly lower than expected by the null hypothesis of no interference (Fig. 3). For *M. odorata*, the observed second donation frequency under interference was 54% lower than the expected frequency under the null hypothesis of no interference, whereas for *M. brachystephana*, the observed second donation frequency under interference was 60% lower than the expected frequency under the null hypothesis. Finally, for the other two species, there was a weak or undetectable effect on second donations by distally attached pollinaria (Fig. 3).

Association between prevention of concatenation and presence of horns

In Oxypetalinae, the most parsimonious reconstruction of the presence of caudicle horns resolved two equally parsimonious reconstructions, one with two events of horn acquisition and one event of horn loss (Fig. 4), with the other tree having three

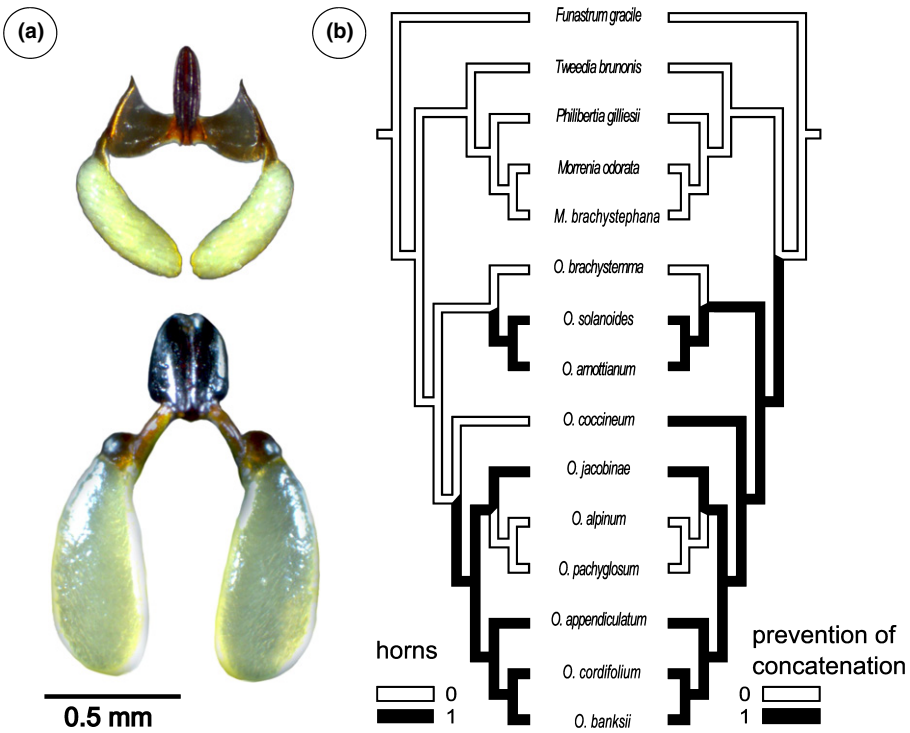

Fig. 4 Pollinarium horns and avoidance of concatenation in the Oxypetalinae phylogeny. (a) Pollinarium of *Oxypetalum arnottianum* (upper) and of *Morrenia odorata* (lower) which do not and do concatenate, respectively; *O. arnottianum* showing horns on the caudicles and a greater development of the corpusculum in relation to pollinium size. (b) One of the two most parsimonious reconstructions of the presence of caudicle horns and the ability to concatenate of 15 Oxypetalinae species; the presence of pollinarium horns is traced in black on the left-hand side of the tree and avoidance of concatenations is in black on the right.

independent events of horn acquisitions (Fig. S1). For the prevention of concatenation the analysis also resolved two equally parsimonious reconstructions, one with two independent losses and one reversal (Fig. 4), with the other tree having one loss and two reversals (Fig. S1). For any of these reconstructions, the overall association between the presence of horns and the absence of concatenation was highly significant across the phylogeny, according to Pagel's correlation test ($P = 0.0020$).

Functionality of the pollinarium horn in *Oxypetalum solanoides*

For manual pollinium insertions performed with nondissected pollinaria to nonvisited flowers, there was no formation of chains. The horn penetrated the guide rail first and drove the pollinium into it, with the caudicle becoming detached near the corpusculum in all attempts, thus leaving the horn and the pollinium inserted in the guide rail. The pollinarium attached to the vector was then released from the guide rail before it could make contact with the corpusculum of the pollinarium located at the end of the guide rail, precluding any possibility of concatenation. Thus, concatenation is apparently avoided by the horn acting as a stopper, keeping the caudicle away and preventing it from being clasped by the corpuscle.

By contrast, 65% of the pollinations performed with dehorned pollinaria resulted in caudicles not being detached, and being conducted into the corpusculum of the pollinarium sitting above the rail, which became clasped and formed a link (Video S2). The homogeneity test between intact and dehorned pollinaria vs concatenation frequency was highly significant ($\chi^2 = 337\,999.4$, $P = 0.00001$).

Discussion

Male competition is apparently severe in milkweed in aspects ranging from pollen dispersal to fertilization. These include: competition for space on limited attachment points on the body of pollinators; the presence of a reconfiguration mechanism that avoids self-pollination and increases spatial interaction among unrelated pollinaria (Peter & Johnson, 2006); the limited space in a flower for pollinia insertion; and the disproportionate number of pollinaria which may be required for a very low fruit set (Queller, 1983).

The proposed explanation of pollinaria chaining as a means to increase pollen removal by pollinators (Coombs *et al.*, 2012) may explain the origin of concatenation in milkweeds from a male competition perspective (see Harder & Johnson, 2008), provided that pollinaria in a chain belong to the same individual. Accordingly, concatenation is the evolutionary outcome of functional coordination amongst pollinaria. Here, we introduce a complementary explanation of concatenations: pollinaria may exploit attachment sites provided by other individuals and thus enjoy a privileged position to deliver their pollinia. Competition among pollinaria would then become inevitable and the differences in the pollinaria with respect to those which attached first would result in varying fertilization success among the pollinaria carried by the same pollinator. We were able to demonstrate that this competition may exist, at least in some species where the donation efficiency of the second pollinium was significantly reduced in proximal pollinaria by spatial interference of distal ones.

We propose that concatenation often has positive impacts, because it implies functional coordination amongst pollinaria of the same plant (see Coombs *et al.*, 2012). However, depending on the degree of cross-pollination, concatenation can easily have negative impacts, implying a competitive rivalry amongst unrelated pollinaria. Thus, as long as interaction among pollinaria is positive, the selection to concatenation would be favored, but as soon as interactions turn negative, the selection would favor the acquisition of horns and avoidance of concatenation. In this way, in some species, where male competition was not strong enough to have evolutionarily suppressed concatenation through the acquisition of horns, we were able to detect interference amongst concatenated pollinaria.

In our opinion, caudicle horns are a product of male competition for space on pollinators and for access to fertilization opportunities. In the Oxypetalinae phylogeny, pollinarium horns were acquired and significantly associated with the loss of concatenation, whereas the loss of horns caused a return to concatenations. Additionally, when the horns in *Oxypetalum solanoides* were experimentally removed, the pollinaria reverted to concatenation, suggesting that horns were evolutionary acquired or lost as a means to prevent or promote concatenations, respectively. The present findings could also provide an explanation for the existence of structures such as horns or tooth-like sterile expansions associated with the caudicles that are known on other milkweed pollinaria (Woodson, 1954; Bruyns, 2005; Farinaccio, 2007), but further observational and experimental data are still needed to confirm their function.

In relation to the function of the horn, it seems that the same pollinarium part which in concatenating species aids the insertion of the pollinium into the guide rail, has become evolutionary modified to work as a stopper, thus keeping the caudicle away from the corresponding corpuscle of the recipient flower. Therefore, a new function – prevention of linkage – may have been acquired by the caudicles in addition to guidance of the pollinium into the guide rail.

The hypothesis of rival interaction amongst pollinaria implies that an evolutionary race may be taking place with, on one side, defensive weapons on the attachment sites (caudicles) and, on the other, the breaching of the defense by the clasping site (corpusculum), with both aiming to be optimized. Future work should now be directed at determining if a coordinated development of these interacting and seemingly offensive and defensive traits is taking place.

The evidence provided here with respect to the evolutionary acquisition of horns corresponding to the prevention of concatenation, along with the interference of distal over proximal pollinaria in a chain, suggests an example of 'interaction dependent' (Murphy, 1998) sexual selection in plants – that is, of the type that Darwin (1871) proposed for animals. To our knowledge, intrasexual interference competition has been previously shown among plants only for wild radish (Marshall *et al.*, 1996), which

probably involved chemical interactions among pollen grains or tubes on the stigma or in the style. The present findings now add to this exceedingly short list an example that is noteworthy in several aspects: first, confrontations take place during pollination in a narrow sense, that is, after pollen removal but before its deposition onto the stigma; secondly, a direct physical interaction is involved; and third, remarkable morphological features such as horns can be identified as presumably evolutionary products of male–male confrontation.

Broadening the original Darwinian definition of sexual selection has allowed the application of the concept to both plants and animals (Willson, 1990; Arnold, 1994; Delph & Ashman, 2006; Moore & Pannell, 2011). However, to our knowledge, neither physical struggle nor intrasexual competition before pollen deposition on the stigma has been previously reported in plants (Willson, 1990; Delph & Ashman, 2006). Here, we further suggest and provide evidence that plants may have weapons of the sort known in animals as means of response to these struggles. Because the horns on the pollinaria of milkweeds are engaged in male physical struggles, they represent the botanical equivalents of the horns in many male vertebrates, such as bucks, and insects (e.g. Darwin, 1871; Andersson, 1994; Andersson & Iwasa, 1996).

From a broader perspective, the present results suggest that neither self-propulsion nor well-developed sensory perception are required for sexual selection to take place through intrasexual struggles. Apparently, only physical contact is enough to influence the reproductive success of competitors and to promote the evolution of defensive and attack weaponry. This should modify the way we think about sexual selection in organisms that, as in the case of plants, are hermaphroditic, have limited or no capabilities for autonomous movement, or do not have developed sensory perception, wherein reproductive success depends on physical interference among individuals to achieve fertilization.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Ancestral state of the two most parsimonious reconstructions.

Table S1 Taxonomic Tribes, species names and location of the study material

Notes S1 Mathematical model of pollen donation efficiency.

Video S1 Simulation of the pollination process showing the concatenation mechanism.

Video S2 Simulation of the pollination process showing the experimental effect of pollinarium dehorning.

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