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Variability in space and time: contrasting fruit distribution patterns in the deceptive orchid *Orchis militaris*

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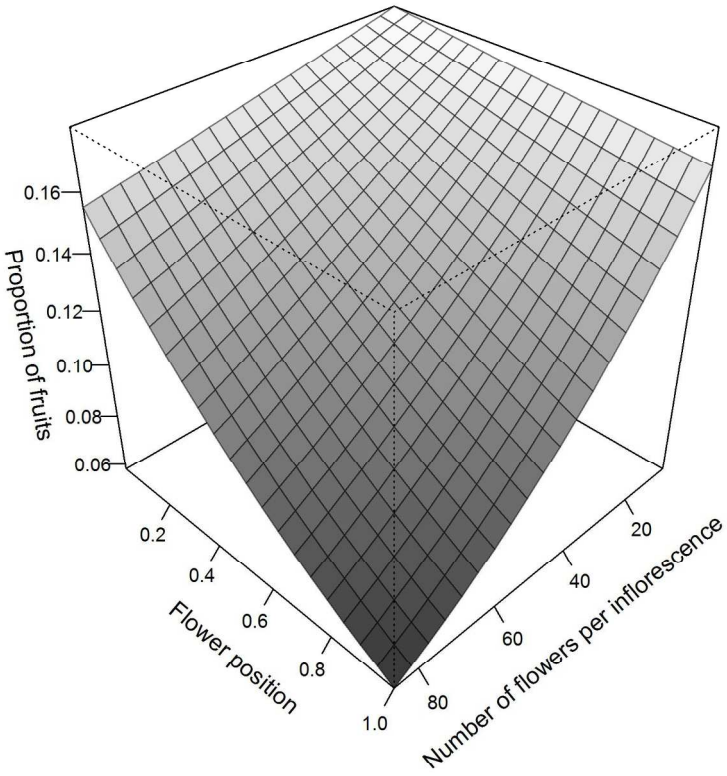

Figure 1. Interaction surface plot: proportion of fruits in relation to relative flower position and floral display size (2015 data).

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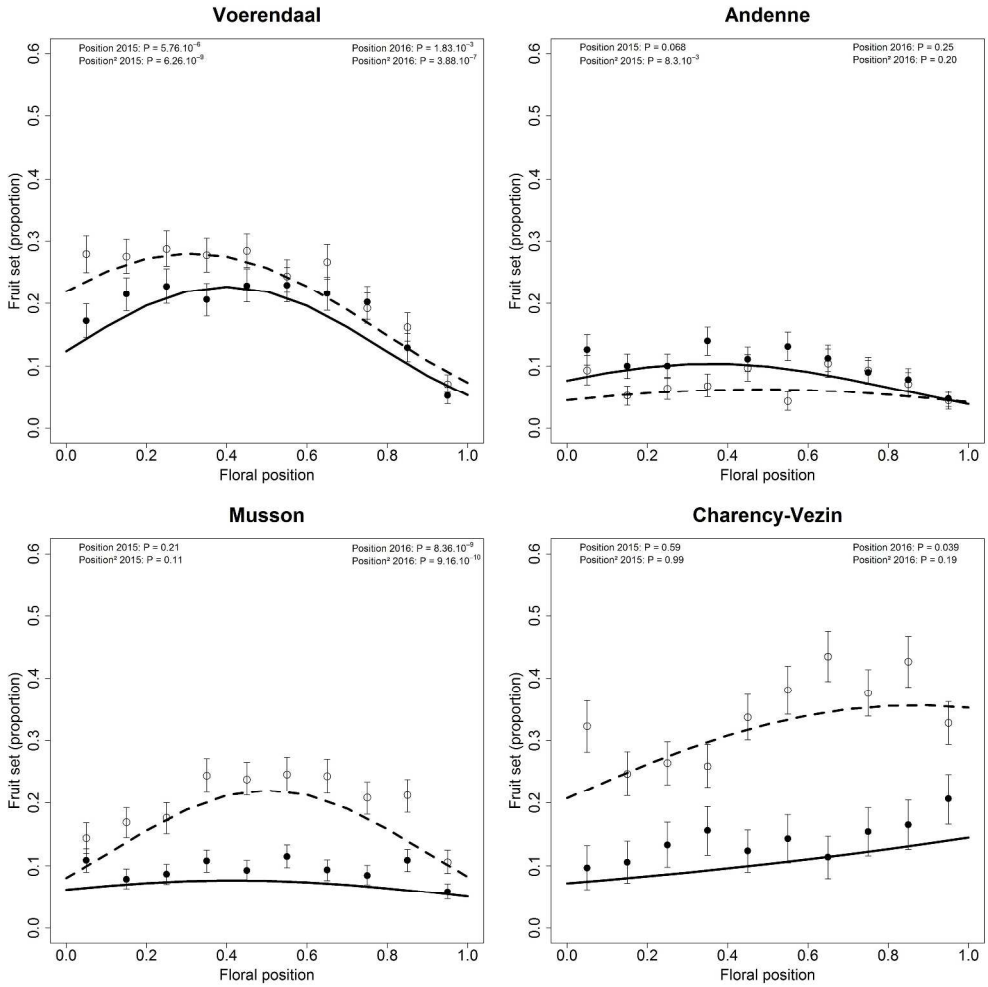

Figure 2. Patterns of variation in fruit production across relative flower position in Voerendaal, Andenne, Musson, Charency-Vezin, Dompcevrin, Bacourt, Longchamps-sur-Aire and Troussey. Regression curves represent fitted relationships based on the final model. For p-values, the significance threshold value is set at 0.025. Solid lines and filled circles (standard error, SE) represent 2015 data, and dashed lines and open circles (SE) represent 2016 data.

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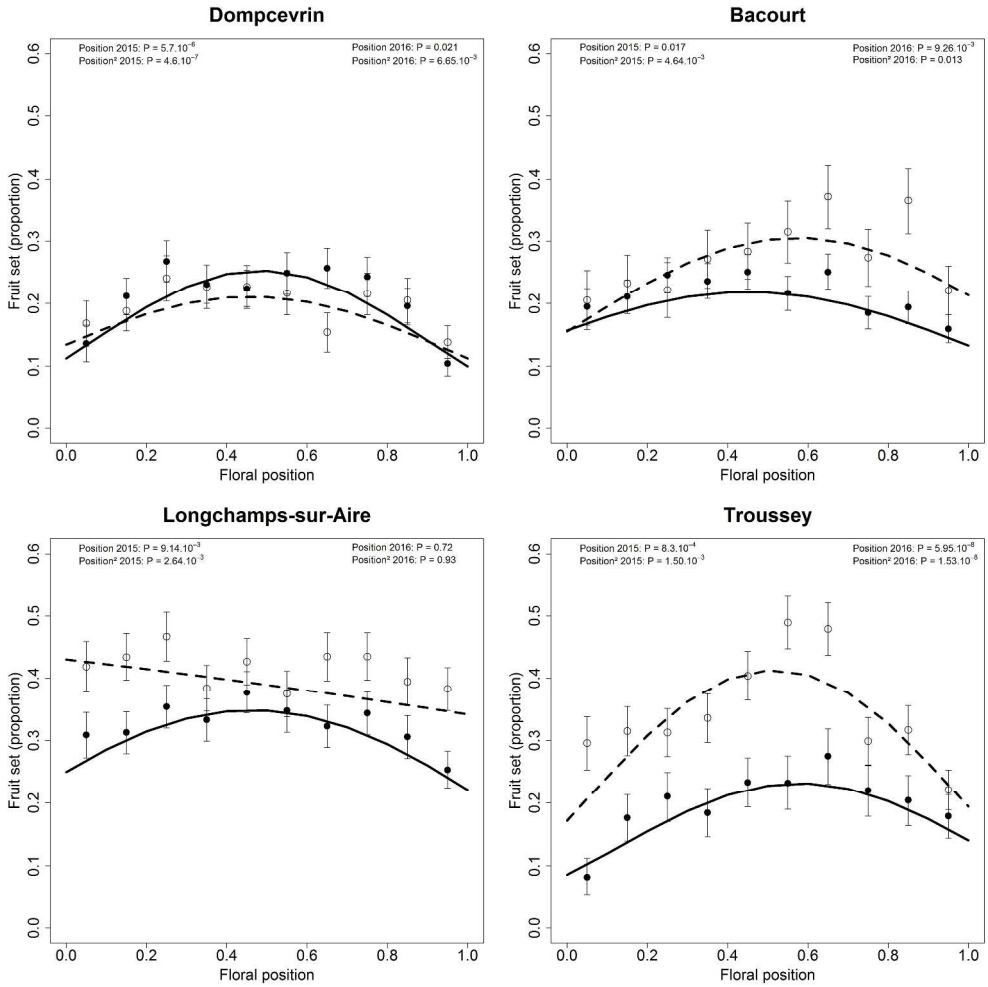

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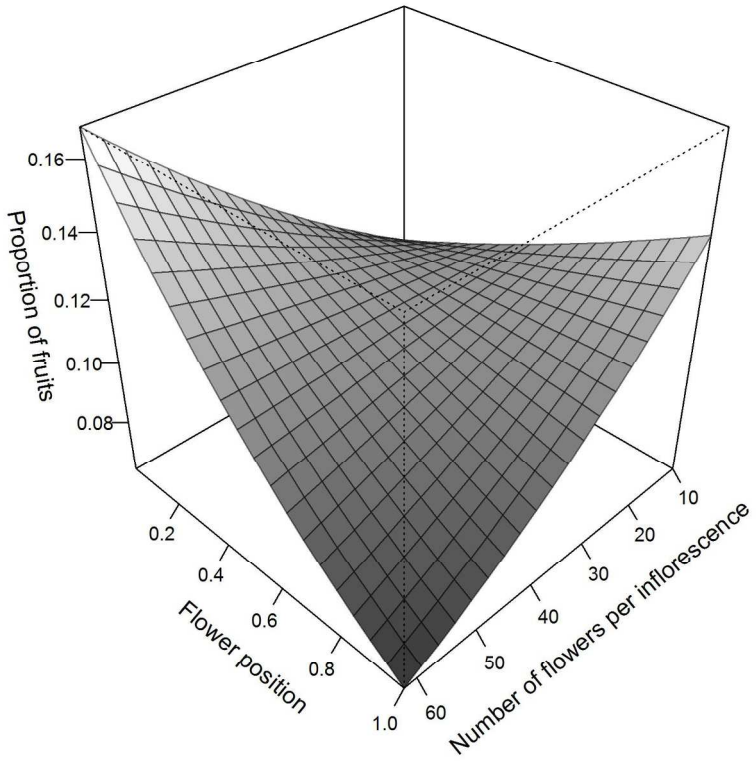

Figure 3. Interaction surface plot: proportion of fruits in relation to relative flower position and floral display size (Musson data).

177x177mm (300 x 300 DPI)

Variability in space and time: contrasting fruit distribution patterns in the deceptive orchid *Orchis militaris*

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Fruit distribution patterns in *Orchis militaris*

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Keywords: deceptive orchid, floral display size, flower position, fruit production, fruit set, Orchidaceae, pollinators

- In Angiosperms, a decrease of fruit production towards the apex of individual inflorescences is usually observed. Orchids are thought to be primarily pollination-limited species and non-uniform pollination could cause this decrease pattern in several species. Fruit production was investigated in relation to flower position and floral display size in *Orchis militaris* (Orchidaceae), a deceptive species.
- During two years, eight populations of *O. militaris* were studied and fruit position along the inflorescence was recorded. Generalised linear models were performed to examine the effect of population, year, flower position and floral display size on fruit production.
- The dominant pattern was characterised by a higher fruit set in the middle part of the inflorescence (parabolic pattern). A non-directional pattern of fruit production was also detected in some populations. Within a given population, patterns were generally consistent among years. In one of the two study years and in one of the eight populations specifically, the proximal-to-distal decrease in fruit production was dramatic in plants with a large floral display but weak or absent in small displays.
- Our study demonstrates the intraspecific diversity of fruit distribution patterns in *O. militaris*. Non-uniform pollination along the inflorescence is likely to be responsible for the parabolic pattern, while irregular visitation could explain the non-directional pattern of fruit production. Pattern variation among years and between populations could arise from spatiotemporal variation in pollinator assemblages. Resource competition effects could explain the interaction effect between display size and flower position.

INTRODUCTION

In flowering plants, fruits are usually not uniformly distributed within individual inflorescences (Lee 1988; Diggle 1995). Natural fruit production is typically higher at the bottom (in early flowers) than at the apex (in late-opening flowers) of the inflorescence in many species with acropetally developing inflorescences (i.e. proximal-to-distal decrease; e.g., Diggle 1995; Torres & Galetto 1999; Hiraga & Sakai 2007; Zeng *et al.* 2009). Although proximal-to-distal decrease in fruit production is the most commonly observed pattern, proximal-to-distal increase patterns (Sutherland 1987; Goldingay & Whelan 1993; Huth & Pellmyr 1997; Arista *et al.* 1999), fruit production peaking in the middle part of the inflorescence (e.g., Webb & Bawa 1985; Berry & Calvo 1991; Harriss & Whelan 1993; Ortiz *et al.* 2003) and non-directional patterns (Rodríguez-Robles *et al.* 1992; Jacquemart 1997; Navarro 1998) have also been reported in the literature. These patterns arise because fruit production is constrained by both biotic (pollinators, predation/disease, etc.) and abiotic (resource limitation, freezing, drought, etc.) factors (Stephenson 1981; Ayre & Whelan 1989; Diggle 1995). For example, resource competition between developing fruits, intrinsic declining investment in distal structures (architectural effects) and non-uniform pollen deposition have been proposed to explain proximal-to-distal decline patterns (Diggle 1995). In addition to flower position, floral display size has been reported to affect fruit production in numerous flowering plant species, larger displays often having positive effects on fruit set (e.g., Willson and Rathcke 1974; Udovic 1981; Bartkowska and Johnston 2014). However, flower position and floral display size can interact and influence fruit formation through resource limitation (Stöcklin & Favre 1994; Iwata *et al.* 2012). Resource competition between proximal and distal structures is likely to be exacerbated in larger displays due the higher number of competing structures that separate distal flowers from the nutrient source.

Orchids are primarily pollination-limited species (Tremblay *et al.* 2005), that is, their flowers primarily fail to set any fruit due to the absence of any pollen deposition on their stigma (Wilcock & Neiland 2002). The influence of flower position on natural capsule production has been studied in a variety of deceptive orchid species and, still, all four aforementioned fruit production patterns have been observed in this group: proximal-to-distal decrease (Nilsson 1980, 1983, 1984; Fritz 1990; Jersáková & Kindlmann 1998; Tremblay 2006; Vandewoestijne *et al.* 2009), proximal-to-distal increase (Vandewoestijne *et al.* 2009), higher fruit production in the middle part of the inflorescence (Vallius 2000) and non-directional pattern (Vallius 2000; Kropf & Renner 2005; Pellegrino *et al.* 2005a,b; Pellegrino *et al.* 2010). To describe natural patterns of fruit distribution in a given species, numerous studies focus on single populations followed during only one flowering season (e.g., Jersáková & Kindlmann 1998; Vallius 2000; Tremblay 2006). Moreover, data from different populations and/or years are often combined into a single dataset, thus eluding the effect of these variables (e.g., Berry & Calvo 1991; Kropf & Renner 2005; Vandewoestijne *et al.* 2009). Consequently, the extent to which fruiting patterns may vary in space (i.e. among populations) and time (i.e. among years) remains largely unknown in natural populations. However, in pollination-limited species, such a variation could exist because of spatiotemporal variation in pollinator assemblages and plant-pollinator interactions (Gómez & Zamora 2006) and differences in foraging behaviours among pollinator groups (Hambäck 2001).

The goal of this work is to contribute to the knowledge of fruit distribution patterns in primarily pollination-limited species with low levels of fruit set (Tremblay *et al.* 2005), namely deceptive orchids. In a multi-year and multi-site study, we aimed to accurately

describe the effect of flower position on fruit production, and taking into account the potential concomitant effect of floral display size. Under open-pollinated conditions, we measured the position of fruits within inflorescences, fruit production being a reliable indirect indicator of pollinator activity (Kull 1998), in the deceptive species *Orchis militaris* L. (Orchidaceae). We studied eight *O. militaris* populations in Western Europe along two consecutive years in order to evaluate both spatial and temporal variation in fruit distribution patterns. We aimed to answer the following questions: (i) What are the naturally occurring patterns of fruit distribution in *O. militaris*? We expected to find a proximal-to-distal decrease pattern in most of the studied populations since it is the commonest observed pattern; (ii) Is there a between-year consistency of fruit distribution patterns at the population level? Consistency could be prevented by temporal variation in pollinator assemblages; (iii) Does floral display size influence the effect of flower position on fruit set? If present, the proximal-to-distal decrease pattern is expected to be more pronounced in many-flowered individuals, because resource competition between proximal and distal structures may be more important in larger displays.

MATERIAL AND METHODS

Study species and sites

Orchis militaris is a perennial herb showing a Eurasian-Mediterranean distribution (Kretzschmar *et al.* 2007). The species ranges 0-2,200 m a.s.l. and grows in chalk grasslands, wet meadows, sand dunes, abandoned cultivated lands, scrub communities or open woodlands. *O. militaris* appears as a moderately long "stem" (not in a botanical sense), usually varying from 20 to 45 cm; plants develop a single acropetally developing raceme bearing ten to forty hermaphroditic and spurred flowers (Farrell 1985; Delforge 2012). In

Western Europe, it blooms from April to June (Delforge 2012). The flowering period lasts about a month in a given population, ten days elapse between the opening of the first proximal flower and the opening of the last distal flower of a given inflorescence and individual floral display size peaks during a week (T. Henneresse, pers. obs.). The species is assumed to be self-compatible (Neiland & Wilcock 1998; Metsare *et al.* 2015), non-autogamous (Delforge 2012) and it depends on the generalised food deception strategy to set fruits (Henneresse *et al.* 2017). According to Vöth (1987), hymenopterans from the genera *Andrena* (Andrenidae) and *Halictus* (Halictidae) are confirmed pollinators, although several potential pollinator species are also known (Henneresse & Tyteca 2016). Low levels of fruit set (< 30%) are common (Claessens & Kleynen 2011; Henneresse *et al.* 2017) and fruit maturation lasts around 30-35 days (T. Henneresse, pers. obs.).

The study was carried out from May to July 2015 and 2016 in eight natural populations at least 10 km apart located in Belgium, France and in the Netherlands (Table 1). Biotopes consisted in more or less open herbaceous formations on calcareous soils: recolonised slag heaps or quarries, semi-dry calcareous grasslands and mesic grasslands more or less colonised by shrubs (see Henneresse *et al.* 2017 for details).

Flower and fruit position

In each population and during each year, we marked between 50 and 150 *O. militaris* inflorescences (= plants), depending on population size. We selected a study individual every five plants when walking in a population. Plants were left to natural pollination. About one month after the end of the flowering period, we counted the number of flowers and capsules and determined the position of the fruits on each inflorescence. We replaced the absolute

flower position (from 1 to x, bottom to apex) by the relative flower position by applying the following formula: absolute flower position/total number of flowers of the inflorescence (Cao *et al.* 2007, 2015). As a result, the relative position varied from 1/x, the lowest position in the inflorescence, to 1, the highest position. Relative position was also used by Huth & Pellmyr (1997), Denham & Whelan (2000) and Tomaszewski *et al.* (2018). This method allows comparing inflorescences with varying number of flowers and it requires taking into account the effect of floral display size in the model analysis. In addition, it allows avoiding abnormally high values of fruit set in distal absolute flower positions, due to the low number of inflorescences displaying large number of flowers. Fruit set per inflorescence was calculated by dividing the number of capsules by the total number of flowers.

Statistical analyses

We used generalised linear mixed models (GLMMs, binomial error, logit link function) to analyse how the investigated factors affected fruit production, As the model failed to converge when year (and its interaction with other fixed effects) was included, we considered 2015 and 2016 data separately. Individual inflorescence (a unique combination of population and inflorescence number) was included as a random effect and the following fixed effects and their interactions were considered: population, position (i.e. relative flower position), quadratic effect of the latter (position²), floral display size (i.e. number of flowers per inflorescence), population × position, population × position² and position × floral display size. Other interactions were not included in the models due to excessive complexity and convergence failure. Wald χ^2 test was used to generate p-values (type II).

In order to investigate the among-year consistency of fruit production patterns, we performed population-specific GLMMs. In each model, we included individual inflorescence, year, position, position², floral display size, year × position, year × position² and position × floral display size. Wald χ^2 tests were used to generate p-values (type II). From these models, we went on to test the significance of position and position² for each year.

To plot the relationship between proportion of fruits and relative flower position, we categorised the latter into ten intervals (0-0.1, 0.11-0.2, …, 0.91-1). Fruit set for each interval was calculated (*y values*). The median of each interval (0.05, 0.15, …, 0.95) was chosen to plot *x* values. Then, we fitted regression curves to data points by calculating the inverse logit of the model equation. All statistical analyses were performed in R version 3.4.1 (R Development Core Team 2016) and the package *lme4* was used to fit GLMMs (Bates *et al.* 2015).

RESULTS

A total of 31,511 flowers, of which 6,677 developed into a fruit, were produced on 1,447 inflorescences. Individual fruit set varied between 0 and 0.97 (mean ± SE: 0.21 ± 0.01) and inflorescences developed 4 to 86 flowers (21.8 ± 0.3) (Table 1). In 2015, population, flower position, position², population × position, population × position² and position × floral display size had a statistically significant effect on fruit production. We detected a significant position × floral display size interaction: fruit production declined dramatically from proximal to distal flowers in larger floral displays; in smaller displays, proximal flowers had little advantage over distal ones (Fig. 1). In 2016, similar results were obtained, except that the interaction between position and floral display size had no statistically significant effect (Table 2). Due to

significant two-way interactions, regression curves were calculated for each population/position combination separately (Fig. 2). According to visual analysis and result summaries (p-values for position and position² are shown in Fig. 2), two patterns of fruit distribution were distinguished: uniform distribution of fruits along inflorescences (5 curves out of 16) and higher fruit set in the middle part of the inflorescence than in proximal and distal flowers (leading to a parabolic shape). The latter type of pattern was dominant and occurred in nearly all populations (11 curves out of 16). We detected significant year × position and year × position² interaction in only two of the eight studied populations (Table 3), meaning that the observed patterns were generally consistent within a population.

DISCUSSION

Our results show that there is a significant intra-inflorescence variation in fruit set in *O. militaris*. Although various patterns of fruit distribution have been described in the literature, a higher fruit production in the middle part of the inflorescence (i.e. parabolic pattern) was the commonest pattern in our study system. A non-directional pattern was also observed in 5 cases out of 16, although visual analysis suggested a directional pattern in some cases (e.g., linear decrease in Longchamps-sur-Aire in 2016: see Fig. 1, but statistically non-significant). Our study demonstrates the importance of including multiple populations and flowering seasons in the analysis to accurately describe the diversity of fruit distribution patterns.

Unlike the proximal-to-distal decrease pattern, the two patterns of fruit production detected in *O. militaris* – i.e. fruit production peaking in the middle part of the inflorescence (parabolic pattern) and non-directional pattern – can hardly be explained by resource competition or architectural effects as they are generally hypothesised (Diggle 2003). As mentioned

previously, orchids are primarily pollination-limited species (Tremblay *et al.* 2005) and pollinator activity (i.e. non-uniform pollination) is probably responsible for the proximal-to-distal decrease pattern in orchids with acropetally developing inflorescences (Tremblay 2006). The non-uniform pollination hypothesis posits that pollen deposition and/or pollen quality differ among stigmas, depending on their position on the inflorescence (Diggle 1995; Medrano *et al.* 2000). For example, if pollinators begin foraging in proximal flowers and move upward on inflorescences, proximal flowers may be more likely to receive higher quantity of pollen (Nilsson 1983) and more cross-pollen than distal flowers, leading to an advantage for fruit formation (Lee 1988). Furthermore, different groups of pollinators can have different food searching behaviours (Hambäck 2001; Matsuki *et al.* 2008; Castro *et al.* 2013), e.g. variation in arrival position and directionality. On vertical inflorescences, pollinators (mostly bumblebees) often begin foraging in proximal positions and move upward (Pyke 1979; Waddington & Heinrich 1979; Best & Bierzychudek 1982; Orth & Waddington 1997; Fisogni *et al.* 2011). This behaviour has been hypothesised to be due to vertical patterning of rewards (Pyke 1979), floral orientation (Wang *et al.* 2014), position adopted during flower visits (Corbet *et al.* 1981) or instinctive behaviour (Valtueña *et al.* 2013). The arrival position can also be located in the middle part of the inflorescence (Arnold 1982; Devlin & Stephenson 1985; Galen & Plowright 1985; Jersáková & Johnson 2007) or pollinators can start foraging at a random point (Valtueña *et al.* 2013) and some taxa move at random along the inflorescence (Fisogni *et al.* 2011).

As mentioned above, *O. militaris* is not pollinated by a single pollinator species, but rather visited by numerous potential pollinator species (Henneresse & Tyteca 2016), and pollination involves more or less effective pollinators. Those with a proboscis longer than the spur are ineffective since they reach the end of the spur before the viscidium adheres to their body

(Vöth 1987; van der Cingel 1995). The two fruit production patterns observed in our study could thus result from differences in pollinator assemblages and foraging behaviour among populations. On the one hand, the non-directional pattern of fruit production can be explained by irregular intra-inflorescence visiting patterns of local pollinators (Pellegrino *et al.* 2010). On the other hand, the parabolic pattern of fruit production could result from repeated pollinator visits in the middle of the inflorescences, potentially leading to higher fruit set (Lammi *et al.* 2003). In several studies focused on European food-deceptive orchids, female bumblebees were the main pollinators and their role was thought to be predominant in shaping the proximal-to-distal fruit production pattern (Nilsson 1980, 1983, 1984; Fritz 1990). Consequently, bumblebees may not be the primary pollinators of *O. militaris* in cases where the parabolic pattern was observed. Instead, solitary bees could be involved since the observations of Vöth (1987) suggest the importance of short-tongued species as confirmed pollinators of *O. militaris*. In several plant species with vertical inflorescences, solitary bees tend to begin foraging in the middle part of the inflorescence (Benedek *et al.* 1973; Arnold 1982; Bernhardt & Burns-Balogh 1986) and move upward less frequently than bumblebees (Fisogni *et al.* 2011; Valtueña *et al.* 2013; but see Kadmon *et al.* 1991). Their intra-inflorescence foraging movements could be responsible for the parabolic pattern, but (in)direct observations and pollination effectiveness studies are needed to confirm this hypothesis.

The two types of pattern were consistent over studied years in most populations. Between-year consistency was already reported in other studies (Arista *et al.* 1999; Pellegrino *et al.* 2005b) and may suggest the existence of time-stable factors (at least over the two study years) responsible for the observed pattern in a given population. By contrast, the absence of among-year consistency in fruit production patterns could imply temporal variation in the proximate

261 causes (e.g., pollinator identity and abundance). Pollinator assemblages vary among sites and
262 years and the diversity of plant-pollinator associations can change across years (Gómez &
263 Zamora 2006; Alarcón *et al.* 2008; Li & Huang 2009; Castro *et al.* 2013). Locally, the relative
264 abundance of different pollinators with different foraging behaviours could thus determine the
265 shape of the fruit production patterns. Differences in microhabitat selection by pollinators and
266 environmental factors (e.g., temperature, rain, etc.) could explain spatiotemporal variation in
267 insect abundance and diversity (Castro *et al.* 2013), but these effects remain to be investigated
268 in our system.
269
270 In 2015 (all populations) and in Musson specifically, the overall effect of flower position on
271 fruit set was influenced by floral display size. This statistically significant interaction effect
272 on fruit formation has apparently rarely been highlighted (Stöcklin & Favre 1994). In 2015,
273 the proximal-to-distal decrease in fruit production was dramatic in large displays, but quite
274 weak in small ones (Fig. 1). In Musson, a dramatic proximal-to-distal decrease only occurred
275 in large displays, whereas a weak proximal-to-distal increase occurred in small displays (Fig.
276 3). These results may reflect a greater resource competition between proximal and distal
277 structures (e.g., Diggle 1995) in many-flowered plants. However, Fritz & Nilsson (1996)
278 suggest that resource limitation is negatively correlated with floral display size in deceptive
279 orchids. In addition, Jacquemyn & Brys (2010) showed that resource limitation does not
280 restrict floral display size in *Orchis purpurea* Huds. since large inflorescences had a higher
281 probability of flowering again the next year than small inflorescences. In 2016 (all
282 populations) and in the seven other sites however, the effect of flower position was not
283 dependent upon display size. This suggests that the hypothetical important disadvantage of
284 distal flowers in large inflorescences due to resource competition is not a consistent feature in
285 *O. militaris*. Multi-site and multi-year studies focused on the quantification of the total

resource input are necessary to better understand resource reallocation within inflorescences (Dai *et al.* 2018).

In summary, our results reveal that fruit production in *O. militaris* depends on flower position and that two patterns coexist at the species level in different populations and among consecutive flowering seasons. The occurrence of the non-directional and parabolic patterns may have been previously underestimated, at least in deceptive species, due to inadequate sampling strategies. Since orchids are thought to be primarily pollination-limited species (Tremblay *et al.* 2005), pollinators and their behaviour are the most likely proximate causes for the observed patterns, which should be extensively investigated in future research. Importantly, our field study not only involves pollinators, but also, potentially, other factors affecting fruit production such as resource competition, diseases and predation. As such, further studies under controlled conditions (e.g., supplemental hand-pollination and addition or removal of pollinators) are needed to unravel the relative contribution of these factors in explaining the observed fruit production patterns. In deceptive species, pollinator visits to flowers are rare events difficult to record (Cozzolino *et al.* 2005; Claessens & Kleynen 2011) and thus time-consuming (Widmer *et al.* 2000). In this regard, accurate information about pollinator foraging behaviour and subsequent effects on plant ecology could benefit from technological advances such as automatic camera recording (e.g., Nakase & Suetsugu 2016; Steen 2017).

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529 Figure 1. Interaction surface plot: proportion of fruits in relation to relative flower position
530 and floral display size (2015 data).
531
532 Figure 2. Patterns of variation in fruit production across relative flower position in
533 Voerendaal, Andenne, Musson, Charency-Vezin, Dompcevrin, Bacourt, Longchamps-sur-
534 Aire and Troussey. Regression curves represent fitted relationships based on the final model.
535 For p-values, the significance threshold value is set at 0.025. Solid lines and filled circles
536 (standard error, SE) represent 2015 data, and dashed lines and open circles (SE) represent
537 2016 data.
538
539 Figure 3. Interaction surface plot: proportion of fruits in relation to relative flower position
540 and floral display size (Musson data).

Appendix 1. Results of the GLMMs used to analyse the effect of population, flower position, flower position², floral display size, and their interactions on fruit production. Tables show variance and SD for the random effect, and estimates and SE for fixed effects, separately for each year.

2015		
Random effect	*Variance*	*SD*
Individual inflorescence	0.603	0.777
Fixed effects	*Estimate*	*SE*
Intercept	-2.443	0.230
Bacourt	0.842	0.297
Charency-Vezin	-0.016	0.430
Dompcevrin	0.417	0.323
Longchamps-sur-Aire	1.295	0.300
Musson	-0.194	0.311
Troussey	0.014	0.387
Voerendaal	0.437	0.303
Flower position	1.758	0.971
Flower position²	-2.565	0.939
Floral display size	0.136	0.067
Bacourt × flower position	-0.149	1.230
Charency-Vezin × flower position	-0.858	1.722
Dompcevrin × flower position	2.250	1.329
Longchamps-sur-Aire × flower position	0.168	1.233
Musson × flower position	-0.725	1.284
Troussey × flower position	2.423	1.545
Voerendaal × flower position	2.044	1.261
Bacourt × flower position²	0.602	1.175
Charency-Vezin × flower position²	2.480	1.592
Dompcevrin × flower position²	-1.756	1.260
Longchamps-sur-Aire × flower position²	0.519	1.177
Musson × flower position²	1.336	1.234
Troussey × flower position²	-0.991	1.442
Voerendaal × flower position²	-2.104	1.223
Flower position × floral display size	-0.247	0.094

2016		
Random effect	*Variance*	*SD*
Individual inflorescence	1.021	1.010
Fixed effects	*Estimate*	*SE*
Intercept	-3.186	0.316
Bacourt	1.332	0.448
Charency-Vezin	1.774	0.388
Dompcevrin	1.277	0.415
Longchamps-sur-Aire	2.983	0.387
Musson	0.574	0.382
Troussey	1.595	0.395
Voerendaal	1.824	0.365
Flower position	1.462	1.268
Flower position²	-1.558	1.180
Floral display size	0.102	0.073
Bacourt × flower position	1.710	1.744
Charency-Vezin × flower position	0.246	1.521
Dompcevrin × flower position	0.936	1.645
Longchamps-sur-Aire × flower position	-1.676	1.513
Musson × flower position	3.312	1.500
Troussey × flower position	3.385	1.552
Voerendaal × flower position	0.748	1.434
Bacourt × flower position²	-1.182	1.605
Charency-Vezin × flower position²	0.520	1.407
Dompcevrin × flower position²	-1.146	1.525
Longchamps-sur-Aire × flower position²	1.494	1.408
Musson × flower position²	-3.018	1.397
Troussey × flower position²	-3.258	1.442
Voerendaal × flower position²	-1.874	1.351
Flower position × floral display size	-0.139	0.090