Functional implications of the staminal lever mechanism in *Salvia cyclostegia* (Lamiaceae)

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**Background and Aims** Flower morphology and inflorescence architecture affect pollinator foraging behaviour and thereby influence the process of pollination and the reproductive success of plants. This study explored possible ecological functions of the lever-like stamens and the floral design in *Salvia cyclostegia*.

**Methods** Flower construction was experimentally manipulated by removing either the lower lever arms or the upper fertile thecae of the two stamens from a flower. The two types of manipulated individuals were intermixed with the control ones and randomly distributed in the population.

**Key Results** Removing the sterile lower lever arms significantly reduced handling time per flower of the main pollinator, *Bombus personatus*. Interestingly, this manipulation did not increase the number of flowers probed per plant visit, but instead reduced it, i.e. shortened the visit sequence of the bumble-bees. Both loss of staminal lever function by removing lower lever arms and exclusion of self pollen by removing upper fertile thecae significantly reduced seed set per flower and seed set per plant. Both the manipulations interacted significantly with inflorescence size for the effect on female reproductive output.

**Conclusions** Though the intact flowers demand a long handling time for pollinators, the reversible staminal lever is of advantage by promoting dispersal of pollen and thus the male function. The particular floral design in *S. cyclostegia* contributes to the floral constancy of *B. personatus* bumble-bees, with the lower lever arms acting as an optical cue for foraging cognition.

**Key words:** Adaptation, *Bombus personatus*, experimental flower manipulation, floral constancy, floral design, foraging behaviour, geitonogamy, *Salvia*.

**INTRODUCTION**

Flowers are widely believed to be functionally integrated organs promoting efficient pollination through their interaction with pollinators (e.g. Endress, 1994; Armbruster et al., 2004; Harder and Barrett, 2006; Ordone et al., 2008). Flower morphology and inflorescence architecture affect pollinator behaviour and pollen transfer efficiency and, thereby, influence both the mating system and reproductive success of plants (Harder and Barrett, 1995; Williams, 2007; Karron et al., 2009; Sandring and Agren, 2009).

It has been demonstrated that plants employ a variety of strategies to manipulate the foraging behaviour of pollinators to their own advantage, i.e. to increase rates of outcrossing and fertility (Iwasa et al., 1995; Harder et al., 2001). To promote pollen dispersal, plants can distribute pollen among pollinators by either a packaging or a dispensing mechanism, i.e. restricting the pollen removed in a single flower visit (Lloyd and Yates, 1982; Harder and Thomson, 1989). In addition, complex floral design has been seen as one of the strategies by which plants manipulate a pollinator to leave the inflorescence relatively early (Ohashi and Yahara, 2001). Thereby, the degree of geitonogamous self-pollination, which is one of the main costs of large floral displays (Darwin, 1876; Harder and Barrett, 1995), can be reduced (Ohashi, 2002). Another important aspect of floral design for adaptation is its association with floral constancy when pollinators visit the flowers of one species, bypassing rewarding flowers of other species (Grant, 1950; Waser, 1986; Goulson and Cory, 1993; Lewis, 1993; Laverty, 1994; Geegar and Laverty, 2001). In the context of adaptation, it has been argued that complex flower constructions reflect the behavioural constraints associated with the cognitive abilities of pollinators (Darwin, 1876; Lewis, 1993; Geegar and Laverty, 2001).

The lever-like stamen in *Salvia* (Lamiaceae) is a particular form of modified stamen, which is derived from the elongation of the stamen’s connective (Hildebrand, 1865; Troll, 1929; Trapp, 1956; Claßen-Bockhoff et al., 2004a). In each flower, two stamens are present with their lower lever arms (LLA) centrally positioned in the entrance of the corolla tube with the fertile anthers located below the upper lip, forming a lever mechanism for pollination (Fig. 1C). Many studies have focused on the morphological and morphogenetic aspects of the lever-like stamens in *Salvia* (e.g. Correns, 1891; Troll, 1929; Trapp, 1956; Claßen-Bockhoff et al., 2004a) and also the systematic and phylogenetic relationships of *Salvia* species (e.g. Cantino, 1992; Wagstaff et al., 1995; Walker et al., 2004; Walker and Sytsma, 2007). Studies on pollination and
functional ecology have explored the lever’s functional significance (e.g. Grant and Grant, 1964; Claßen-Bockhoff et al., 2004b; Reith et al., 2007; Wester and Claßen-Bockhoff, 2007). However, little is known about the functional interplay between the lever mechanism and a particular flower design, and no experimental manipulations have been done to explore this relationship.

In the present study, flowers of *Salvia cyclostegia* were manipulated to investigate the influence of variation in floral design on aspects of pollinator foraging. First, the sterile LLAs of the two stamens in each flower, which act as a barrier blocking access of visitors to nectar, were removed. We predicted that removal of the LLAs would reduce the probing time per flower and would also affect the sequence of pollinators visiting (i.e. the number of flowers probed per plant visit). Secondly, the fertile anthers hidden below the upper lip were removed. As no own pollen was now present, all seeds would be the product of outcrossing.

With these manipulations, the hypothesis was tested that variation in flower structure affects the foraging pattern and floral constancy of pollinators and thereby the reproductive success of the plants. Specifically, three questions were addressed. (1) Does the removal of LLAs reduce the handling time per flower and increase the number of flowers probed per plant visit? (2) Does the change in floral design affect the preference (or floral constancy) of the pollinators probing flowers of *S. cyclostegia*? (3) Does the staminal lever mechanism influence the female reproductive success, i.e. does its loss have any consequence for seed set?

**MATERIALS AND METHODS**

**Study site and species**

The study was conducted during the flowering season of 2009 at Wenhui village, Lijiang City, Yunnan province, south-west
Salvia cyclostegia E. Peter. (Lamiaceae; Fig. 1C), a species endemic to China, is a small perennial herb distributed mainly in north-west Yunnan and south-west Sichuan provinces. It inhabits hillsides, meadows and pine forests, usually around 3000 m a.s.l. The study population is about 30 × 50 m² and includes approx. 4500 individuals, located on a slope next to a pine forest. The flowering season is usually in May. Each plant flowers for 20–25 d, i.e. the total flowering time is about 4–5 weeks.

Inflorescences are 30–50 cm tall, usually unbranched with verticillasters of five or six flowers each. Flowers are zygomorphic, white or white-yellow, with a hooded upper lip (Fig. 1B). Nectar is produced at the base of the ovary and accumulates in the narrow corolla tube. The style is exerted and protrudes out of the upper lip, thus contributing to a well-developed approach herkogamy (Fig. 1B). The connective of each stamen is widened, separating the two thecae of each anther from each other. The upper theca of each stamen is fertile and hidden below the upper lip, while the lower one is mostly reduced and acts as a barrier on the pollinator’s way to nectar (Fig. 1C). The filaments are relatively short and thick; a joint is formed at each of their distal tips enabling the reversal movement of the lever-like connective. When a pollinator pushes its head into the flower for nectar, the reversal movement of the lever-like connective. When a pollinator leaves, the two fertile thecae move back below the upper lip.

Experimental manipulations of flower structure

At the beginning of the 2009 flowering season, 120–150 plants of S. cyclostegia, randomly distributed in the population, were labelled with plastic tags. Almost all of the sampled plants flowered synchronously and possessed only one unbranched inflorescence per plant. The labelled plants were randomly assigned among three treatments, with the treatments intermixed and randomly distributed throughout the population, and each treatment involving 40–50 plants.

(1) LLA-removal treatment. To test the functional significance of the lever mechanism, in all freshly opened flowers on an inflorescence the LLAs were removed with a pair of small curved scissors (Fig. 1D). This treatment disabled the staminal lever mechanism, preventing pollen grains from being deposited on the pollinator’s back.

(2) Upper fertile thecae (UFT)-removal treatment. To test the consequence of the loss of staminal lever’s function for female reproductive success without a change to floral design, in all freshly opened flowers on an inflorescence, the UFT were removed by gently unwrapping the upper lip without touching the stigma (Fig. 1E). The UFT-removed flowers are apparently similar in their construction to the intact ones because the fertile thecae are enclosed in the upper lips and are not exposed to the insects.

(3) Control treatment. All flowers on an inflorescence were left intact (Fig. 1C) and exposed to insects for natural pollination.

Each day during the experimental period, these treatments were performed repeatedly until all the flowers on the treated inflorescences were withering. Because flowers of S. cyclostegia are usually open early in the morning, the treatments were usually started at 0730 h (sunrise) and finished by 0900 h when pollinators begin their activities. Finally, all treated inflorescences were collected about 20 d after flower withering. Each one was stored in a bag. The numbers of fruits and seeds per inflorescence were counted, and then the total flower number on each inflorescence determined by counting the number of pedicels which remain on the inflorescence after flower withering. In this way it was possible to estimate the percentage fruit set and seed set per flower to measure maternal reproductive success.

Pollination observation

Almost every day in the experiment, pollination was observed for 3–5 h after flower manipulations. In a defined area of the population, the frequency of each species of pollinator that appeared was recorded. When observing the behaviour of the visiting pollinators, one of the bumble-bees was randomly followed that appeared in the population at a time, recording the pollinator’s visitation sequences on the differently treated inflorescences, i.e. the number of flowers probed per plant visit. Meanwhile, the handling time per flower visit was also measured for each type of treated flowers, i.e. the duration from landing on the lower lip and crawling into the corolla tube to leaving the flower after taking nectar. To investigate pollinator foraging preference among treatments, the frequency of each type of flower encounter (probing: land and feed successfully; non-probing: approach closely and/or land but reject without attempting to feed) was recorded. The preference was determined by calculating the percentage of probing times in observed approaches for each type of treated inflorescence.

Five to seven individuals of each pollinator species were collected for species identification and for morphometric measurements, which included body length, thorax thickness, thorax width and tongue length (naturally extended length).

Data analysis

The probing time per flower was compared among treatments with ANOVA, prior to which the data were log-transformed to meet the assumptions of ANOVA; then Tukey multiple comparisons of means were performed.

The proportion of flowers probed per plant visit was calculated by dividing the number of flowers probed per plant visit (visitation sequence) by the number of open flowers on the inflorescence (floral display). The treatment effects on the proportion of flowers probed in floral display were tested by binomial regression with a logit link function.

The pollinators’ probing preference was tested with Pearson’s chi-squared test for the data on the frequencies of real probing or non-probing when pollinators approached each type of treated inflorescence.

Fruit set was calculated as fruit number per inflorescence and seed set as seed (nutlet) number per flower per inflorescence. For these data, linear regression was used to test the difference between the control and each of the two manipulating treatments. The overall statistical significance of the
treatment effects was determined using ANOVA with treatment as categorical variable and total number of flowers per inflorescence as random variable. Prior to analysis, arcsin square root transformation for fruit set, and sqrt-transformation for both seed set per flower and seed output per inflorescence was performed. For all data analyses, R version 2.7.2 (R Development Core Team, 2008) was used.

RESULTS

Pollinator assemblage

In the study population of *Salvia cyclostegia*, the pollinator assemblage mainly consisted of bumble-bees of three species: queen *Bombus personatus*, worker *B. friseanus* and queen *B. remotus*. Of the three species, *B. personatus* is the largest, with a mean (± s.e.) body length of 27.3 ± 0.96 mm (n = 5), thorax thickness 7.2 ± 0.09 mm (n = 5), thorax width 8.3 ± 0.21 mm (n = 5) and tongue length 12.5 ± 1.01 mm (n = 5). In terms of body size this species fits the flower proportions of *S. cyclostegia* very well. When bumble-bees probe flowers, they first touch the stigma and are then loaded with pollen on their back by contact with the anthers. *Bombus personatus* occurred with the highest frequency (>90%) of all pollinators and was thus the dominant pollinator in the population. The other two species, *B. friseanus* and *B. remotus*, are almost identical in body size and considerably smaller than *B. personatus*. Their mean (± s.e.) body length is 15.6 ± 0.72 mm (n = 7), thorax thickness 5.1 ± 0.35 mm (n = 7), thorax width 5.6 ± 0.37 mm (n = 7) and tongue length 5.2 ± 0.47 mm (n = 7). Individuals of the two species only appeared at the end of the flowering season with a very low frequency (<10%). When probing flowers, bumble-bees of the two species have to enter the flower completely to access nectar, with pollen being deposited on their dorsal abdomens. All bumble-bees of the three species visit flowers mainly for nectar, but occasionally the two small species also collect pollen.

Effect of manipulations on the dominant pollinator’s visitation

Removal of the staminal lower lever arms (LLA-removal treatment) significantly reduced the probing time per flower of *B. personatus* individuals (ANOVA, $F_{2.194} = 15.4, P < 0.001$; Fig. 2). Mean probing time per flower (± s.e.) was shortened from 3.66 ± 0.14 s (n = 102) of the control to 2.70 ± 0.14 s (n = 58) of the treated flowers ($P < 0.001$). For UFT-removal treatment, mean probing time per flower (± s.e.) was 3.74 ± 0.23 s (n = 37), very similar to the control, and also significantly longer than in the LLA-removal treatment ($P < 0.001$).

The percentage of flowers probed per plant visit of *B. personatus* individuals was significantly different among treatments (Fig. 3; n = 221, $P < 0.001$). For the LLA-removed inflorescences, the mean proportion of flowers probed per visit was 52.4%, significantly >63.2% of the control ($Z = -4.732$, $P < 0.0001$), while the UFT-removal treatment did not differ from the control ($Z = -0.006$, $P > 0.5$). The mean number of flowers probed per plant visit (visitation sequence) was 2.53 ± 0.19 (mean ± s.e., n = 62) for the LLA-removed, 3.28 ± 0.322 (mean ± s.e., n = 49) for the UFT-removed, and 3.37 ± 0.19 (mean ± s.e., n = 110) for the control inflorescence, respectively.

With respect to the pollinators’ visiting preference (Fig. 4), removing LLAs of the stamens significantly reduced the likelihood of a flower being probed ($\chi^2 = 8.90$, $P = 0.003$), i.e. the percentage of probing times in the total approaching times for the flower; while the treatment of removing UFT did not impose obvious effects on the visit selectivity compared with the control ($\chi^2 = 0.23$, $P = 0.632$).

Consequences of experimental manipulations on female reproduction success

Fruit set was significantly different among treatments (Fig. 5A; $F_{2.98} = 3.10$, $P = 0.049$). Fruit set of the controls (mean ± s.e.: 0.79 ± 0.019, n = 33) was significantly higher than that of the LLAs-removed inflorescences (mean ± s.e.: 0.73 ± 0.016, n = 37; $t = -2.57$, $P = 0.012$); that of the
FIG. 4. Percentages of successful probes and/or non-probes in *B. personatus* approaches of inflorescences with the intact (Control), simplified (LLA, stamens’ lower lever arms removed), and emasculated (UFT, upper fertile theca removed) flowers, respectively. LLA removal significantly reduced the probability of the manipulated flower being probed ($\chi^2 = 8.90$, $P = 0.003$).

FIG. 5. Mean ($\pm$ s.e.) fruit set (A), seed set per flower (B) and seed set per plant (C) on the intact (Control), LLAs-removed (LLA, stamens’ lower lever arms removed) and emasculated (UFT, upper fertile theca removed) inflorescence, respectively. *, Significantly different from the control at $P < 0.05$.

UFT-removed (mean $\pm$ s.e.: 0.75 $\pm$ 0.020, $n = 32$) was intermediate and not significantly different from the others.

Seed set per flower also differed significantly among treatments (Fig. 5B; $F_{2,98} = 3.19$, $P = 0.045$). Seed set per flower of the control (mean $\pm$ s.e.: 1.26 $\pm$ 0.057, $n = 33$) was significantly higher than that of both the LLAs-removed inflorescences (mean $\pm$ s.e.: 1.06 $\pm$ 0.073, $n = 37$; $t = -2.22$, $P = 0.028$) and the UFT-removed ones (mean $\pm$ s.e.: 1.06 $\pm$ 0.097, $n = 32$; $t = -2.34$, $P = 0.021$).

Seed set per plant (total number of seeds, i.e. of nutlets, per inflorescence) differed significantly among treatments (Fig. 5C; $F_{2,98} = 4.26$, $P = 0.017$). Mean seed number per plant ($\pm$ s.e.) was closely similar for the LLAs and UFT treatments (32.8 $\pm$ 2.69, $n = 37$ and 32.1 $\pm$ 2.75, $n = 32$, respectively) and both were significantly lower than the control (40.4 $\pm$ 2.38, $n = 33$), respectively (each $P < 0.03$).

For effects on both seed set per flower and seed set per plant, the interaction between treatment and inflorescence size was significant (seed set/flower: $F_{2,96} = 3.396$, $P = 0.038$, seed set/plant: $F_{2,96} = 3.948$, $P = 0.023$; Fig. 6). Total seed number per plant was significantly positively correlated with flower number per inflorescence both for the control (Fig. 6D; Pearson’s correlation, $R^2 = 0.30$, $t = 3.69$, $n = 33$, $P < 0.001$) and for the LLAs treatment (Fig. 6E; Pearson’s correlation, $R^2 = 0.26$, $t = 3.52$, $n = 37$, $P = 0.001$), but not for the UFT treatment (Fig. 6F; Pearson’s correlation, $R^2 \approx 0$, $t = -0.37$, $n = 32$, $P = 0.717$). Seed set per flower was significantly negatively correlated with the number of flowers on the inflorescence for the UFT treatment (Fig. 6C; Pearson’s correlation, $R^2 = 0.21$, $t = -2.80$, $n = 32$, $P = 0.009$), but not for the control nor for the LLAs treatment (Fig. 6A, B).

DISCUSSION

*Salvia* consists of over 900 species distributed world-wide and is characterized by modified lever-like stamens and a dorsal pollination mode (see Claßen-Bockhoff et al., 2003). In this study, the flower construction was changed by means of experimental manipulation, and the results indicated that the removal of the sterile LLAs and the resulting change in floral design generated significant effects on the foraging behaviour of pollinators with consequences for female reproductive success.

Manipulation of floral design and pollinator visitation

The interaction between plants and bees is based on conflicting interests (Jong and Klinkhamer, 2005; Westerkamp and Claßen-Bockhoff, 2007), as plants evolved to receive and export pollen as efficiently as possible, i.e. at the lowest cost, while bees evolved to collect the highest rewards in the most economical ways, i.e. with the lowest possible efforts and within the shortest possible time.

In this study, it was shown that the removal of LLAs significantly reduced the probing time per flower of the *Bombus personatus* individuals by over 30 %. This result is similar to previous studies (see Harder, 1983; Laverty, 1994; Gegear and Laverty, 1995; Ohashi, 2002). It implies that the presence of LLAs makes access to nectar more difficult and demands more handling time for pollinators. Although the particular
floral design reduces the pollinator’s handling efficiency, the staminal lever in \textit{S. cyclostegia} is of advantage as a mechanism for dispensing pollen. Given many pollen-removing visits, an individual plant would maximize its total pollen dispersal by presenting only a small portion of its pollen at one time (packaging mechanism) and/or by limiting the amount of the presented pollen removed during a single visit (dispensing mechanism) (Harder and Thomson, 1989). The repetitiveness of the staminal lever movement in \textit{S. cyclostegia} ensures pollen is removed by a few successive visits and thus enhances male function.

Pollinators tend to act as efficiently as possible for reward by, for example, visiting flowers of one species sequentially (floral constancy; Darwin, 1876; Gegear and Laverty, 2001) and probing more flowers per plant visit (Heinrich, 1979; Klinkhamer and de Jong, 1993). However, this will increase the opportunity for geitonogamy, reducing efficiency of pollen export for plants (Jong and Klinkhamer, 2005). It has been demonstrated that complex floral design evolves partly as one of the strategies to reduce the degree of geitonogamy by manipulating pollinators to probe fewer flowers per plant visit (Ohashi and Yahara, 2001). Ohashi (2002) found that the reduction in floral complexity in \textit{S. nipponica} through removing the whole stamens of all flowers increased the number of successive probes of pollinators within the inflorescence, i.e. extended the visit sequence. However, in the present study, the removal of LLAs, although removing the obstacle for the pollinator entering the corolla tube, did not encourage the bumble-bees of \textit{B. personatus} to probe more flowers in sequence than did the control, but instead shortened the visit sequence. One possible reason is that \textit{B. personatus} individuals have formed floral constancy to flowers of \textit{S. cyclostegia} (see Lewis, 1993; Gegear and Laverty, 1995, 2001). In the population studied, the LLAs-removed plants were comparatively rare and were randomly distributed among the other two types of plants whose flowers were intact in appearance. Therefore, when landing on the manipulated plants with non-LLA novel flowers, it is most likely for flower-constant foragers to leave early for other familiar-flowered ones where they can skillfully probe. Consequently, the LLAs-removed plants received an overall smaller number of successive probes than the control ones in the same population.

Furthermore, the finding that pollinators have forage selectivity further supported the view for floral constancy. When encountering LLAs-removed flowers, the \textit{B. personatus} bumble-bees were more likely to choose to ignore rather than probe than they did with the intact one, although the treated flowers were easier to probe. Also, the finding elucidates the role staminal lower arms play in the pollinators’ foraging cognition. Probably they serve as a key cue in forming a search image for the flowers of \textit{S. cyclostegia} when a pollinator is moving within and among plants (e.g. Wilson and Stine, 1996; Goulson, 2000). This viewpoint is also in agreement with the proposition that the staminal lower arms in \textit{Salvia} may function as a guiding and/or attractive structure for pollinators (Claßen-Bockhoff et al., 2004a). Apart from these aspects of the staminal lever’s function discussed above, the clipping manipulation possibly resulted in some other impacts, e.g. on odour emission of flowers, which could also affect pollinators’ response to the change in floral design.
Consequences of manipulation with flower structure on reproduction success

In the present study, it was shown that LLA manipulation incurred remarkable consequences for female reproductive success in *S. cyclostegia* at the individual level. The LLAs-removed individual has a significantly lower fruit set and seed output than the control. Most likely, this can be attributed to the reduction in pollinator attractiveness due to LLA removal as *B. personatus* showed remarkable discrimination against the manipulated flowers. Besides, LLA removal distinctly reduced both the probing time per flower and the number of flowers probed per plant visit. Consequently, the treated individuals received an overall reduced probing rate per flower and reduced pollen deposition on the stigma (Thomson, 1986; Harder, 1990; Young and Stanton, 1990; Conner et al., 1995). Furthermore, given that fertile pollen in the upper thecae cannot be effectively deposited on a pollinator’s back as a result of the lever function’s loss, LLA removal will ultimately lead to insufficient pollen availability for female reproduction due to the exclusion of self-pollen.

It was found that UFT removal (i.e. emasculation) neither affected probing selectivity nor probing time per flower and the number of flowers probed in sequence by bumble-bees, i.e. the UFT-removed individuals had the same opportunity to receive out-crossing pollen for female reproduction as the intact ones. Nevertheless, the UFT-removed inflorescences had a significantly lower seed set per flower and seed output per inflorescence than the control. Therefore, it is suggested that geitonogamy makes an indispensable part of female fitness realization in a population of *S. cyclostegia*. This result also implies that *S. cyclostegia* has a limited pollen carry-over in the population of the present study (see Robertson, 1992). Hence, when a bumble-bee probed several flowers on a large floral display, it is likely that the stigmas of flowers sequentially probed received self pollen, thus resulting in a high-degree of geitonogamy (also see Karron et al., 2009).

Finally, with inflorescence size being taken into account, it was suggested that only for the UFT-removed inflorescences did seed output not increase linearly with increasing flower number per inflorescence, and thereby only their seed set per flower significantly decreased with inflorescence size. According to field observations, it was found that the individuals of *B. personatus* mostly performed mop-up probing to various-sized inflorescences (also see Ohashi and Yahara, 1998, 2001), i.e. different-sized inflorescences largely stood an equal chance of receiving cross pollen. Even if large inflorescences have a high visitation rate, the probing rate per flower rarely increases (Ohashi and Yahara, 1999), and even decreases with increasing floral display (Jong et al., 1992; Wang et al., 2005). Therefore, it is highly probable for the UFT-removed inflorescences that exclusion of self pollen speeded up the reduction in pollen availability per flower with increasing inflorescence size and, consequently, led to seed set per flower decreasing with increasing inflorescence size.

In summary, as predicted, the removal of LLAs reduced pollinator’s handling time per flower, but it did not result in a higher number of flowers probed in sequence within an inflorescence. The change in floral design and the loss of lever function resulting from LLA removal incurred a distinct effect on the foraging cognition of *B. personatus*, with significant consequences for the female reproduction success. It is concluded that the staminal lever, although reducing pollinator’s handling efficiency, to some extent, is of advantage as a mechanism for dispensing pollen, and that this particular floral design is associated with a remarkable floral constancy, with LLAs contributing to pollinator attractiveness and/or foraging cognition in *S. cyclostegia*.

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**LITERATURE CITED**


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