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Is plastic cleistogamy an adaptive reproductive strategy? *A study of the annual species* Lamium amplexicaule.

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Summary

INTRODUCTION

1. Mixed mating systems

Reproductive systems are characterized by an extraordinary diversity of mating strategies throughout all taxonomic levels of living organisms. First of all, there is a distinction between sexual and asexual reproduction, then in sexual reproduction there are hermaphroditic species and species with separate sexes (dioecy). In hermaphroditic species reproduction can be achieved through outcrossing (mating with other individuals), selfing (mating with oneself), or a variable mixture of both (mixed mating) (Charlesworth, 2006).

Mixed mating (i.e. reproduction through selfing and outcrossing) has been documented in plants as well as in animals (Jarne and Charlesworth, 1993, Jarne and Auld, 2006, Goodwillie et al., 2005). All of the organisms capable of mixed mating are necessarily simultaneous hermaphrodites in order to be able to self fertilize. The frequency of hermaphrodites is remarkably high in plants, but not all hermaphroditic plant species are capable of selfing. In order to avoid the negative effects of selfing (inbreeding depression), hermaphroditic plants have developed various mechanisms for the avoidance of their own pollen – self incompatibility, heterostyly or dichogamy (Bawa and Beach, 1981). An important fraction of the hermaphroditic plants remains however capable to produce selfed progeny (Goodwillie et al., 2005).

Selfing in plants can be achieved through different mechanisms of pollen transfer (Lloyd and Schoen, 1992). Pollen could be either transferred from the anthers of one flower to the stigma of another flower of the same plant using pollen vectors (geitonogamy) or to the stigma of the same flower with or without the mediation of a pollen vector (facilitated and autonomous selfing respectively). In autonomous selfing we further distinguish prior, competing and delayed selfing, defined by the timing of self pollination relative to cross pollination, whereas facilitated selfing is obligately occurring at the same time as outcrossing since pollen vectors are needed for both pollination types. The evolutionary significance of each of these selfing mechanisms and their consequences for the fitness of the plant are different and depend on the ecological context. For instance, geitonogamy is considered as a byproduct

of floral adaptations aiming to attract more pollinators and thus should not be adaptive (Charlesworth and Charlesworth, 1987b, De Jong et al., 1993); facilitated selfing could be disadvantageous if there is gamete discounting (Lloyd, 1992), whereas delayed selfing, and in some conditions prior, competitive and facilitated selfing could increase individual seed set when outcrossing fails (Lloyd, 1979, Lloyd, 1992, Schoen and Brown, 1991b). The benefits and disadvantages of different selfing types depend on many other factors such as the quality of selfed and outcrossed progeny (inbreeding depression, Lloyd, 1979, Charlesworth and Charlesworth, 1987a), the production costs of the two progeny types (resource allocation, Iwasa, 1990) or the limited quantity of male and female gametes (pollen and ovules discounting, Lloyd, 1992, Holsinger, 1991). Thus determining the extent to which selfing provides fitness advantage for a mixed mating individual is a complex task, and a consistent amount of theoretical explanations has been developed in order to do so. A non exhaustive review of this theory, ranging from basic models that study simple genetic consequences of selfing versus outcrossing to more complex explanations, including the ecological context in which mixed mating occurs is presented in the following section.

2. Models for the evolution of mixed mating

2.1. Costs of outcrossing versus inbreeding depression

Individuals capable of partial selfing in an outcrossing population have an automatic transmission advantage over outcrossing individuals – they leave three copies of their genes (two through selfed seeds, i.e. self pollen and ovules and one through outcross pollen), contrary to individuals that only outcross which leave only two copies of their genes (one through their outcrossed ovules and one through outcrossing pollen (Fisher, 1941). This cost of outcrossing should inevitably lead the evolution of the mating system to complete selfing in the absence of opposing forces. The main opposing force to the cost of outcrossing is inbreeding depression, which reduces the fitness of inbred (issued from

selfing) relative to non inbred progeny (issude from outcrossing, Charlesworth and Charlesworth, 1999). Inbreeding depression is calculated as the difference of fitness of outcrossed (w_{out}) to selfed progeny (w_{self}) relative to that of outcrossed progeny: $\delta = \frac{w_{out} - w_{self}}{w_{out}}$. Thus if selfed progeny fitness is reduced to less than half the fitness of outbred progeny $(\delta < 1/2)$, complete selfing should evolve because of the high mortality/fitness loss in inbred progeny. Inversely, when selfed individuals are at least half as good as outcrossed ones the population should evolve towards complete outcrossing (Lande and Schemske, 1985). According to this model, any form of mixed mating is only a transition towards a stable, single mating type strategy. Schemske and Lande (1985) collected available estimates for the outcrossing rate of 55 species in order to test this hypothesis. Their data had a bimodal distribution with one modality for complete selfers and one for complete outcrossers and very few species in between. They conclude that the data corroborated the theory of unstable mixed mating (Figure I.1).

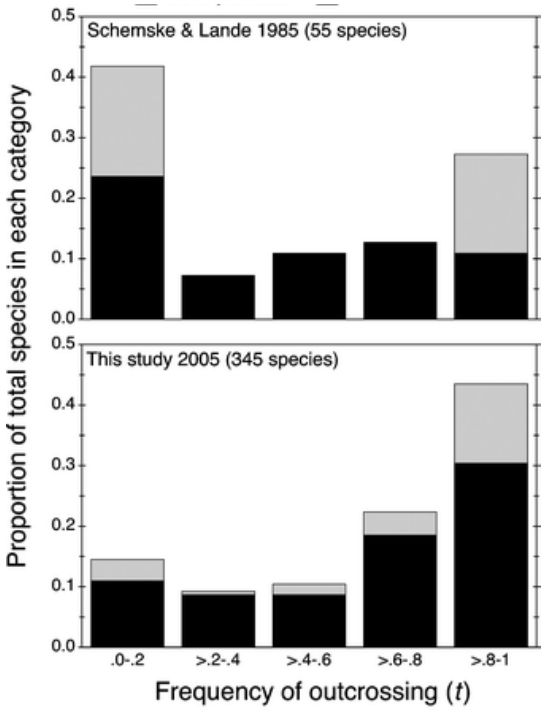

Figure I. 1. Distribution of the frequency of outcrossing through biotic (black) and abiotic (grey) pollinated species). Upper panel correspond to data for 55 species collected by Schemske and Lande (1985), while lower panel corresponds to data for 345 species collected by Goodwillie et al. (2005). Reproduced from Goodwillie et al. 2005.

Twenty years after the publication of Schemske and Lande's meta analysis, Goodwillie et al. (2005) updated it with new outcrossing rate estimates adding almost 300 new species (Figure I.1). This new distribution of mixed mating is different than the one observed by Schemske and Lande, with a significant decrease in the number of complete selfing and an increase of mixed mating species. Goodwillie et al. (2005) warn that their estimates can be biased because of the overrepresentation of some families, and because outcrossing rates are generally not estimated in species that are expected to have a single mating type. However, species with mixed mating represent about 50% of the studied data, which is a rather high occurrence for a supposedly unstable mating system. Therefore, incorporating other evolutionary of ecological factors in the theory of mixed mating could help to improve the predictions of Schemske and Lande's model and explain the frequent occurrence of mixed mating in plants.

2.2. Models predicting stable mixed mating in a non variable environment

Most of the models of mixed mating in plants include some effects of inbreeding depression. There are models that focus on explicit genetic mechanisms of inbreeding depression and its relation to the breeding system with no other evolutionary forces, and models that in addition to the effects of inbreeding depression include factors which either reduce the automatic advantage of selfing so that outcrossing could be maintained, or confer s advantages to selfing (different than the cost of outcrossing) up to a certain rate, beyond which increasing the selfing rate is unfavorable for individual fitness. For the latter category, there is a subset of models that also include the effect of ecological factors and environmental variation.

Models with explicit mechanisms for inbreeding depression

Deleterious effects of inbreeding could be due to the accumulation of at least partly recessive alleles which are more or less deleterious and revealed by inbreeding (partial dominance hypothesis), or to the lack of heterozygote loci that increase individual fitness (overdominance hypothesis, Charlesworth and

Charlesworth, 1999). Inbreeding depression caused by overdominance can increase with selfing (because of the increased homozygosity in selfed progeny) and under certain conditions allow for maintenance of mixed mating (Charlesworth and Charlesworth, 1990). However, it is known nowadays that the deleterious effects of inbreeding depression are mostly due to partial dominance rather than overdominance thus this model has limited application. When due to partial dominance, inbreeding depression can decrease with consecutive generations of selfing, a phenomenon called "purging" of the deleterious alleles. This is a consequence of the selective decrease of the recessive deleterious alleles frequency, which are revealed in homozygous individuals produced by selfing (Crnokrak and Barrett, 2002). Most often, theory supposes that inbreeding depression magnitude decreases with consecutive generations of selfing because of the purging of deleterious alleles (e.g. Lande and Schemske, 1985, Uyenoyama and Waller, 1991). This should generally favor the evolution towards complete selfing because of the positive feedback created by the purging of deleterious alleles.

Inbreeding depression can sometimes increase with consecutive generations of selfing if purging is not efficient, as it has been observed in some species (Schuster and Michael, 1976, Good and Hallauer, 1977, Frankham et al., 2001, Williams and Savolainen, 1996). If selfing increases inbreeding depression, this could limit the evolution towards complete selfing and maintain mixed mating under certain conditions. For instance, if inbreeding depression due to weakly deleterious mutations is not very pronounced after one generation of selfing (i.e. less than 0.5 for selfed individuals), but considerably increases under several consecutive generations of selfing because of the inefficient purge of mildly deleterious alleles, locally stable mixed mating systems can be maintained (Latta and Ritland, 1994). Finally, some models considering modifier and fitness genes show that under certain assumptions, modifier genes could behave as neutral loci when inbreeding depression is near 0.5, thus allowing for mixed mating (Charlesworth et al., 1992).

Factors that moderate the effects of automatic transmission advantage or the effect of inbreeding depression

Models based solely on the effects of inbreeding depression are often related to uncommon features of this parameter (e.g. overdominance hypothesis) and cannot explain more general cases of mixed mating. Therefore the basic effects of inbreeding depression and cost of outcrossing are often combined with other factors that modulate the basic effects of these two factors.

Pollen discounting - The model of Schemske and Lande (1985) assumes that selfing requires very small amounts of pollen, and in consequence a selfer has as much pollen available for outcrossing as an outcrosser (i.e. no pollen discounting). It is however plausible that increasing the selfing rate could impact the quantities of exported pollen through changing flower morphology for instance rendering flowers less attractive for pollinators (Ritland, 1991), or decreasing the quantity and quality of pollen produced (Holsinger, 1992). Pollen discounting reduces the automatic advantage of selfing and thus reduces the inbreeding depression threshold required for the evolution of outcrossing (Holsinger et al., 1984). Lloyd (1979) incorporated pollen discounting in a basic analytical model of phenotypic selection (inbreeding depression vs. cost of outcrossing). His results show that when the selfing rate affects the outcross pollen fertilization success, an intermediary parameters zone in which mixed mating could be stable for a range of inbreeding depression magnitudes exists. Holsinger (1991) used a mass action model that supposed frequency dependence of the selfing rate, i.e. individual selfing rate depends not only on the number of self fertilized ovules but also on the number of cross fertilized ovules which in turn depend on individual pollen export and population pollen export. The results of this model show that mixed mating can be favored in the absence of inbreeding depression. Several studies estimate pollen discounting in species with mixed mating systems (reveiwed in Busch and Delph, 2012) and they find different results according to the species and its life history. However, empirical data about pollen discounting are still very scarce, probably because of the difficulties encountered in measuring this

parameter (Holsinger and Thomson, 1994) thus further work is needed in order to evaluate its effect on mixed mating.

Biparental inbreeding - In populations with low dispersal, outcrossing could result in biparental inbreeding (mating among relatives). Biparental inbreeding increases the number of gene copies passed on the offspring through outcrossing, which decreases the cost of outcrossing and stabilizes mixed mating provided that inbreeding depression suffered by this type of inbred progeny is not lower compared to that suffered by selfed offspring (Uyenoyama, 1986).

Reproductive assurance - In addition to the cost of outcrossing, one of the most commonly cited advantages of selfing is reproductive assurance. Reproductive assurance is based on the fact that pollination of selfers is independent of external pollen contribution (i.e. independent of mating partners availability) and pollen vectors. Therefore selfing individuals should have higher offspring output than outcrossing individuals whenever outcrossing fails (Busch and Delph, 2012). Provided that selfing does not decrease the number of ovules available for outcrossing (no ovules discounting) or the outcross pollen exported by an individual (no pollen discounting), and that at the same time it increases the total seed set of a given individual, selfing can only be beneficial. This is the case with delayed selfing in annual plants (Lloyd, 1979, Lloyd, 1992).

2.3. Models for stable mixed mating in variable environments

The models presented in the previous section suppose that environmental conditions remain constant throughout an organism lifespan and across generations. For instance, Lloyd's models of reproductive assurance supposed a general, constant lack of outcross pollen. Pollen availability, along with many other environmental factors can vary in time and in space, changing the selection of reproductive assurance. Several models consider ecologically variable conditions that select for mixed mating as a "best of both worlds" strategy (bet-hedging; Philippi and Seger, 1989).

Reproductive assurance and pollinator ecology - Morgan and Wilson (2005) studied the effect of temporal variable pollination on the mating system in an adaptive dynamics model. They estimate fitness as the geometric mean of successfully produced selfed and outcrossed progeny to which the negative effects of inbreeding depression add up. Their model shows that mixed mating could be stable when reproductive assurance is provided by prior selfing. The parameters range for stable mixed mating in this model broadens when the variance of pollination effectiveness increases.

Reproductive assurance in novel habitats - Colonization of new habitats is another situation in which outcross pollen is scarce because of the lack of mating partners. Baker (1955) suggested that in such cases selfing should be favored. This hypothesis has been largely considered since (see in Cheptou, 2012). For instance, in a metapopulation model, Pannell and Barrett (1998) show that self fertilization is advantageous in novel populations in which outcross pollen availability is scarce, but this advantage shifts to outcrossing when population density increases. However, the opposite results have been observed in some models. Assuming evolutionary syndromes (sets of traits) that combine dispersal strategies and mating types in a metapopulation with unpredictable pollination success, Cheptou and Massol (2009) show that selfing should be rather associated to non-dispersal strategies and outcrossing should be associated with dispersal strategies. Though results of different models about the effect of dispersal on mixed mating are seemingly contradictory, the global conclusion is that dispersal to new habitats with different qualities than the native habitat can maintain mixed mating.

Environment dependent inbreeding depression - The idea that inbreeding depression depends on environmental variation has recently been confirmed by numerous empirical studies (reviewed in Armbruster and Reed, 2005). A remarkable number of studies measuring inbreeding depression in two or more environments show that inbreeding depression is in general more pronounced in stressful than in benign environments (Armbruster and Reed, 2005, Fox and Reed, 2011). Based on these observations, recent models show that environment dependent inbreeding depression can maintain stable mixed

mating (Cheptou and Mathias, 2001, Cheptou and Schoen, 2002). For instance, in a phenotypic model Cheptou and Mathias (2001) show that spatial or spatio-temporal variation of inbreeding depression results with higher fitness averaged over time in mixed mating individuals compared to individuals that reproduce through a single mating strategy.

Resource allocation - Sakai (1995) developed an analytical model of phenotypic selection in which increasing flower size or floral display result in non linear increase of flower costs. Mixed mating is maintained as a balance between the costs of selfing (inbreeding depression) and the costs of large floral displays: in order to reduce the costs of large floral displays necessary for pollinator attraction, the individual increases the selfing rate. In another phenotypic selection model, Iwasa (1990) developed a similar model which includes costs of selfed and outcrossed seeds and costs of floral display. In this model the relationship between selfing rate and resource availability depends on the shape of the costs function, and mixed mating is maintained when costs of floral display increase exponentially.

Most of the mixed mating models that consider environmental variation suppose a single, invariable outcrossing rate. In other words, mixed mating is a bet-hedging strategy which allows optimizing the average fitness of a given genotype/phenotype over time, compared to a single mating genotype/phenotype (Cheptou and Mathias, 2001, Cheptou and Schoen, 2002, Morgan and Wilson, 2005, Holsinger, 1986), but some models suppose variable outcrossing rate that can adjust to environmental conditions (Pannell and Barrett, 1998, e.g. Sakai, 1995). If a plant is capable of adjusting its outcrossing rate according to environmental variation, its fitness would be additionally increased compared to plants with fixed outcrossing rates. Variable outcrossing rates can increase seeds production in some environments, and/or can allow the production of the most appropriate progeny type in each environment thus decreasing the number of maladaptive individuals. Furthermore, if the resources not spent on maladaptive progeny are reinvested, more adapted offspring in the focal

environment can be produced. (Figure I.2). However, several conditions need to be fulfilled in order for plasticity to be adaptive and to be more beneficial than a fixed mixed mating type.

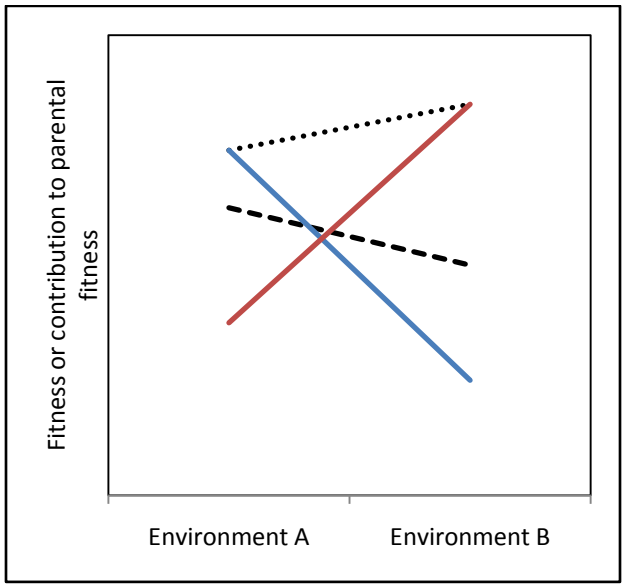

Figure I. 2. The advantage of plastic versus fixed mating types in variable environments. When individuals experience environmental variation, selfed and outcrossed progeny contribution to parental fitness may differ across environments. Here, selfed progeny (in blue) contribute more to parental fitness in environment A, and inversely, outcrossed progeny (red) contribute more to parental fitness in environment B. Thus a parent with a stable mixed mating strategy (dashed lines) will yield higher average fitness in variable environments than parents that reproduce only through selfing or outcrossing. Furthermore, if the parent is capable of modifying its outcrossing rate thus reproducing only to the appropriate mating type in the focal environment (dotted lines), its fitness would be even higher compared to that of a parent with a single, fixed mixed mating strategy.

3. Plasticity

Plasticity is the ability of a given genotype to modify its phenotype in response to environmental variation (Bradshaw, 1965). The effect of the environment on phenotypic expression has been known for a long time, though in the past it has been considered as a nuisance for the study of living organisms, hence the necessity of controlled experimental conditions (Sultan, 2000). Nowadays, phenotypic plasticity in response to environmental change is fully acknowledged as an important mechanism taking part in the evolution and adaptation of living organisms which could influence individual fitness and the interactions between an organism and its environment as well (Miner et al., 2005, Via et al., 1995). Plasticity has been detected for morphological, physiological, phenological, live-history traits (Sultan, 2000) and molecular mechanisms (Callahan et al., 1997, Pigliucci, 1996), in plants as well as in animals (Sultan, 2000, Whitman and Agrawal, 2009, Beldade et al., 2011).

Plasticity can be visually represented with reaction norms that depict phenotypic values of a given genotype measured in different environments (Sultan, 2000; Figure I.4, Schlichting, 1986). However, reaction norms are only informative about the phenotypic variation of the studied trait in the particular set of environmental conditions. Thus it is possible that certain traits, often morphological, seem non plastic (i.e. canalized, sensu Debat and David, 2001) whereas in reality there are other, "invisible" variations (physiological or molecular) that maintain a seemingly stable phenotype in response to environmental variation (Bradshaw, 1965, Whitman and Agrawal, 2009, Nylin and Gotthard, 1998).

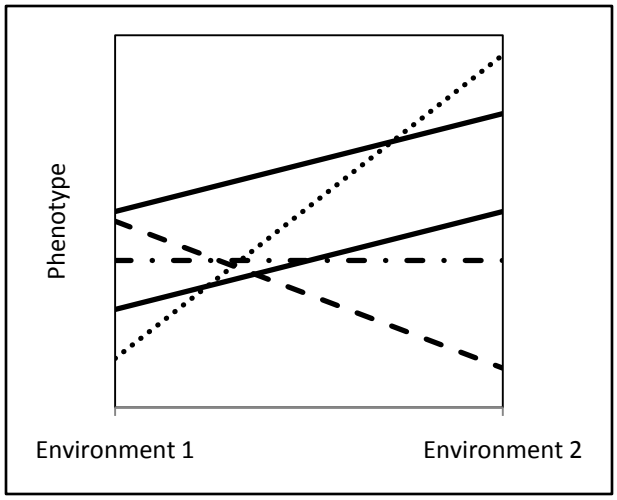

Figure I. 3. Reaction norms. Reaction norms depict the phenotypic plasticity of a given genotype across environments. Estimating phenotypic plasticity is ideally achieved through placing clones of one genotype in variable environments. If the species cannot be cloned, then genetically similar individuals can be used for the estimates, such as selfed progeny. The magnitude of plasticity for a give trait can differ among different genotypes. In this figure, the dotted line represents the most plastic genotype (highest slope) whereas the dot-dashed line corresponds to a non plastic phenotype (null slope). Genotypes in solid line have the same reaction norms (i.e. same plasticity) though the average phenotype they produce in each environment is not the same. The dashed line corresponds to a genotype that has the same magnitude of plasticity as the solid line genotypes, but the direction of the plastic response is reversed.

The magnitude of plasticity depends on the organism and the trait (or set of traits) considered. Plasticity can range from continuous phenotypic variation for traits like size, growth rate, photosynthetic activity in plants, to discrete phenotypes for other traits such as leaf or seed heteromorphism in plants or sexual phenotypes in reptiles (Roff, 1996, Whitman and Agrawal, 2009, Nijhout, 2003, Charnov and Bull, 1977). Lloyd (1984) named these two types of phenotypic variation labile phenotypes and conditional strategies. The magnitude of the plastic response can differ between species, populations of the same

species and even between genotypes within a population, hence the importance of the genotype x environment interaction in plasticity studies (Schlichting, 1986, Scheiner, 1993). Finally, some traits are known to be generally more plastic (e.g. leaf number, plant size) than others (Via et al., 1995) and there are even traits for which virtually no phenotypic plasticity has been observed, like petal number, leaf venation or fruit type (Givnish, 2002).

4. Adaptive plasticity

4.1. Adaptive plasticity theory

Since plasticity is triggered by environmental variation, the question about its adaptive character has attracted much attention. Theory predicts that under some circumstances of environmental variation, plastic mechanisms should be favoured, whereas in other cases non plastic individuals are more adapted (Lloyd, 1984, Gabriel et al., 2005). For instance, if environmental changes are rapid and unpredictable, organisms should respond by rapid and reversible phenotypic changes such as physiological responses (labile phenotypes, Gabriel et al., 2005, Lloyd, 1984, Schlichting, 1986). An example of such responses are the regulation of transpiration in plants through stomatal conductance in response to daily temperatures variation, or leaf orientation towards sunlight in order to optimize photosynthetic activity (Franks et al., 2009). If the organism is not able to respond quickly enough to the environmental variation a bet-hedging, non plastic phenotype should be more appropriate. When environmental changes occur on a larger temporal scale and are predictable and non reversible, morphological or life history plasticity can be adaptive (Whitman and Ananthakrishnan, 2009).

4.2. Conditions for the evolution of adaptive plasticity

It is important to notice that not all plastic variation is adaptive (Sultan, 1995, Bradshaw, 1965, van Kleunen and Fischer, 2005). Phenotypic variation could be a result of the inability of an organism to maintain a canalized phenotype in changing environments (Debat and David, 2001). Moreover, if the

environmental change is not correctly perceived inappropriate (maladptive) phenotypes can be produced (Langerhans and DeWitt, 2002). Several conditions need to be fulfilled in order for phenotypic plasticity to be adaptive.

Plasticity increases average fitness across environments - Plastic phenotypes must have a fitness advantage over non plastic ones. This can be achieved through environment dependent selection, i.e. selection that favours different phenotypes in different environments (Via and Lande, 1985). Thus each phenotype should increase individual fitness in the environment that induces its production, and inversely, decrease individual fitness in the environment(s) that induce other phenotypes (Via and Lande, 1985). Another possibility is that plastic individuals have significantly improved fitness in at least one environment compared to non plastic ones, which increases fitness averaged through all environments as in the case of induced predator defences (DeWitt, 1998).

Plasticity must not be too costly - If the costs necessary to produce a plastic phenotype are higher than the fitness benefits of plasticity, then non plastic phenotypes should be favoured (Schlichting, 1986, Scheiner, 1993, DeWitt et al., 1998, Auld et al., 2010). Inversely, plasticity can reduce the production costs of certain traits such as predator defences or toxin resistance if their permanent expression is too costly (Karban, 2011, DeWitt et al., 1998). The costs of plasticity could be due to the expensive mechanisms that have to be maintained in order to produce plastic responses (i.e. assessing environmental variation information, processing the information acquired, then producing the appropriate phenotypic response), the production of a particular phenotype that is very costly, or the genetic interaction between plasticity genes and other genes important for individual fitness (Auld et al., 2010, DeWitt et al., 1998).

Reliable assessment of environmental variation – To be adaptive, the phenotype produced by a plastic individual must be appropriate to the given environment. This includes correctly assessing

environmental variation but also an appropriate time lag of the response so that the phenotype produced matches the environment (Whitman and Agrawal, 2009, Karban et al., 1999, Langerhans and DeWitt, 2002, Scheiner, 1993). There are different ways to assess environmental variation which depend on the time necessary to produce the adequate phenotypic response. For instance, if an organism adapts to the new environment by rapid, physiological (or behavioural) plastic changes, then the phenotypic response can be induced by the particular event or set of events that the organism is supposed to adapt to (Karban et al., 1999). When the phenotypic change takes more time (i.e. developmental or morphological changes) then the trigger of the response is an environmental cue – an event that does not directly affect individual fitness, but is highly correlated with environmental changes that do so (Karban et al., 1999, Nijhout, 2003, Whitman and Agrawal, 2009). In plants, photoperiod is an universal cue that is informative about the habitat quality, average temperatures, risk of frost or drought (Bradshaw, 1965, Whitman and Agrawal, 2009). Cues less predictable than photoperiod are good indicators for more stochastic environmental variation (Bradshaw, 1965). For example, temperature could indicate pollinators activity (Scaven and Rafferty, 2013), or moisture levels could indicate risk of pathogen infestation in plants (Karban et al., 1999). When relying on environmental cues there is a risk of wrongly assessing the environment (Miner et al., 2005). Thus in plastic traits that are important for individual fitness, their variation can be more finely tuned if they depend on several such cues that increase the reliability of the environment prediction (Givnish, 2002).

5. Plasticity in mating systems

Though mating system theory has rarely explored the conditions for the evolution of reaction norms, empirical data have frequently revealed plastic mating systems. Plasticity has been documented for sex expression (Korpelainen, 1998, Diggle, 1994, Miller and Diggle, 2003, Charnov and Bull, 1977), self incompatibility (Vogler et al., 1998, Vogler and Stephenson, 2001) or mixed mating (see hereafter). Reaction norms or temporal variation of the outcrossing rate are known for several mixed mating

organisms (Kalisz et al., 2004, Herlihy and Eckert, 2007, Kalisz et al., 1999, Lobo et al., 2013, Ruan and da Silva, 2012, Jacquemyn and Brys, 2008, Piper et al., 1984) and in others variation of the outcrossing rate was revealed in reciprocal transplant or controlled conditions experiments (Brys and Jacquemyn, 2012). The relationship between variation of ecological factors and variation of the outcrossing rate has been demonstrated in some of these studies (Kalisz et al., 2004, Brys and Jacquemyn, 2012, Jacquemyn and Brys, 2008, Piper et al., 1984).

Often, plastic mating strategies are adaptive. For instance, low resources availability favours male plants because of the lower production costs for this sex (no investment in seeds, Korpelainen, 1998). The evolution of adaptive plasticity for mating traits is more complex than for non reproductive traits because of the inherent frequency dependent selection acting on mating systems. A consequence of a frequency dependent selection is that individual fitness is determined not only by the expressed phenotype, but also by the frequencies of the other phenotypes encountered in the population. This implies that there are no environment dependent optimal phenotypes and constrains the evolution of plasticity in reproductive traits (Shaw and Mohler, 1953, Ernande and Dieckmann, 2004). For instance, in dioecious species there is negative frequency dependence that maintains the sex ratio to 1:1. If female frequency increases in a given environment, male reproductive success will automatically increase because there will be more available female mates. In addition the female-biased sex ratio results in pollen limitation which decreases female reproductive success. Each sex is favoured when rare and disadvantaged when more frequent, which prevents the emergence of highly biased sex ratios in the population. Inversely, Charnov and Bull (Bull and Charnov, 1989, Charnov and Bull, 1989) showed that if the fitness of a given sex is not dependent on its frequency, then stable sex ratios different than the 1:1 ratio expected under negative frequency dependence could emerge, provided that there is an optimal sex for each environment. They support their data with the biased sex ratios observed in some reptilians for which it has been shown that a wrongly induced sex (i.e. producing females in conditions that usually

favour male production) has lower fitness that the normally induced sex (Bull and Charnov, 1989, Charnov and Bull, 1977).

In mixed mating systems, frequency dependence results from the automatic advantage of selfing. The relative fitness of selfing to that of outcrossing depends on the automatic advantage provided that the exported outcrossing pollen is successful. In the absence of pollen discounting and inbreeding depression, the magnitude of automatic advantage depends on the number of ovules available for outcrossing in the population and thus on the proportion of outcrossers. The result of this type of frequency dependent advantage is selection towards complete selfing if inbreeding depression is low or complete outcrossing if inbreeding depression is high Pollen discounting reduces the automatic advantage of selfing by reducing the number of gene copies left by the selfers compared to the outcrossers. Therefore frequency dependant selection on selfing will change with pollen discounting, leaving a possibility for the emergence of a stable mixed mating system.

6. Cleistogamy

Cleistogamy is the ability to produce closed, cleistogamous flowers (CL) which never reach anthesis, thus they can neither receive external pollen (obligate selfing) nor export pollen (complete pollen discounting), as opposed to open, chasmogamous flowers (CH) which are potentially outcrossed. Most of the cleistogamous species produce both floral types, thus cleistogamy is considered as a particular type of mixed mating.

Cleistogamy has been recorded for 693 species in 50 different families. Half of the cleistogamous species belong to the Poaceae (326 species), and it is also frequent in the Violaceae and Fabaceae families (Culley and Klooster, 2007). Cleistogamy is represented equally in herbaceous annual and perennial species, but it is rare among woody annuals (Oakley et al., 2007). The geographical distribution of cleistogamous species is quite large, and they are encountered in various and contrasting habitats

(Plitmann, 1995a). All of these observations suggest that cleistogamy has repeatedly evolved in different circumstances; it is thus plausible that cleistogamy is an adaptive response to a wide range of evolutionary and ecological conditions affecting the mating system and individual fitness.

6.1. Costs and benefits of reproduction via CL and CH flowers

Flower costs - In general, cleistogamous flowers are cheaper to produce (Schemske, 1978, Waller, 1979). This is due to their smaller size, the lower pollen production and the absence of auxiliary structures that serve to attract pollinators. The production costs of CL flowers can be up to 100 times lower than those of CH flowers in some species of Impatiens (Schemske, 1978, Waller, 1979), though the difference is in general less pronounced in other species for which this type of data is available (see Oakley et al., 2007).

Reproductive assurance – Most of the studies comparing the costs of reproduction via CH and CL flowers find that reproduction via CL is less costly than the reproduction via CH flowers. This is partly due to the lower investment in the floral structures composing CL flowers, but also because CL flowers have higher fruit and seed set (Oakley et al., 2007) and sometimes they produce larger seeds as well (e.g. Trapp and Hendrix, 1988). The higher seed set is partly due to the autonomous pollination of CL flowers in absence of pollen vectors, which provides reproductive assurance (Waller, 1980, Albert et al., 2011) but for some cleistogamous species it has been observed that even in conditions of pollen supplementation on CH flowers, CL seed set remained higher (Bernstrom, 1950).

Other associated traits – Though it is not as frequent as the differences in flower costs and seed production, several cleistogamous species present different dispersal and germination strategies for the two floral types. Some cleistogamous species have subterranean CL flowers and aerial CH flowers (Cheplick, 1987, McNamara and Quinn, 1977, Trapp and Hendrix, 1988) or aquatic CL and aerial CH flowers (Jiang and Kadono, 2001). In these cases, the dispersal distances of the two seed types are considerably different – while CL seeds stay near the parent, CH seeds are potentially dispersed further.

Dispersal differences between CL and CH seeds have been also noted in species with aerial CL and CH flowers (Berg, 2000, Beattie and Lyons, 1975), in which ballistic dispersal distances differ between the two fruit types, and could be combined with differences in myrmecochorous dispersal (Beattie and Lyons, 1975); whereas in other species, auxiliary dispersal structures (pappus) vary between CL and CH flowers (Porras and Munoz, 2000). Germination timing also differs between CL and CH seeds in some species (Campbell et al., 1983). Moreover, the difference in germination timing between CL and CH seeds can be more or less pronounced according to different years (Huebner, 2011) or environmental conditions. Finally, whereas germination timing differences are usually due to morphological differences between the two types of seed, special cases of cleistogamy dependent dormancy were observed as well (McNamara and Quinn, 1977, see also Cheplick, 2007, Ares et al., 1970).

Most of the differences listed here provide a net advantage to CL flowers. The disadvantages of reproducing through CL flowers are based on the genetic consequences of selfing – potential effects of inbreeding depression and low genetic diversity (Culley and Klooster, 2007). Cleistogamous species are in general self compatible, and except rare cases in which there are separate sex CH flowers (e.g. Schemske, 1978) or those in which the floral structure prevents contact between the anthers and the stigma within the same flower (Schmitt et al., 1985), it is highly likely that some degree of selfing occurs in CH flowers thus diminishing the genetic advantage of CH progeny. Estimates of outcrossing rate in CH flowers are generally lower than one (Mitchell-Olds and Waller, 1985, Waller and Knight, 1989, Culley, 2002) and empirical observations of flower morphology or pollinator behavior show that CH flowers are capable of delayed selfing or geitonogamy as well (Culley, 2002, Stewart, 1994). This means that CH flowers progeny potentially suffer from inbreeding depression, which lowers their genetic advantage. However, the consequences of inbreeding depression on individual fitness differ from those classically encountered in monomorphic floral type species because of the total pollen discounting associated with selfing through CL flowers. Since CL flowers do not benefit the automatic advantage of selfing, CH

flowers progeny would have advantage over the CL flowers whenever inbreeding depression is higher than zero.

6.2. Different types of cleistogamy

The morphological differences between CL and CH flowers can be more or less pronounced. All CL flowers are characterized by lack of anthesis, but there are other differences in some species. In most species anthers from CL flowers are reduced and produce less pollen (Cruden, 1977). More particular changes have been observed in some species – the stamens can change their form or position in order to facilitate the pollen transfer (Lord, 1981), and there are also species in which pollen tubes can develop and fertilize ovules without anther dehiscence (Mayers and Lord, 1983, Anderson, 1980, Berg and Redbo-Torstensson, 1998) . The corolla of CL flowers is generally smaller than that of CH flowers, and this difference is due to the fewer cell divisions and smaller cells of the corolla, and in some species there are substantial shape differences between CL and CH flowers (Lord and Hill, 1987). CL flowers often lack auxiliary structures serving to attract pollinators, since they pollinate autonomously (Pacini et al., 2003).

The morphological classification of cleistogamous species is a rather complex task, since the differences between CL and CH flowers can vary importantly between different genera. Instead, a distinction based on the development pathways of the CL flowers has been proposed (Culley and Klooster, 2007, Lord, 1981), with three distinct cleistogamous types.

Dimorphic or true cleistogamy (Lord, 1981, Culley and Klooster, 2007) is a result from different development pathways for the two floral types. The determination of the floral type is made prior to floral development and CL cannot switch to CH afterwards, resulting with substantial morphological differences between the two floral types. This is the most frequent type of cleistogamy with 536 dimorphic cleistogamous species (Culley and Klooster, 2007).

Induced or pseudocleistogamy (Lord, 1981, Culley and Klooster, 2007) in which the floral type is determined during the development phase by environmental conditions, with CL flowers produced if environmental conditions are unfavorable for anthesis, is present in 61 species out of the 693 known. Induced CL flowers are distinguished from CH flowers merely by the lack of anthesis and their smaller corolla.

Complete cleistogamy (Culley and Klooster, 2007, Lord, 1981) in which only CL flowers are produced was recorded for 72 species. This type of cleistogamy remains controversial, since it has only been observed in experimental conditions, meaning that genetic and environmental variation which could both influence CH production were rather limited. Empirical observations show that most of the cleistogamous species produce some CH flowers, either on the same plant or within a given population.

It is important to notice that the term "induced" in the definitions of dimorphic and induced cleistogamy is relative to the timing of the flower development determination, whereas the ecological properties of dimorphic and induced cleistogamous species can resemble. The production of CL and CH flowers in dimorphic species is often induced by environmental factors, but this "induction" needs to be made sufficiently in advance of the flowering season, whereas in induced species the "induction" occurs during flowering. For example, *Viola pubescens* is an understory perennial dimorphic cleistogamous whose CH proportion is responsive to light intensity. If light intensity suddenly declines, the plant switches from CL to CH production by aborting CH buds and forming new, CL buds (Culley, 2002).

6.3. Plastic cleistogamy

In most cleistogamous species, CH proportion varies with variable environmental conditions such as photoperiod, temperature, nutrients and water availability, humidity, pollination success, herbivory, competition, or different combinations of these factors (Lecorff, 1993, Redbo-Torstensson and Berg, 1995, Schemske, 1978, Steets et al., 2006, Stewart, 1994, Bell and Quinn, 1987, Cortes-Palomec and

Ballard, 2006, Paoletti and Holsinger, 1999, Lord, 1982). The impact of these cues on the direction and the magnitude of the CH proportion variation depends on the species, and can also vary in time, but in general, they are indicators either of environmental quality or of environmental features that could influence the fitness contribution of either floral type. CH proportion plasticity can therefore be an adaptive response to environmental variation. For instance, long days can either increase or decrease the proportion of CH flowers. In understory or shade-tolerant plants, such as *Microstegium vimineum* (Cheplick, 2007) or *Viola pubescens* (Culley, 2002), long photoperiods favor the production of CL flowers. Inversely, in grassland or cultivated species, such as *Amphicarpum purshii* (Cheplick, 2007) or soybean (*Glycine max*, (Takahashi et al., 2001)), long photoperiods favor the production of CH flowers. In all these species, photoperiod is a cue that indirectly indicates the habitat quality. For instance, in *V. pubescens*, long photoperiods are correlated with low canopy densities and high temperatures, which in turn induce low pollinators activity. In soybean, long photoperiods indicate low probability of frost, meaning that the plant can invest in more costly modes of reproduction. In *A. purshii*, whose size and CH proportion is decreased by intraspecific competition, long photoperiods indicate low density populations, hence more resources available for plant growth and reproduction.

6.4. Cleistogamy mating models

Models for the evolution of cleistogamy are scarce and, in general, focus on the distinct features of CH and CL flowers and the benefits they could provide when the environment varies. More rarely, some models consider the evolution of cleistogamy within the scope of classical mixed mating theory. Finally, some of the models for cleistogamy evolution are restrained to a subsample of CL species in which seed and fruit heteromorphism is highly pronounced. Only a few mathematical or simulation models exist about the evolution of cleistogamy (Schoen and Lloyd, 1984, Lu, 2002, Masuda et al., 2001), but based on empirical or experimental observations, many verbal models were formulated.

Genetic differences between selfed and outcrossed progeny

Since CL and CH flowers produce genetically different progeny (selfed vs. outcrossed), there is no doubt about the genetic differences between the two progeny types. These differences could include inbreeding depression, heterosis, or higher genetic variability in outcrossed genotypes. Nevertheless, theory on the evolution of cleistogamy has rarely focused on the effects of inbreeding depression. This is mostly due to the idea that since selfing rates are generally high in cleistogamous species, inbreeding depression should be low because of the purge of deleterious alleles, and also because there important non genetic advantages of CL flowers which could compensate the deleterious effects of inbreeding (Oakley et al., 2007). Estimates of the inbreeding depression magnitude are very scarce in cleistogamous species, and the few data available confirm the idea that inbreeding depression is very low (Oakley and Winn, 2008, Culley, 2000, Trapp and Hendrix, 1988), which could explain the negligence of this factor in theory of cleistogamy evolution.

Heterosis or increased genetic variability in CH progeny was the focus of one mathematical model (Lu, 2002). This model considers inbreeding depression, pollination success of the two floral types and non genetic differences between CL and CH progeny, and opposes purging of deleterious alleles through CL flowers and heterosis of outbred progeny in CH flowers: CL flowers purge recessive lethal alleles but constant selfing could fix some mildly deleterious alleles in the inbred lineages; whereas CH flowers provide crosses between different lineages creating heterosis that reduces the load of mildly deleterious alleles. Thus when the selfing rate (CL production) increases the benefits of outcrossing between selfed lines also increase, which favors CH production. According to other, verbal models, heterosis or increased genetic variation in CH progeny is a bet-hedging strategy (sensu Philippi and Seger, 1989) that allows adaptation to periodical environmental variation which stabilizes CH and CL production (Waller, 1984, Waller, 1980). Finally, some classical mixed mating models treat special case scenarios that could

apply to cleistogamy. For instance in his mixed mating model Lloyd (1979) included a special case with morphological adaptation for increased selfing which results in stable mixed mating.

Reproductive assurance and pollination success

Because of the different pollination mechanisms and seed set success for CL and CH flowers, cleistogamy is often considered to provide reproductive assurance in response to variation in pollinator abundance. In general, this type of models is more specific about the differences in the two floral types, and some of them include genetic effects of the two floral types as well. Perhaps the most complete model for the evolution of cleistogamy is the one suggested by Schoen and Lloyd (1984), which incorporates differences in flower types (costs of flowers, pollen discounting), progeny fitness (flower type and inbreeding degree/inbreeding depression), and homogeneous or heterogeneous environments. In absence of environmental variation, cleistogamy evolution follows classical predictions for the evolution of mixed mating in single floral morphs, with either complete selfing or complete outcrossing as the only stable states of the mating system (Lloyd, 1992, Lloyd, 1979). However, when environmental variation is temporal (i.e. variation in pollinators abundance), each flower type contributes more to parental fitness at different times of the flowering period, plastic cleistogamy is stable. This is true provided that the environmental variation could be properly assessed; otherwise the mixed strategy (CL and CH production) is not stable and the single mating strategy that yields higher global fitness is selected. This model shows that variable cleistogamy could be a plastic adaptive trait if the conditions necessary for the emergence of adaptive plasticity are fulfilled (see plasticity section). In addition to Schoen and Lloyd's analytical model, several verbal models consider cleistogamy as an adaptive plastic mechanism (Redbo-Torstensson and Berg, 1995, CaraDonna and Ackerman, 2012, Albert et al., 2011) or a bet-hedging strategy (Waller, 1984, Waller, 1980), and they all deduce that CL production should be enhanced when pollinators are scarce or when pollinator abundance varies in an unpredictable manner.

Avoidance of sibling competition

In mixed mating systems with a single floral morph it has been suggested that increasing the outcrossing rate could provide more fit progeny which helps to avoid competition between highly related progeny (full sibs) or the deleterious effects due to habitat crowding. This theory has often been supported with examples of cleistogamous species (Schmitt and Ehrhardt, 1987, Schmitt et al., 1987, Clay, 1982). For instance, Waller (1980) suggests that the size threshold necessary for the onset of CH production in *Impatiens capensis* is an indicator of the competition levels in the offspring – large plants produce more offspring, and should therefore favor the production of CH progeny which are more fit and less related, whereas small plants that produce little progeny should favor CL production because of the lower production costs of this floral type/progeny.

Geitonogamy avoidance

Geitonogamy is considered as a non adaptive byproduct of pollinator behavior facing large floral displays. Indeed, geitonogamy provides selfing at the same expenses of outcrossing (floral display, pollinator awards, Charlesworth and Charlesworth, 1990). Single floral morph species avoid geitonogamy by reducing floral display at a particular moment of their phenology or by reducing overall flower production in order to diminish the probability for a single pollinator to visit several flowers on the same plant. However, this could also reduce the plant attractiveness and decrease overall outcrossing rates. Cleistogamy could be a mechanism of geitonogamy avoidance without paying the cost of pollinator absence. Masuda et al. (2001) proposed a phenotypic selection model in which they account for genetic (i.e. inbreeding depression, pollen discounting in CL flowers) and non genetic differences (i.e. lower costs and higher pollination reliability of CL flowers) between CH and CL flowers and geitonogamy between CH flowers as well. They show that inbreeding depression could limit the evolution towards complete CL production, whereas geitonogamy, which increases with the CH floral display, should constrain the evolution towards complete CH production. A similar verbal model was suggested by Stewart (1994).

Differential production costs of CL and CH flowers and progeny

One important observation in the geitonogamy avoidance model is that CH proportion should decrease with increased plant size. This is true for some cleistogamous species, most of which are perennials (e.g. Berg and Redbo-Torstensson, 1998, Mattila and Salonen, 1995), but in general CH proportion increases with increased plant size (Oakley et al., 2007). This observation was translated into the cost of flowers hypothesis – when plants experience favorable environments, they can invest in showy, CH flowers that are more costly to produce, and inversely, when environmental conditions are harsher, CL production is favored (Schemske, 1978, Waller, 1979). However, the difference in production costs cannot sustain CH production on its own – because CL flowers are less costly, they should be favored in all circumstances, unless CH flowers confer some advantage which is absent in CL flowers (Oakley et al., 2007).

Dispersal and germination differences

Since association between amphicarpy (production of aerial and subterranean seeds) and cleistogamy are commonly observed (Gopinathan and Babu, 1987, Cheplick and Quinn, 1983, Zeide, 1978, Trapp and Hendrix, 1988), the difference in dispersal properties of CL and CH progeny was incorporated in some models. These models consider that CL seeds stay in the maternal habitat whereas CH seeds disperse further. Cleistogamy is then adaptive if non dispersers (CL) have higher fitness in the maternal habitat and at the same time dispersers (CH) yield higher fitness in novel habitats. This could be the case if there is spatial environmental variation, and if parent fitness is the highest in its local environment. CL progeny, being genetically more similar to their parent than CH progeny will therefore yield higher fitness by not dispersing, whereas the increased genetic variability of outcrossed CH progeny provides higher potential for adaptation in novel environments (Schoen and Lloyd, 1984, Iwasa, 1990, Schmitt et al., 1985). The difference in germination rates of CL and CH flowers was also proposed as a mechanism that stabilizes cleistogamy. For instance, dormant CL seeds, regardless of the mechanism that delays their germination, could constitute a seed bank that allows the persistence of the population after a habitat disturbance (Cheplick and Quinn, 1983).

7. About this thesis

In the past 30 years, the theory of mixed mating has been continuously expanding, followed by accumulating experimental and empirical studies of mixed mating and its adaptive value. Nevertheless, some aspects of mixed mating are still undermined, such as its plastic character. Though evidence about plastic selfing rates and reaction norms of mixed mating in various organisms have accumulated recently, the adaptive character of plastic mixed mating is rarely considered in theoretical approaches. Because of the differences in the two floral morphs, several properties of the cleistogamous species render them an interesting model for the study of plastic mixed mating. First, CH proportion could translate in outcrossing rate provided that CH flowers substantially outcross. If this is the case, the assessment of the mating type (i.e. outcrossing rate) of cleistogamous species is quite a simple task: the outcrossing rate is proportional to CH proportion. Second, CH proportion is at least partly determined in advance of the pollination timing, especially in dimorphic cleistogamous species. Therefore, the variation of the selfing rate in cleistogamous species is a genuine adaptive adjustment to environmental variation, as opposed to monomorphic flower species in which variable selfing rates are sometimes a byproduct of pollinator behavior causing facilitated selfing or geitonogamy. And finally, CL flowers do not benefit the automatic advantage of selfing because of the complete pollen discounting, but on the other hand they are cheaper to produce, their pollination is more reliable and in general they yield higher seed sets. Therefore, the evolutionary forces that maintain outcrossing once selfing evolves should be even more pronounced in cleistogamous species. Studying cleistogamous species could provide insightful information about the general evolutionary mechanisms operating in mixed mating systems.

However, the place of cleistogamy in mixed mating theory is still enigmatic, mostly because some of the characters that are systematically examined in monomorphic mixed mating systems were neglected in the case of cleistogamy. For instance, to what extent does plastic CH translate into plastic outcrossing?

Estimates of the outcrossing rate in cleistogamous species are still very rare. Then, if plastic cleistogamy is an equivalent for plastic mixed mating, is it an adaptation to environmental variation? Which evolutionary forces promote the adaptive character of plastic cleistogamy? Can we explain the maintenance of cleistogamy through classical evolutionary forces such as inbreeding depression, or should other forces, related to the particularities of the two floral morphs be taken into account, and which ones?

All of these questions are addressed in the scope of this thesis through the study of *L. amplexicaule*, an annual cleistogamous species. Since little is known about the ecological factors that could influence the production of the two floral types in this species, we first conducted a two-year field survey of natural populations of *L. amplexicaule* in France. Based on some of the data obtained at the beginning of the field survey, and on some previously published data about the physiological control of CH production and the life cycle of *L. amplexicaule*, we then continued the study of this species combining common-garden experiments, molecular biology and theoretical approach in order to bring answers to the questions asked in the previous paragraph.

CHAPTER 1. Ecology of cleistogamy in *Lamium amplexicaule*: a field study

Introduction

Lamium amplexicaule L. is a weedy annual plant belonging to the mint family (Lamiaceae). The species is native from Europe and Asia and documented as invasive in all other continents (Holm et al., 1979). Populations of *L. amplexicaule* can be found at variable altitudes and latitudes and in different habitat types ranging from cultivated lands to rocky mountains and walls. It is characterized as a ruderal plant, with low tolerance for shading, drought or competition (Baude et al., 2011, Tutin et al., 1993, Baskin and Baskin, 1984) and it is thus most commonly observed in gardens, cultivated lands or recently abandoned agricultural fields, before the emergence of any other species.

Several subspecies have been identified for *L. amplexicaule* (Mennema and Natho, 1989), most of which have a geographical distribution restricted to Eastern Europe and Asia. The most widespread subspecies is *L. amplexicaule* ssp. *amplexicaule*, and even within this subspecies, important morphological differences exist among geographic locations (Bernstrom, 1952), which has led to the use of several synonyms to describe them (Mennema and Natho, 1989, The Plant List, 2010). The differences between varieties and lineages are mainly morphological and phenological (plant size, leaf size, shape and color, flower shape and color, pollen color, cleistogamy levels, flowering date), but cross incompatibilities have also been observed in some cases (Bernstrom, 1952).

Seeds of *L. amplexicaule* can be highly dormant. Ødum (1965) tested soil samples from a site that has not been disturbed for over 400 years, and found viable *L. amplexicaule* seeds. The dormancy of *L. amplexicaule* seeds has been extensively studied, showing that there are both primary innate dormancy, which prevents seeds from germinating immediately after they have matured and fallen off the mother plant, and secondary conditional dormancy, which depends on environmental conditions (Baskin and Baskin, 1998, Jones and Bailey, 1956). Primary dormancy is probably due to a soluble germination inhibitor component that decreases at dry storage, (Jones and Bailey, 1956), whereas environmental factors that can enhance secondary dormancy are low levels of moisture, low light intensity, short

photoperiod and extreme (low or high) temperatures (Baskin and Baskin, 1981, Baskin and Baskin, 1984, Jones and Bailey, 1956). Based on the environmental conditions necessary to ripen dormant seed, *L. amplexicaule* can have a life cycle that corresponds to a summer or a winter annual (Baskin and Baskin, 1981, Baskin and Baskin, 1984). Summer annuals germinate in the late winter and have a short life cycle that ends at the beginning of the summer, whereas winter annuals germinate in the late summer to late autumn, producing flowers in the autumn if they germinated early enough, or during the following spring if they germinated in the late autumn. These two life cycles are timed so that the plant minimizes the risk of competing with other species in the same habitat during its development, and further avoids summer drought (Baskin and Baskin, 1984).

L. amplexicaule is a dimorphic cleistogamous species. Its open or chasmogamous (CH) flowers have a purple pink zygomorphic corolla, formed by a long tube ending with two labia. Closed or cleistogamous (CL) flowers present corolla morphology similar to the CH flowers but they are three to four times smaller, lighter colored and they never reach anthesis (Figure T.1).

Figure T. 1. *L. amplexicaule* plant bearing chasmogamous and cleistogamous flowers. CL flowers are the dark purple bud-like structures pointed by the white arrows; all other bud-like structures are actual CH buds. *Copyright Avinoam Danin, The Hebrew University of Jerusalem*.

Both floral types have four stamens that closely surround the stigma, and four ovules lodged at the base of the calyx, but CL flowers have lower pollen counts compared to the CH flowers (Lord, 1980b). Flowers are forming cymules disposed by whorls. Flowering is ascending by whorls, and the developmental timing of each flower is determined by its position between whorls and within a whorl (Lord, 1979a). At the beginning of the flowering period, plants produce CL flowers only (constitutive cleistogamy, Lord, 1979a, Lord, 1980a, Lord, 1980b), whereas later in the flowering season plants can produce CH and CL flowers in variable proportions (induced cleistogamy or induced chasmogamy). Complete cleistogamy has been observed in some specimens, whereas the maximal CH proportion observed was never higher than 50% (Lord, 1979a).

Though CH flowers are potentially outcrossed, *L. amplexicaule* is classified as a predominantly selfing species (Fryxell, 1957). Bernstrom (1950) showed that CH flowers seed set is very high for unmanipulated plants kept in an isolated greenhouse, and explained this as a consequence of the anthers brushing the stigma during anthesis and the high proximity of stigma and anthers in CH flowers. However, self-fertilization within CH flowers was never complete, averaging 90% (compared to 98-100% in CL flowers), and this even if CH flowers were pollen-supplemented by hand. Other authors have interpreted this result as a type of partial cryptic self-incompatibility mechanism (Lord, 1981), but comparisons of self and outcross pollen germinations were not made for this species.

Little is known about the genetic determinism of cleistogamy in *L. amplexicaule*. Correns (1930) managed to select “CH rich” and “CH poor” lineages, and crossing these lineages resulted in a Mendelian segregation of the two characters. However, Correns (1930) also noticed that the CH proportion of a given lineage depended on environmental conditions, an observation that was later confirmed by experimental studies and empirical observations (Lord, 1982, Lord and Mayers, 1982, Baskin and Baskin, 1984, Allard, 1944). Lord (1982) showed that photoperiod and temperature could influence the

production of CH flowers, with high temperatures and long days increasing the production of CH flowers, and conversely, low temperatures and short days enhancing the production of CL flowers. She further showed that the production of CL and CH flowers was determined prior to flower development, since a single external application of gibberellins led to the opening of all subsequently produced flowers, including those that were not yet formed at the time of the application (Lord and Mayers, 1982).

All observations of *L. amplexicaule* show that the species produces more CH flowers in spring than in autumn (Lord, 1982, Lord and Mayers, 1982, Baskin and Baskin, 1984, Allard, 1944). One possible explanation for this pattern is that the production of the two floral types is an adaptation to variation in pollinator abundance. In long, warm days of spring, when pollinators are abundant, *L. amplexicaule* produces more CH flowers that can be outcrossed, while in short, cold autumn days, when pollinators are scarce, CL flowers, whose pollination is autonomous, are more frequent (Lord, 1982). A recent study showed that the CH proportion in *L. amplexicaule* can also be influenced by the presence of a closely related species, *L. purpureum*, with frequency, but not density of *L. purpureum* in the habitat resulting in a decrease of the CH proportion of *L. amplexicaule* (Sato et al., 2013). The authors of this study suggested that because interspecific crosses of these two species are ineffective (Bernstrom, 1954), the decrease of the CH proportion (or rather the increase of the CL proportion) is a mechanism for interspecific pollination avoidance that can nevertheless maintain high seed sets.

Despite extensive studies of the life cycle of *L. amplexicaule*, especially of seed dormancy, and studies of the physiological control of cleistogamy, this species has rarely been considered as a model of cleistogamy within an ecological context. Except for the qualitative observations of CH proportion variation, little is known about the precise effect of seasonal variation on CH production and even less about the adaptive character of this trait regarding seasonal variation. Moreover, *L. amplexicaule* has

often aroused interest as an invasive species, but few data about its ecology in its native geographical zone are available. In this study, we present the results of a two years survey of natural populations of *L. amplexicaule* in two French regions. Our goals were to get an insight of the flowering phenology of this species and detailed data about the production of CH and CL flowers. We therefore compared the flowering phenology of induced CH flowers (statistics of its distribution, including duration, mean, and maximal values of CH proportion) across geographical regions and habitats, as well as across seasons and years. We also studied the overall CH proportion variation across seasons, years, regions and habitats to find out possible environmental factors that could influence this trait in natural populations (photoperiod, temperature, and habitat quality).

Materials and methods

Populations studied

For our field study we chose two French regions, one in Southern France, near Montpellier, and one in Northern France, near Dijon. The regions were chosen because of the contrasting climates – Mediterranean in Montpellier with high temperatures and irregular precipitation, and temperate in Dijon with moderate temperatures and slightly higher and more regular precipitation. Natural populations surveys were made in spring 2010, autumn 2010, spring 2011 and autumn 2011. In each season, the surveys began shortly after the CH flowering started and lasted until the complete senescence of the plants. During each survey session, all population sites previously followed were checked for new plants. In 2010, we monitored two populations in Dijon and two in Montpellier in the spring and then three populations in the autumn in Dijon. In the spring of 2011, we surveyed the same populations as in the spring of 2010, and three additional populations in each region. In the autumn of 2011 only one of the previously studied populations remained, thus we chose four new populations in Dijon (Table T.1). A few populations found in vineyards in Dijon had all their plants damaged by early

Table T. 1. Populations studied during the field survey in 2010 and 2011. Habitat: type of habitat in which populations were found; O: favorable habitats, C: unfavorable habitats. Population DMos habitat has been recently colonized by other species, thus vegetative cover density was lower than that in the other unfavorable habitats. X: population observed and monitored during the indicated season. Ploughed: plants were observed on the site but the field was ploughed before the study, n/a – no plants were found on the study site for the indicated season.

Region	Habitat	Population	Pop	2010 Spring	2010 Autumn	2011 Spring	2011 Autumn
Montpellier	O	Cucule	ML	x	n/a	X	n/a
Montpellier	C	PicStLoup	MS	x	n/a	X	n/a
Montpellier	O	CombaillauxOuverte	MCO			x	n/a
Montpellier	C	CombaillauxFermée	MCF			x	n/a
Montpellier	O	Lauret	MLa			x	n/a
Dijon	O	Marsannay	DL	x	x	x	X
Dijon	C	Canal	DS	x	n/a	x	n/a
Dijon	O	LongéroisHaut	DLH		x	x	Ploughed
Dijon	O	Bouvier	DBo		x	n/a	Ploughed
Dijon	O	LongéroisBas	DLB			x	Ploughed
Dijon	O/C	Mosson	DMos			x	n/a
Dijon	O	Brochon	DBr				X
Dijon	O	Vougeot	DVt				X
Dijon	O	MSDJeune	DMJ				X
Dijon	O	MSDVieille	DMV				X

plough that occurred before the survey (Table T.1). The preliminary results in spring 2010 showed that the habitat type in which populations grew had a significant effect on the plant size and CH proportion. We thereafter distinguished two habitat types: (i) open favorable habitats, which correspond mainly to vineyards that are regularly ploughed (i.e. recently disturbed habitats), and bear large populations (several hundred individuals) and virtually no other vegetation than *L. amplexicaule*; and (ii) closed unfavorable habitats, which correspond to lawns and fallow lands bearing small populations (less than 50 individuals) and have dense vegetation covers composed of multiple grass species (undisturbed habitats, Figure T.2). We aimed at having several favorable habitats and several unfavorable habitats per survey session whenever possible. Population ML in Dijon (Table 1) was the only one where we were able to monitor both spring and autumn plants for two years.

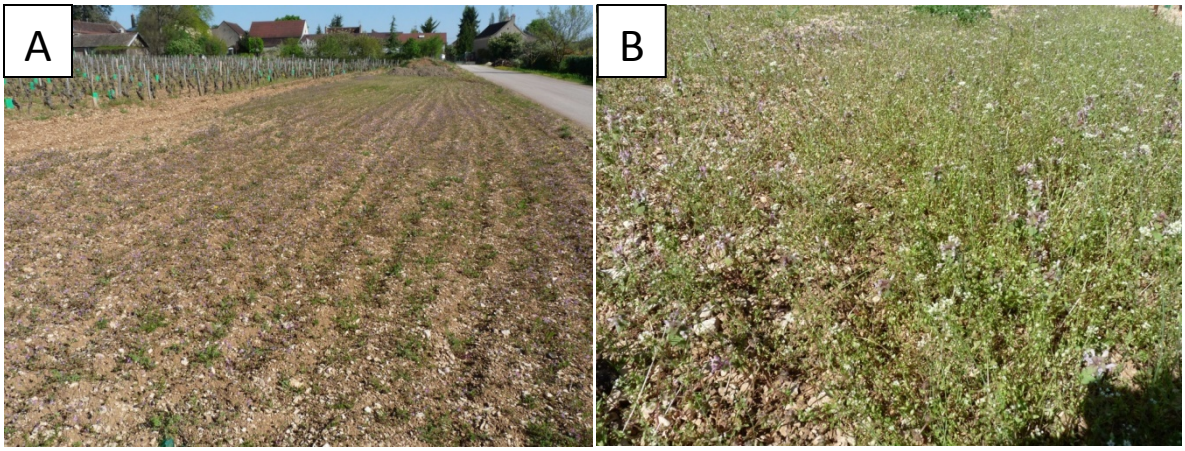

Figure T. 2. Habitat description: Open, favorable habitats (A) and closed, unfavorable habitats (B) for *L. amplexicaule*. All ground vegetation observed in A. is *L. amplexicaule*. In B. *L. amplexicaule* is more difficult to distinguish (purple flowers that are easily visible correspond to *L. purpureum*).

Data about the mean temperatures and daylength near Montpellier and Dijon during each flowering period were obtained from online databases (ptaff, 2005, Meteorologic, 2013). Mean daily temperature was averaged for each survey period. It has been shown that photoperiod can enhance flower production for up to two weeks prior to the flower formation (Lord and Mayers, 1982), thus we considered daylength data for the period two weeks prior to the beginning of each survey until its end. Because of the unusually high temperatures of the autumn 2011, CH flowering in this year began earlier than expected, and we thus missed the maximum of the induced CH proportion for most of the populations. However, two of the studied populations (DL and DMJ) were characterized as “late” because they started producing CL flowers at the beginning of the survey, and we were thus able to record their entire flowering period.

Flower counts

Within each population 20 to 25 plants were randomly chosen and their principal axis was tagged with colored tape. In large populations, plants were sampled along one or two linear transects with minimal distance of 50 *cm* between two plants, whereas in small populations we tried to maximize the distance between plants without following any particular spatial pattern. CH and CL flowers on the principal axis

were counted every four days in the spring of 2010. Since the lifetime of a CH flower is about four days (pers. obs.) only freshly emerged flowers were counted, thus avoiding recounting the same flower. CL flowers are distinguishable from CH flower buds because of the lighter color of their corolla and their pointed tip. Whenever there was a doubt about the identification of a CL flower, it was counted at the following survey date after it has completely developed (for CH flowers) or wilted (for CL flowers). Old, wilted CL flowers were removed from their calices on each count date to avoid recounting them. For the other three seasons (autumn 2010, spring and autumn 2011), populations were surveyed weekly, and flowers that looked older (with flaccid, but not completely wilted corollas) were counted as well. At the end of the flowering season, tagged plants were collected and the number of calices on the principal axis was counted.

The flower counts per date were used to calculate the variation of the induced CH proportion during the survey. For each population, the induced CH proportion was calculated at each date as $n_{CH}/(n_{CL} + n_{CH})$ with n_{CH} and n_{CL} being the number of CH and CL flowers recorded in the population on the particular date. These temporal estimates of the CH proportion were used for phenology illustrations (Figure T.4, T.5). In order to study the CH proportion variation, overall CH proportion for each individual was estimated as N_{CH}/N_{cal}, with N_{CH} being the total number of CH flowers recorded during the survey session and N_{cal} the total number of flowers.

Statistical analysis

We tested for the effect of the year, season, region and habitat type on the CH proportion and on plant size (number of calices) with ANOVAs. Since not all populations were surveyed for each of these explanatory variables, we used different subsamples of our data for the different analysis. All of the explanatory variables and their interactions were declared as fixed factors. Proportions (CH proportions and pollination success) were arcsin-square-root transformed to ensure normality and homoscedasticity of the residuals. Significance levels in all models were tested using Fisher's F statistic.

Effect of the year – Because environmental conditions differed between 2010 and 2011, we first analyzed the effect of the year on the number of calices and the CH proportion. We analyzed separately the data obtained in spring and in autumn. In spring, we used the data of the number of calices and CH proportion from populations ML, MS, DL and DS and year, region, year x region and population nested in region as fixed explanatory variables. It is important to notice that because the flower count protocol was different between years in spring, the year effect in spring also included effects due to these differences. In autumn, the only available data for 2010 and 2011 were for the population DL (Table 1). We analyzed number of calices and CH proportion with year as explanatory and fixed variable.

Effect of the season – The effect of the season was tested for the population DL, which was surveyed in both seasons in 2010 and 2011, and separately for the population DLH, which was surveyed during the autumn 2010 and the spring 2011 (Table 1). Data from population DL were analyzed with year, season and their interaction as fixed explanatory variables, and data from population DLH were analyzed with season as the single fixed explanatory variable.

Effect of the region and the habitat – Because the Montpellier populations did not have an autumn generation, the effect of the region and the habitat type were studied for populations in spring only. We used year, region, habitat and their second degree interactions, as well as population nested within year x habitat for this analysis.

Results

Temperature and photoperiod variation

The mean temperatures during the field survey period were about 2°C higher in 2011 than in 2010 in both seasons. Within a given year, average spring temperature was lower than the average autumn temperature (Figure T.3). Photoperiod was about 1h longer in Dijon than in Montpellier during spring, independently of the year. Photoperiod is ascendant in spring and descendant in autumn, but the average daylength recorded during the surveys was similar for both seasons (Table T.2).

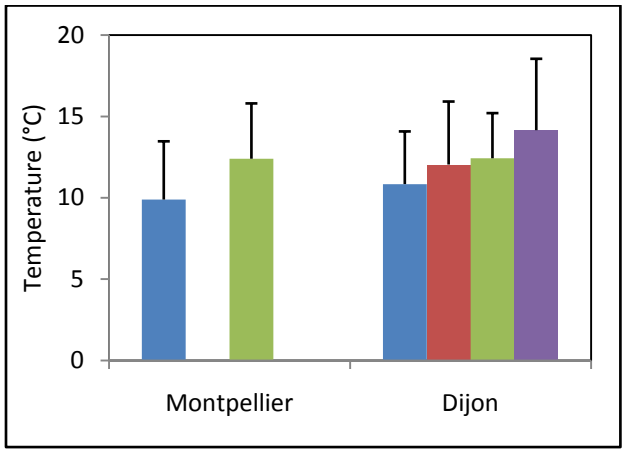

Figure T. 3. Average daily temperatures (in Celsius degrees) measured near Montpelier and Dijon during the field survey of *L. amplexicaule*. Blue: spring 2010, red: autumn 2010, green: spring 2011, violet: autumn 2011. Vertical bars represent standard deviation.

Table T. 2. Daylength during the field surveys. The start dates are two weeks before the beginning of the survey, since CH flowers production can be enhanced 2 weeks prior to the bud production. The end dates correspond to the last survey date.

Region/year	Spring		Autumn	
	Date	Daylength	Date	Daylength
Montpellier				
2010 start	10.03.2010	11h39		
2010 end	22.04.2010	13h45		
2011 start	01.03.2011	11h12		
2011 end	8.04.2011	13h05		
Dijon				
2010 start	25.03.2010	12h26	01.09.2010	13h22
2010 end	25.03.2010	14h09	21.10.2010	10h35
2011 start	15.03.2011	11h52	10.09.2011	12h53
2011 end	25.04.2011	14h09	04.11.2011	09h52

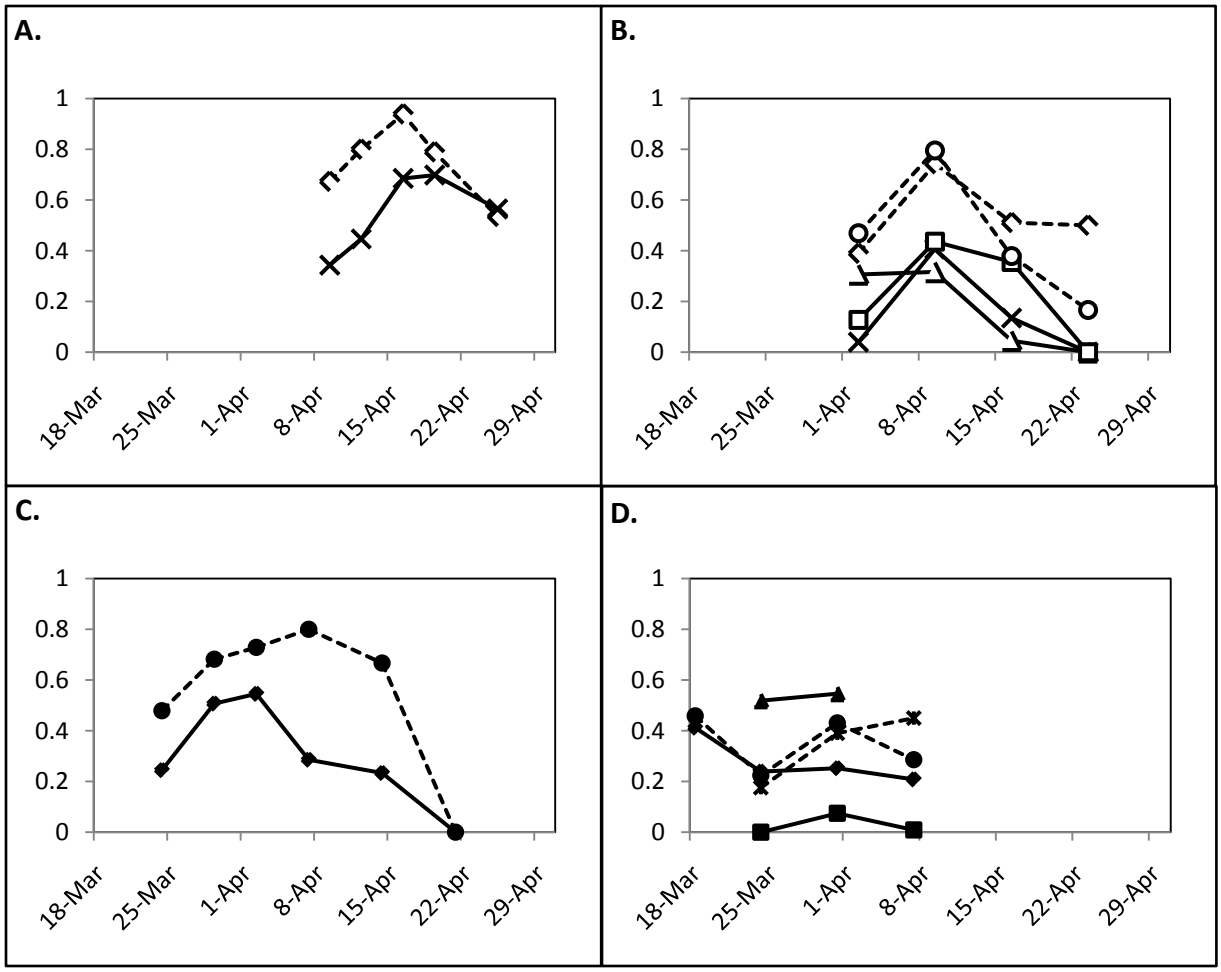

Figure T. 4 Induced CH proportion through time for spring populations. A. Dijon in 2010, B. Dijon in 2011, C. Montpellier in 2010, D. Montpellier in 2011. Solid lines: open, favorable habitat populations, dashed lines: closed, unfavorable habitat populations. Empty circles: DMos, empty diamonds: DS, X – DL, empty squares: DLH, empty triangles: DLB, full circles: MS, stars: MCF, full diamonds: ML, full triangles: MCO, full squares: MLa

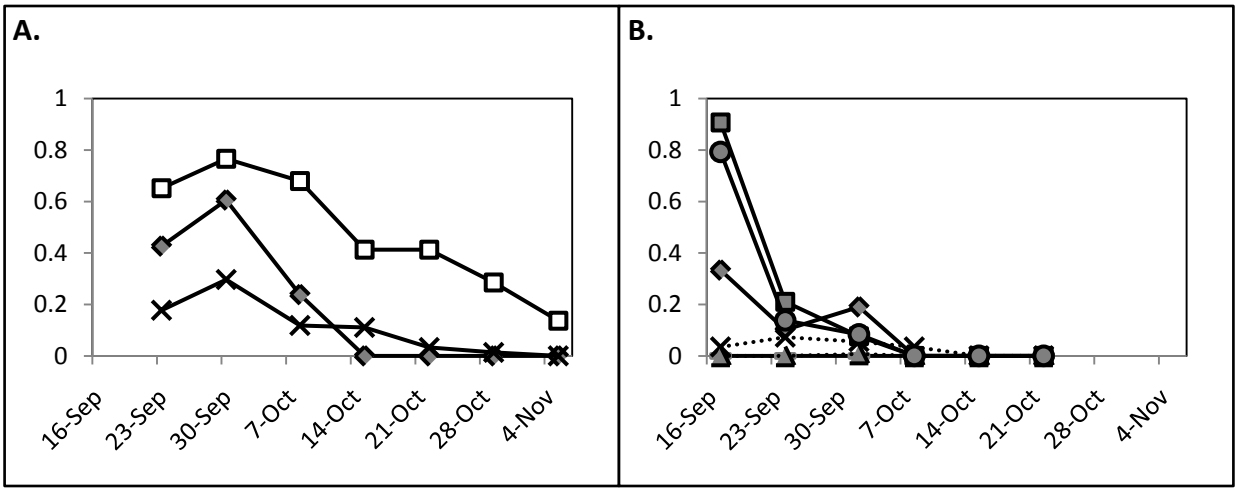

Figure T. 5. Induced CH proportion through time for autumn populations in Dijon in A. 2010, and B. 2011. Dotted lines: “late” populations in autumn. X: DL, empty squares: DLH, gray diamonds: DBo, grey triangles: DMJ, grey squares: DMV, grey circles: DVt. Population DBr produced no CH flowers so it was not illustrated.

Flowering phenology

The maximal induced CH proportion in spring was delayed by about one to two weeks in Dijon compared to Montpellier in both years (FigureT.4). Populations in unfavorable habitats had higher maximal induced CH proportions compared to populations in favorable habitats (Figure T.4). An exception is observed for the population MCO in 2011, whose induced CH proportion is close to that of populations from unfavorable habitats, but this population has only two survey points, and it is thus difficult to ascertain we correctly estimated the maximal CH proportion (Figure T.4.D). In general, the maximal induced CH proportion was easy to identify in spring, except for some of the Montpellier populations (ML, MCF, FigureT.2.D). The flowering period in spring was rather short, though we do not have enough data at the beginning of flowering or the beginning of the production of CH flowers. However, after the maximal induced CH proportion, plants continued producing high proportions of CH flowers for about one to three weeks before becoming completely senescent and completely and abruptly stopping flower production.

The patterns of induced CH proportion in autumn were different than those in spring (Figure T.5). None of the Montpellier populations had an autumn generation, and neither did the plants of populations from unfavorable habitat in Dijon (Table T.1). In populations that did have an autumn generation induced CH proportions were the highest at the first (in 2011) or the second (in 2010) survey date in autumn and declined afterwards. Plants continued producing flowers for about six weeks afterwards. Population DBr produced no CH flowers during the survey in the autumn of 2011, though the possibility that CH flowers were produced prior to the beginning of our survey cannot be excluded.

Variation of plant size and global CH proportion

Variation between years - In spring the number of calices did not vary significantly between years, but it did vary between populations, and there was a significant year x region interaction (Table T.4, see Appendix T.1 for the mean values). In the autumn, the number of calices was significantly higher in 2010 (mean ± sd: 58.90 ± 14.93) than in 2011 (mean ± sd: 37.79 ± 18.93, $F_{1,71}$ = 8.50, p-value = 0.004).

Table T. 3. Analysis of variance of the effect of year, geographical region and population on the total number of calices and the global CH proportion for populations MS, ML, DS and DL in spring.

Variable	Model	DF	F value	Pr(>F)
Calices				
	Year	1	0.525	0.46987
	Region	1	0.0297	0.86342
	Pop_{region}	2	36.3615	1.38E-13
	Year x region	1	3.5965	5.98E-02
	Year x pop_{region}	2	0.817	0.44371
	Residuals	149		
Global CH proportion				
	Year	1	69.0188	6.56E-14
	Region	1	0.1008	0.7514
	Pop_{region}	2	15.0177	1.19E-06
	Year x region	1	0.0006	9.81E-01
	Year x pop_{region}	2	1.8563	0.16
	Residuals	144		

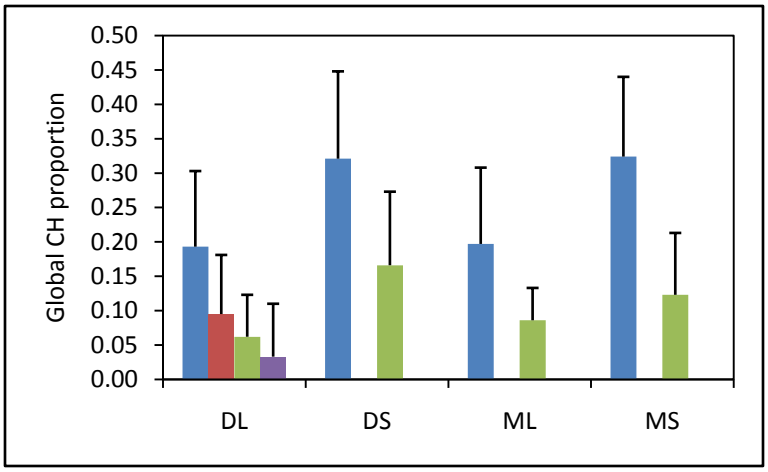

Figure T. 6 Global CH proportion for two populations in Montpellier and two in Dijon, which were surveyed in 2010 and 2011. Blue: spring 2010, red: autumn 2010, green: spring 2011, violet: autumn 2011. Vertical lines represent standard deviation.

CH proportion varied significantly between years for both seasons (Table T.3 for the spring analysis, $F_{1,69}$ = 20.40, p-value <0.0001 for population DL in autumn, FigureT.6). Region had no significant effect on CH proportion in spring, but population and the interaction year x region did. Given that year had a significant effect on the CH proportion in both seasons and on the number of calices in autumn, this variable was included in the subsequent analyses.

Variation between seasons – In the population DL year and season x year had a significant effect on the total number of calices, as expected from the previous analysis, but season had none (Table T.4, see Appendix T.1 for mean values). In the population DLH, the number of calices did not vary between seasons ($F_{1,37}$ = 0.0072, p-value = 0.932, see Appendix T.1 for mean values). All explanatory variables included in the analysis had a significant effect on the population DL overall proportion (Table T.4, Figure T.6). CH proportion in this population DL was higher in 2010 than in 2011, and within a given year it was higher in spring than in autumn (Figure T.6, Table T.4). In the population DLH, season had a significant effect on the CH proportion ($F_{1,37}$ = 52.85, p-value<0.0001), but plants had higher CH proportions in the autumn 2010 than in the spring 2011, which is likely due to differences across years rather than seasons (see Appendix T.1 for mean values).

Table T. 4. Analysis of variance in the number of calices and CH proportion across seasons in population DL, which was surveyed during four growth seasons.

Variable	Model	DF	F value	Pr(>F)
Calices				
	Year	1	9.5431	2.89E-03
	Season	1	0.0548	0.815548
	Year x season	1	10.7548	1.63E-03
	Residuals	69		
Global CH proportion				
	Year	1	23.4504	7.90E-06
	Season	1	11.1138	1.40E-03
	Year x season	1	0.1842	0.669192
	Residuals	67		

Variation between regions and habitats - The number of calices varied significantly across years, regions, habitats and populations for populations surveyed in spring (Table T.5, Figure T.7). In general, unfavorable habitat populations led to fewer calices (Figure T.7). The interaction year x region also had a significant effect on the number of calices (Table T.7). Global CH proportion varied significantly across years, being higher in 2010 than in 2011 (Table T.5, Figure T.7). Habitat and population nested within year x region had a significant effect on the global CH proportion, with plants from unfavorable habitats bearing higher CH proportions than plants in favorable habitats (Table T.5, Figure T.7). Neither region nor any of the remaining interaction terms had a significant effect on this trait.

Table T. 5. Analysis of variance on number of calices and CH proportion in spring across regions and habitats.

Variable	Model	DF	F value	Pr(>F)
Calices				
	Year	1	4.0194	0.04612
	Region	1	4.7482	0.03031
	Habitat	1	51.5796	8.84E-12
	Year x region	1	18.4911	2.50E-05
	Year x habitat	1	2.1633	0.14266
	Region x habitat	1	1.0825	0.29919
	$Pop_{year\ x\ habitat}$	6	6.0205	7.03E-06
	Residuals	237		
Global CH proportion				
	Year	1	142.681	< 2.2e-16
	Region	1	3.662	0.0569
	Habitat	1	60.2486	2.66E-13
	Year x region	1	0.0092	0.9239
	Year x habitat	1	0.078	0.7803
	Region x habitat	1	0.7989	0.3724
	$Pop_{year\ x\ habitat}$	6	8.5553	2.09E-08
	Residuals	232		

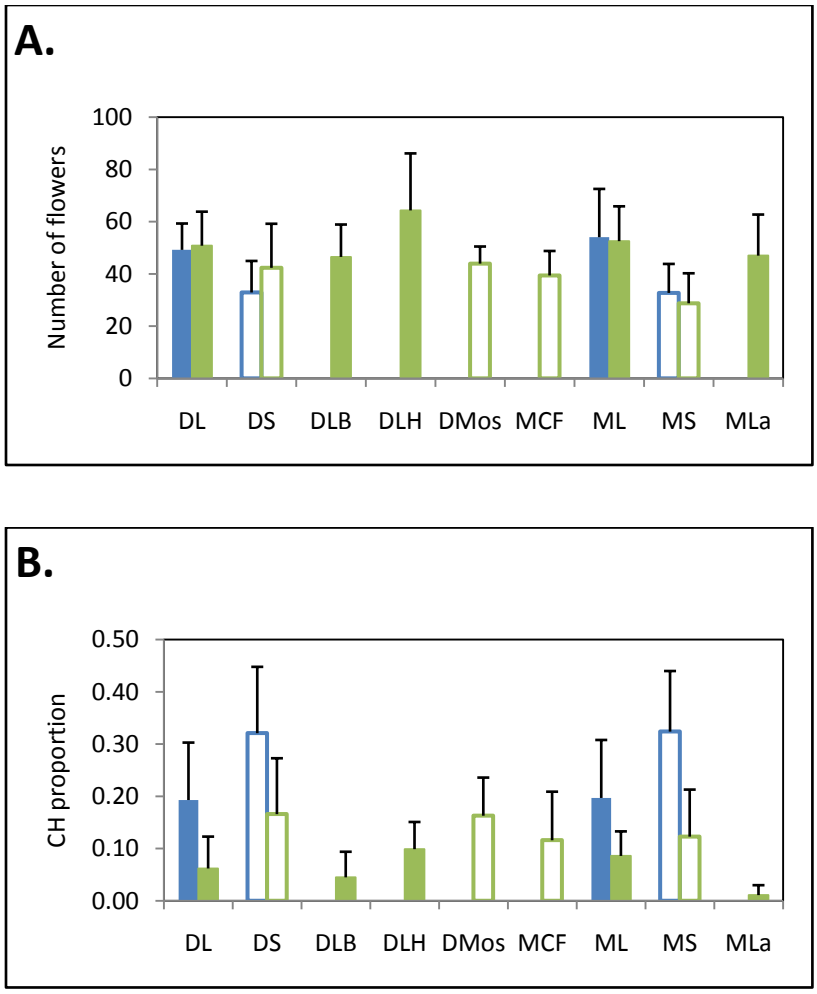

Figure T. 7. Mean number of calices (A) and CH proportion (B) for populations surveyed in the spring in 2010 and 2011. Blue: spring 2010, green: spring 2011. Full bars represent favorable habitat populations while empty bars represent unfavorable habitat populations. Vertical bars represent standard deviation.

Discussion

In this study we present data from surveys of 15 populations of *L. amplexicaule* during a two-year period. We show that *L. amplexicaule* did not systematically have two generations per year, contrary to observations made in populations in North America. In the populations that had two generations per year, flowering phenology differed between seasons. Furthermore, flower number did not vary between seasons or years, but the habitat quality had a strong effect on the flower type production. Finally, CH

proportion varied in time, but the effect of the season was not as clear as in previous studies (Lord, 1982), and CH proportion also varied between habitats, but not between regions.

Winter and summer annual cycles of *L. amplexicaule*

There were substantial differences in the global life cycle of *L. amplexicaule* both between regions and between habitats. Populations from favorable habitats in Dijon had a spring and an autumn generation, whereas populations from unfavorable habitats in Dijon as well as all populations in Montpellier had only a single spring generation. The absence of an autumn-flowering cohort in Montpellier could be explained by the unfavorable, dry summer conditions that prevent seed germination. A single population in Montpellier (MS) seemed to behave as a winter annual according to the description of Baskin and Baskin (1984) of several populations in Kentucky, USA. Small plants in vegetative stage were observed on the site of population MS in November 2012, which could have spent the winter in that vegetative stage and then produced flowers at the same time as the other spring populations in Montpellier. The absence of an autumn cohort in unfavorable habitat populations in Dijon is probably due to the abundance of other species in these habitats. It is known that germination of ripen seeds of *L. amplexicaule* depends on phytochromes that can be inactivated by exposition to high ratios of far red:red light (Jones and Bailey, 1956). High densities of other species present in unfavorable habitats are known to increase the far red:red light ratio and can therefore inhibit the emergence of new seedlings during the autumn season (Smith and Whitelam, 1997).

Flowering phenology

Though we may have missed some of the maximal induced CH proportion our results show that the phenology in autumn was different from that observed in spring. After the peak of the CH proportion in autumn, plants continued flowering for about five to six weeks, which is twice as long as for the spring populations. During this period, flowering gradually decreased until it ceased completely and plants became senescent. In spring, however, flowering was abruptly terminated about two to three weeks

after the CH peak with plants becoming completely senescent as well. These phenologies correspond to changes in the photoperiod and temperatures throughout the season. Temperature increase is known to accelerate plant development and shorten phenologies in several other species (Badeck et al., 2004), and this could be the case in *L. amplexicaule*. In favor of this observation is the fact that in general post peak flowering was shorter in 2011, which was a warmer year than 2010, and also the fact that the total number of flowers was not significantly different across seasons.

Effects of temporal environmental variation

The environmental conditions varied between seasons and years. Photoperiod was ascending in spring and descending in autumn, temperatures were warmer in autumn than in spring, but they were also globally warmer in 2011 than in 2010. Temporal variation did not seem to have an important effect on total flower production, except for the population DL in autumn that produced significantly less flowers in the autumn 2011. This could be explained by the unusually late phenology in the autumn 2011, resulting in unfavorable conditions for plant growth. Temporal variation had an important effect on the CH proportion. Globally, CH proportion was higher in autumn than in spring, but it was also higher in 2010 compared to 2011 suggesting that there could be more than one environmental cue that influences CH production. The joint action of two or more environmental factors has been studied in other cleistogamous species. In *Dichatelium clandestinum* for example, long photoperiods induce CH production, but only if associated with high moisture levels, otherwise only CL flowers are produced (Bell and Quinn, 1987). In *L. amplexicaule*, Lord (1982) observed that photoperiod and temperature have additive effects on CH proportion with long photoperiods (LD) and warm temperatures (+) inducing higher CH proportions, and conversely, short photoperiods (SD) and lower temperatures (-) inducing lower CH proportion. We also detect additive effects of photoperiod and temperature in our study. However, the temperature effects detected in Lord's growth chamber experiment have an inverse pattern than those observed in our field study. There are probably other environmental factors and cues

that influence the CH production in *L. amplexicaule* whose identification would require more detailed assessment of the environmental variation.

Effects of spatial environmental variation

Two levels of spatial variation were considered in this study – geographical regions with contrasting climates, and habitats within a region, both in spring. The phenology of CH proportion was delayed by one to two weeks in Dijon compared to Montpellier in both years, which resulted in longer photoperiods in Dijon compared to Montpellier, but the mean temperatures were similar in the two regions. Other factors, such as precipitation or light intensity, which were not included here, could also have been different. Despite environmental differences between the two regions, populations from Montpellier had only slightly lower total number of calices and did not differ in their global CH proportion from those observed in Dijon in spring. We could therefore reject the hypothesis that plants with similar flower production would have similar CH proportions since this is not true when comparing flower production and CH proportion variation within seasons (see above). There was, however, important variation in plant size and CH proportion between habitats in both regions, and this variation followed the same pattern everywhere – plants from unfavorable habitats produced less flowers but had higher CH proportions compared to plants from favorable habitats that produced considerably more flowers but had lower CH proportions. This observation is rather unusual, since in most annual cleistogamous species the inverse pattern was observed – high CH proportions are associated with larger plants (Oakley et al., 2007). If CH proportion variation is adaptive, this suggests that similar selection mechanisms operate in unfavorable and favorable habitats which are over 500 *km* apart. Some of these mechanisms are discussed in the following chapters.

Studying the effect of environmental variation on CH proportion/plant size variation could provide insightful information about the ecology of cleistogamy in *L. amplexicaule*. However, there are several difficulties that need to be overcome to better understand this cleistogamous system. First, disentangling the joint effect of different environmental factors is a rather difficult task in the absence of controlled conditions. Second, observations in natural populations give information about the variation of traits across natural populations and through time, but we do not know whether this variation is due to population differentiation or phenotypic plasticity. Third, the adaptive character of this variation cannot be assessed without reciprocal transplant experiments used for the geographic variation or common garden experiments used for the temporal variation. All of these issues are addressed and answers are provided in the following chapters.

Appendix 1. Mean ± sd number of calices, CH and CL flowers and global CH proportion for all of the populations studied. n/a: population did not emerge in the given season though it has been previously observed. pl: population emerged in the given season but the trait could not be estimated because the plants were destroyed before harvest or prior to the survey.

	Calices				Induced CH flowers				Induced CL flowers				Global CH proportion			
Population	**2010 Spring**	**2010 Autumn**	**2011 Spring**	**2011 Autumn**	**2010 Spring**	**2010 Autumn**	**2011 Spring**	**2011 Autumn**	**2010 Spring**	**2010 Autumn**	**2011 Spring**	**2011 Autumn**	**2010 Spring**	**2010 Autumn**	**2011 Spring**	**2011 Autumn**
Montpellier																
ML	54.05 ± 18.49	N/A	52.54 ± 13.34	N/A	11.2 ± 8.59	N/A	4.79 ± 3.45	N/A	22.9 ± 10.96	N/A	11.57 ± 5.55	n/a	0.197 ± 0.111	n/a	0.086 ± 0.047	n/a
MS	32.72 ± 11.1	N/A	28.75 ± 11.49	N/A	11.05 ± 6.04	N/A	3.33 ± 2.93	N/A	7.05 ± 3.91	N/A	6.21 ± 3.2	n/a	0.324 ± 0.116		0.123 ± 0.09	n/a
MCO			pl	N/A			4.45 ± 3.02	N/A			3.9 ± 3.97	N/A			pl	n/a
MCF			39.42 ± 9.36	N/A			4.4 ± 3.72	N/A			9.4 ± 5.1	N/A			0.116 ± 0.093	n/a
MLa																
Dijon																
DL	49.21 ± 10.09	58.9 ± 14.93	50.8 ± 13.03	37.79 ± 18.93	9.84 ± 6.39	5.75 ± 5.14	3.05 ± 3.19	1.63 ± 3.56	9.58 ± 4.27	32.35 ± 11.94	17.15 ± 5.84	20.58 ± 9.39	0.193 ± 0.11	0.095 ± 0.086	0.062 ± 0.061	0.033 ± 0.077
DS	32.89 ± 12.07	n/a	42.33 ± 16.87	n/a	11.1 ± 6.54	n/a	6.6 ± 4.22	n/a	3.9 ± 2.65	N/A	6.35 ± 4.37	n/a	0.321 ± 0.127	n/a	0.166 ± 0.107	N/A
DLH		63.65 ± 26.72	64.32 ± 21.82	pl		17.55 ± 9.37	5.9 ± 3.34	pl		13.6 ± 7.24	15.4 ± 10.02	pl		0.274 ± 0.087	0.099 ± 0.052	Pl
DBo		pl	pl	27.45 ± 21.1		5.7 ± 4.74	pl	1.3 ± 1.56		10.2 ± 5.58	pl	5.2 ± 3.11		pl	pl	0.045 ± 0.058
DLB			46.5 ± 12.41	pl			2.15 ± 2.46	pl			5.7 ± 3.56	pl			0.045 ± 0.049	Pl
DMos			43.95 ± 6.54	n/a			7.25 ± 3.65	n/a			6.1 ± 2.9	n/a			0.163 ± 0.073	n/a
DBr				19.4 ± 10.27				0 ± 0				3.07 ± 1.58				0 ± 0
DVt				42.5 ± 20.9				3.25 ± 2.7				6.13 ± 4.5				0.085 ± 0.075
DMJ				25.38 ± 11.54				0.06 ± 0.25				19.69 ± 10.45				0.002 ± 0.007
DMV				41.35 ± 19.99				3.1 ± 2.25				3.1 ± 2.07				0.078 ± 0.056

CHAPTER 2. Does cleistogamy variation translate into outcrossing variation in the annual species *Lamium amplexicaule* (Lamiaceae)?

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Abstract

The maintenance of cleistogamy, the ability to produce closed, obligately selfing flowers (CL), and open, potentially outcrossed flowers (CH), in different proportions, is classically explained through different morphological/physiological properties of the two floral types, but rarely as a mechanism of adjusting the outcrossing rate. We explore the link between CH proportion and overall outcrossing rate in natural populations of *Lamium amplexicaule*. We assessed number of calices, CH proportion, CH and overall outcrossing rate in two natural populations around Montpellier and two around Dijon (France). In each region we had one favorable and one unfavorable habitat population. Unfavorable habitats produce smaller plants (with fewer calices) with higher CH proportions compared to favorable habitats, regardless of the geographic origin of the populations. CH outcrossing rate did not change significantly among populations. Thus the overall outcrossing rate in *L. amplexicaule* is mainly determined by the CH proportion. Contrary to the classical view, unfavorable environments in our study are associated with higher rate of chasmogamous flowers, supposedly more costly to produce. We propose that cleistogamy variation can be considered as a variation of the outcrossing rate and could be explained by classic forces driving the evolution of mating systems (inbreeding depression, pollinators abundance).

ADDITIONAL KEYWORDS: cleistogamy variation; inbreeding depression; microsatellite; mixed mating; pollination success.

Introduction

The maintenance of mixed mating systems, which combine selfing and outcrossing to different extents, remains one of the central questions in reproductive biology studies (e.g., Goodwillie et al., 2005). Classic theory predicts that complete selfing or complete outcrossing should be the only stable states of the mating system evolution. These models study the effects of different evolutionary forces, such as inbreeding depression and automatic transmission advantage of selfing, in a stable environment (Lande and Schemske, 1985, Lloyd, 1992, Lloyd, 1979). Recently, several attempts have been made to take into account the environmental variation when explaining the evolution of mixed mating strategies (Morgan and Wilson, 2005, Cheptou and Mathias, 2001, Cheptou and Schoen, 2002). These models are based on the observation that inbreeding depression or pollinator activity can vary among environments either spatially or temporally. They show that temporal or spatio-temporal variation, but not spatial variation by itself, could maintain stable mixed mating systems, with each reproductive type providing an advantage in a particular environment. However, the mating systems described in these theoretical approaches are considered as stable throughout variable environments, though the variation of reproductive traits has been demonstrated in several empirical systems such as gastropods (Tsitrone et al., 2003) and angiosperms (Kalisz et al., 2004, Herlihy and Eckert, 2007) to cite a few. These variations are partly plastic, but traits differentiation has also been observed across different populations of the same species.

Cleistogamy is a particular case of a mixed mating system in which individuals produce two distinct floral types: closed flowers (cleistogamous, CL) which are obligately self-pollinated and open flowers (chasmogamous, CH) which are potentially outcrossed. In many cleistogamous species, the CH proportion per individual varies according to different environmental cues such as photoperiod (Lord, 1982), light intensity or light availability (Bell and Quinn, 1987, Cortes-Palomec and Ballard, 2006), water availability (Bell and Quinn, 1987, Schemske, 1978), herbivory (Schemske, 1978, Steets et al., 2006),

competition levels (Schmitt et al., 1987, Steets et al., 2006) or nutrient availability (Cortes-Palomec and Ballard, 2006). The among populations variation of the CH proportion in these studies is likely to be due to plasticity as well as to genetic differentiation among populations (Paoletti and Holsinger, 1999).

Different models and hypotheses were proposed in order to explain the maintenance of cleistogamy, based on the production cost differences between the two floral morphs (Schemske, 1978), fruiting success (Cortes-Palomec and Ballard, 2006), flowering phenology (Winn and Moriuchi, 2009, Culley, 2002), inbreeding depression (Stewart, 1994) or pollination success (Redbo-Torstensson and Berg, 1995, Masuda et al., 2004). For instance, measures of the production costs of CL and CH flowers have been made for several species of *Impatiens*, showing that CL flowers are less costly to produce (Schemske, 1978, Waller, 1979) and are more abundant in field observations than in greenhouse experiments (Schemske, 1978). Based on these observations, Schemske suggests that CL flowers should be favored in environments that reduce plant growth (low resources, lack of light, or other stressful conditions) because of the lower cost of CL flowers, and whenever growth conditions are favorable to plant development, CH flowers should be produced in order to provide an opportunity for outcrossing and pollen donation (Schemske, 1978, reviewed in Oakley et al., 2007). In other cleistogamous species it has been suggested that the pollinators abundance should determine the production of CH and CL flowers (Redbo-Torstensson and Berg, 1995). The pollinators abundance hypothesis assumes that CL flowers should be produced in environments where pollinators are scarce, avoiding the risk of not being pollinated, and CH flowers should be produced in environments where pollinators are abundant, leaving a possibility for outcross pollination (Schoen and Lloyd, 1984, Lord, 1982).

CH proportion variation translates into outcrossing rate variation if CH flowers substantially outcross. CH outcrossing rate has been estimated in a few cleistogamous species (*Viola pubescens* (Culley, 2002), *Impatiens capensis* (Waller and Knight, 1989, Mitchell-Olds and Waller, 1985), *Impatiens pallida*

(Stewart, 1994)), suggesting that there can be substantial outcrossing in CH flowers, which can vary from 0.3 to 0.97 among populations or across generations within populations (Waller and Knight, 1989, Mitchell-Olds and Waller, 1985, Culley, 2002). Though variation of the CH proportion across environmental conditions was recorded for all three species (*Impatiens pallida* (Gross et al., 1998), *Impatiens capensis* (Steets et al., 2006), *Viola pubescens* (Culley, 2002)), the link between CH proportion and CH outcrossing rate variation has not been explored so far. It is therefore hard to determine to what extent variation of cleistogamy translates into variation of the outcrossing rate. The connection between the overall outcrossing rate (which takes into account the proportion of both floral types, as well as the outcrossing rate in CH flowers) and environmental variation requires information about how the CH proportion and the CH outcrossing rate vary across environments. For instance, if CH flowers maintain a stable outcrossing rate throughout a range of variable environmental conditions, then variation of cleistogamy translates into variation of outcrossing rate which could be a mating system adaptation to environmental variation. If this is the case, supposing that plants produce more CL flowers when conditions are less suitable for plant development, the outcrossing rate of a cleistogamous species should be higher in favorable than in unfavorable environments. On the other hand, if the CH outcrossing rate fluctuates among environments, the link between CH proportion and outcrossing rate is less obvious, leaving the possibility for other factors, such as the costs of CH flower production, to explain better the variation of the CH proportion.

In this paper, we analyse the production of CH flowers and the outcrossing rate in four contrasted populations of the cleistogamous species *Lamium amplexicaule* L. These populations came from two different French regions with different climates. Within regions, one population came from a habitat favorable to the species development (early successional stage) and the other came from less favorable environment because of more intense competition. These populations were monitored during the spring of 2010 and progeny from CH flowers was used in order to estimate their outcrossing rate. More

specifically, our goal is to identify the major determinant of the outcrossing rate on the population level – CH proportion or outcrossing rate - in the four contrasted populations. For that purpose, we i) estimate the CH proportion in our populations in the field; ii) estimate the outcrossing rate of CH flowers and iii) estimate the overall outcrossing rate for each population.

Materials and methods

Study species

Lamium amplexicaule is a weedy annual of the mint family (Lamiaceae) native to Europe and Asia but documented as an invasive species in all other continents (USDA-NRCS, 2002). It is known as both winter annual (seeds dormant through autumn and winter, flowering in spring) and summer annual (seeds dormant through spring and summer, flowering in autumn) (Baskin and Baskin, 1981). Even though nectar production and pollinator visits of CH flowers have been documented for this species (Orueta and Viejo, 1999), the proportion of CH ovules that set seed can be up to 90% in the absence of pollinators (Bernstrom, 1950). Flowering in this species is ascending within whorls – in the beginning of the flowering season CL flowers are produced on the lowest whorls (true cleistogamy, sensu Lord, 1981), followed by CH flowers in variable proportions in subsequent whorls (Lord, 1980b). The percentage of CH flowers produced during the flowering season can vary from 0 to 50% in response to environmental cues such as photoperiod and temperature (Lord, 1982), with long photoperiod and high temperatures increasing the CH proportion, and short photoperiod and cold temperatures decreasing the CH proportion.

Study sites

In the spring of 2010, four French populations of *L. amplexicaule* were monitored during the flowering season – two populations near Dijon, in central eastern France, and two populations near Montpellier, in southern France. In each geographical region, one of the populations monitored was characterized as

"large" – with several hundred individuals (populations DL in Dijon, GPS coordinates 47°15'58.88"N, 4°59'11.51"E, and ML in Montpellier, 43°44'56.28"N, 3°51'06.49"E), and the other population was characterized as "small", bearing no more than 40 individuals (population DS in Dijon, 47°16'37.14"N, 5°03'43.37"E and population MS in Montpellier, 43°46'16.36"N, 3°47'28.03"E). For each of the four populations a voucher has been deposited in the Botanical Institute of Montpellier herbarium collection (voucher accession numbers: population DS - MPU000543; population ML - MPU000544; population DL - MPU00054; and population MS - MPU000546). Large population habitats were ploughed vineyards, with virtually no other vegetation, while small population habitats were lawns which were densely colonized with other species. Plants observed in large populations were visually bigger than plants in small populations and indeed the number of flowers (Table A. 1) as well as the number of secondary axes (data not shown) per plant are higher in large populations than in small populations. We therefore refer to large population habitats as "favorable" (for plant development) as opposed to the "unfavorable" habitats of small populations. Flowering phenology is delayed by about two weeks in Dijon compared to Montpellier (mean flowering peak in Montpellier – 5 Apr. 2010, mean flowering peak in Dijon – 18 Apr. 2010, personal observation). Since *L. amplexicaule* has up to two generations per year (Baskin and Baskin, 1981), the study sites were checked in late summer/early fall for new plants. Population DL is the only one that produced an autumn generation in 2010, while population DS and the two Montpellier populations had no plants at all in the autumn of 2010. The same growth pattern was observed in 2011 (data not shown) – population DL was the only one to have a spring and an autumn generation, while the other three had only a spring generation.

Total flower production and CH proportion on the principal axis

In each of the four populations monitored, twenty individuals were tagged with colored tape on the principal axis. The production of CH flowers on the principal axis was monitored twice a week during the flowering season. Since CH flowers wilt in about 4 days, only recent, fresh flowers were recorded at each

monitoring date, thus avoiding recounting the same flower. At the end of the flowering season (around the 5th May 2010 in Montpellier, around the 15th May 2010 in Dijon), when signs of advanced senescence were visible (i.e., no flowers produced for at least 7 days, the majority of the plant axes wilted), tagged plants were collected, dried, then stored in paper bags at room temperature until DNA extraction (see below). Before drying, calices on the principal axis were counted. The CH proportion on the principal axis of each individual (p_{CH}) was calculated as its number of CH flowers during the entire flowering season (N_{CH}), divided by the total number of calices on this axis (i.e., total of open and closed flowers, N_{cal}). This estimate has been used in previous studies of the production of CH and CL flowers *Lamium amplexicaule* (Lord, 1982). Moreover, the correlation between the CH proportion on the principal axis and the CH proportion of the whole plant is high and significant (spearman's rho = 0.6777139, p-value < 0.0001) in an independent sample of 54 plants coming from another Dijon population of *Lamium amplexicaule*. The number of CL flowers, N_{CL}, was not directly assessed, but it was estimated as $N_{cal} - N_{CH}$.

CH proportion (p_{CH}) and total number of flowers (N_{cal}) on the principal axis were analyzed using type I General Linear Model (GLM). The explanatory variables in both analyses were region, habitat type and the interaction region x habitat. The CH proportion was analyzed assuming a binomial distribution with CH flowers considered as a success, and the total number of flowers was declared to be following a normal distribution. In such models, the residual deviance follows a Chi-square distribution. When the residual deviance is higher than the expectation of the Chi-square distribution (overdispersion), the probability value of the factors tested could be over estimated. We then scaled the explained (deviance/explained df) with (residual deviance/residual df) for each explanatory variable and significance levels were tested by calculating a quasi *F*-test, which is more conservative than a χ^2 (Saporta et al., 2005). Since the order of introduction of the explanatory variables can influence the estimates in a type I analysis, we checked that introducing the habitat as the first explanatory variable

did not significantly affect our estimates (data not shown). All analyses were performed with software R version 2.13.1. using the GLM function.

Seed collection for progeny arrays and seed set estimates

During the monitoring period, several calices bearing CH and CL flowers on each of the 20 tagged individuals were marked with acrylic paint. After corollas fell out, calices were closed using acrylic paint. At the end of the flowering season, the seeds produced in each marked calyx were counted. In large populations additional 20 – 25 individuals had at least one CH flower calyx marked in order to increase the number of progeny arrays used for the estimation of the outcrossing rate (see below). For each flower type, seeds collected on the same plant were pooled together in an Eppendorf® tube and stored in a dry place at room temperature for 12 months.

Seed sets obtained from the 20 tagged plants were used to estimate the pollination probability for CL and CH ovules. Since the maximal number of seeds per flower is invariably four, the proportion of fertilized CH and CL seeds (G_{CH} and G_{CL} respectively) in each population was estimated as the total number of seeds counted in all marked CH or CL flowers, divided by four times the total number of marked CH or CL flowers respectively. These estimates were used to calculate the overall outcrossing rate on the population level (see below), as well as seed production of CH relative to CL (G_{CH}/G_{CL}) in order to compare the pollination success of CH flowers relative to CL flowers among populations. The G_{CH}/G_{CL} ratio was analyzed the same way as p_{CH} and N_{cal} using a type I GLM supposing a binomial distribution, and habitat, population and their interaction as explanatory variables.

Assessing genotypes in progeny arrays

In the spring of 2011, 12 CH seeds per individual were randomly selected and sown in a sterile Petri dish with two pieces of regularly humidified Whatman paper. Petri dishes were placed in growth chambers with 12h light exposure at 25°C, and 12h in the dark at 13°C. Successfully germinated seeds were planted in 14 x 10 wells plates. A small leaf fragment (less than 1 cm^2) was sampled from each plant

around 6 weeks after the germination. At this stage, no juvenile mortality was recorded in the plants. DNA was immediately extracted from the samples using the DNeasy 96 Plant Kit (Qiagen) following the manufacturer protocol for fresh tissue with an additional lysis step of 2h at 65°C. DNA of the plants monitored in the field (the parents of the progeny arrays) was also extracted. Plants monitored in the spring of 2010 were collected at the end of the flowering season and the tissue has been stored in paper bags in a dry place at room temperature for 18 months. DNA was extracted from these plants using the DNeasy 96 Plant Kit (Qiagen) following the manufacturer protocol for dry plant tissue with an additional lysis step of 2h at 65°C.

In order to assess neutral genetic diversity of each parent and its progeny, we performed PCRs for 13 microsatellite loci grouped in three multiplexes as described in Stojanova et al. (2013a). PCR products were sized using an ABI PRISM 3100 sequencer (Applied Biosystems) and the software GENEMAPPER v. 4.1. The number of parents whose genotype was assayed was 39, 19, 31 and 19 in populations ML, MS, DL and DS respectively. Since some of the parents produced no CH flowers or their seeds did not germinate, the number of successfully genotyped families was 38, 13, 31 and 14 for populations ML, MS, DL and DS respectively. Mean offspring number per parent was 5.42 ± 0.52; 7.46 ± 0.73; 5.77 ± 0.57 and 8.85 ± 0.39 in populations ML, MS, DL and DS respectively. Heterozygosity in parents and in progeny was calculated using GenAlEx 5.1 software (Peakall and Smouse, 2006).

Estimating the open flower and overall outcrossing rates

The CH outcrossing rate on the population level, t_{CH}, inbreeding coefficient in maternal parents, F_{is}, biparental inbreeding and paternity correlations were estimated with Ritland's multilocus progeny array approach using software MLTR version 3.4 (Ritland, 2002). Maternal F_{is} were calculated from maternal genotypes directly, while CH outcrossing rate, biparental inbreeding and paternity correlation were estimated using progeny and maternal genotypes.

Unless otherwise stated, the Newton-Raphson algorithm was used for maximum likelihood estimates (Ritland, 2002). Pollen frequencies estimates calculated by the software did not significantly differ from ovule frequencies (not shown), thus we constrained them to be equal in order to increase the estimation precision of the other parameters. Standard deviation for t_{CH}, F_{is}, biparental inbreeding, and paternity correlations was calculated using 500 bootstraps. All progenies available were used for the estimates, since excluding the arrays that had less than 7 progenies did not significantly affect the estimated values (data not shown). Based on our estimates of the CH outcrossing rate, and given that the CL outcrossing rate is null, the overall outcrossing rate, $t_{overall}$, was calculated as:

$$t_{overall} = t_{CH} \frac{p_{CH} G_{CH}}{p_{CH} G_{CH} + p_{CL} G_{CL}}$$

Results

Flower production, CH proportion and pollination success

The mean number of calices per plant counted on the principal axis varied from 33 to 54 according to populations (mean ± standard error for populations ML, MS, DL and DS respectively: 54.05 ± 4.13; 32.72 ± 2.62; 48.7 ± 2.31; 32.89 ± 2.77, Fig. 1.A). Analysis of deviance showed that habitat type significantly affected the total flower production (Fisher's $F_{1,73}$ = 37.24, p-value < 0.001, Table A.1), with plants in favorable habitats producing more flowers than plants in unfavorable habitats (Fig. A.1.A).

Table A. 1. I General Regression Analysis of the mean number of flowers per individual. Bold F-values are associates with p-value <0.05

Model	Deviance	DF	Residual Deviance	Residual DF	F-value
Null			20040	75	
Region	150	1	19890	74	0.56
Region + Habitat	6720	1	13170	73	37.25
Region x Habitat	120	1	13050	72	0.66

This result was not surprising, given that plants in favorable habitats were bigger than plants in unfavorable habitats, and the number of calices in the principal axis was highly correlated with plant size indicators such as the number of axes (Spearman's rho = 0.71, p-value < 0.001). Neither the region nor the interaction region x habitat affected significantly the flower number (Table A.1). Mean individual CH proportion varied from 0.19 to 0.32 according to populations (mean ± standard error for populations ML, MS, DL and DS respectively: 0.197 ± 0.025; 0.324 ± 0.026; 0.193 ± 0.025; 0.321 ± 0.029; Fig. A.1.B). GLM results showed that the CH proportion was significantly higher in unfavorable habitats than in favorable habitats ($F_{1,73}$ = 11.25, p-value < 0.001, see Table A.2, Fig. A.1.B), but neither the region nor the interaction region x habitat had a significant effect.

Pollination success of CH flowers relative to CL flowers varied among populations, but it was significantly higher in unfavorable habitat populations (mean ± standard error for populations MS and DS respectively: 1.109 ± 0.04; 1.185 ± 0.017) than in favorable habitat populations (mean ± standard error for populations ML and DL respectively: 0.968 ± 0.014; 0.869 ± 0.023) ($F_{1,61}$ = 6.79, p-value = 0.02) (Fig. A.1.C).

Table A. 2. Type I General Regression Analysis of the mean CH proportion per individual. Bold F-values are associates with p-value <0.05

Model	Deviance	DF	Residual Deviance	Residual DF	F-value
Null			330	75	
Region	<0.01	1	300	74	0
Region + Habitat	77.8	1	252	73	**11.25**
Region X Habitat	<0.01	1	252	72	0

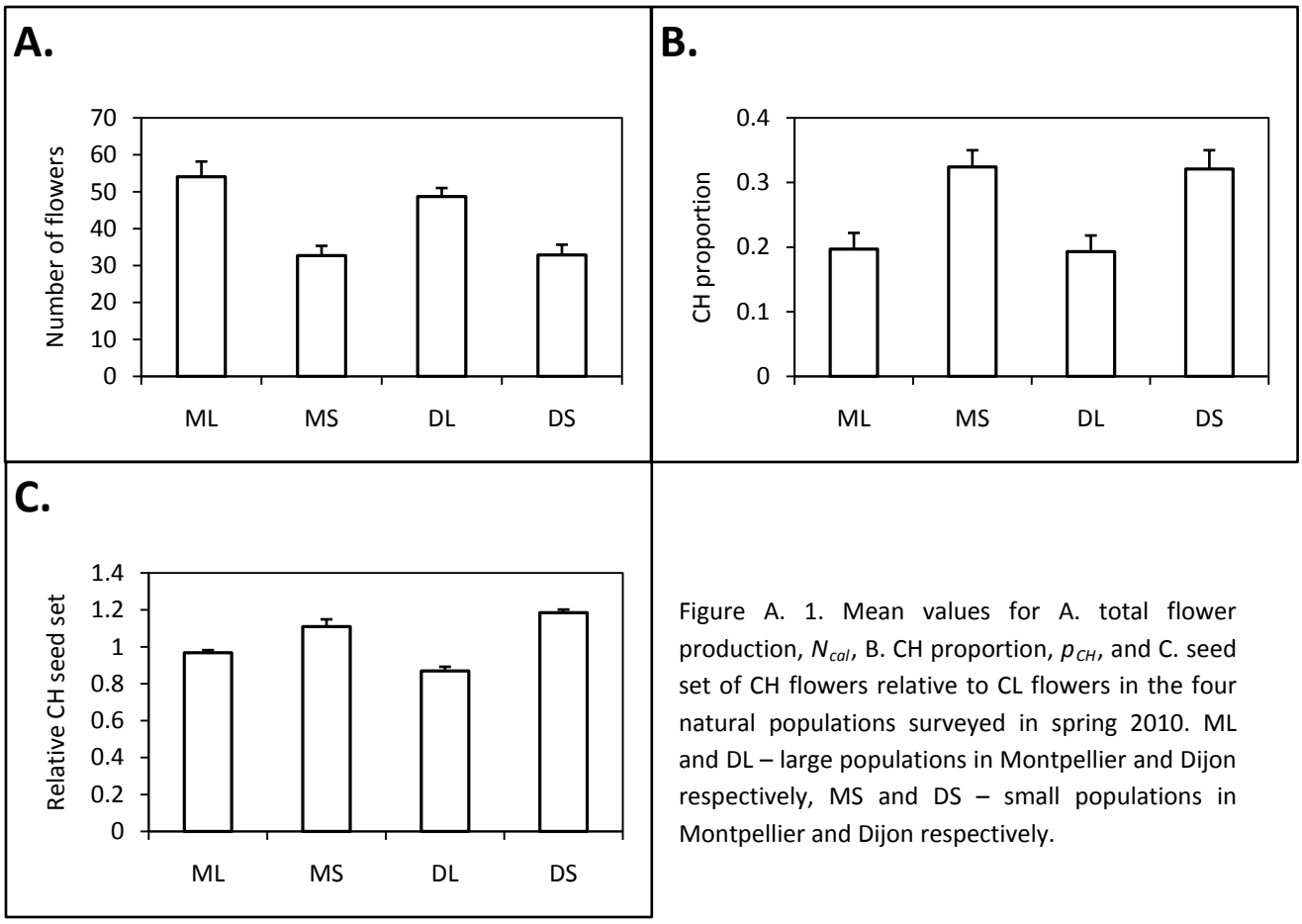

Figure A. 1. Mean values for A. total flower production, N_{cal}, B. CH proportion, p_{CH}, and C. seed set of CH flowers relative to CL flowers in the four natural populations surveyed in spring 2010. ML and DL – large populations in Montpellier and Dijon respectively, MS and DS – small populations in Montpellier and Dijon respectively.

Genetic diversity of populations

All loci were successfully amplified in all four populations, except loci LA-Di05, LA-Tri08, LA-Tri11 and LA-Di03 in population MS (Appendix A). These loci were excluded for further analysis in population MS only. Polymorphism levels were not very high: locus LA-Tri07 had eight and ten alleles in populations ML and DL respectively, and all other loci had allele numbers ranging from one to four across populations. Population DS was highly monomorphic (11 monomorphic loci out of 13). In all four populations there was at least one locus with more alleles in the progeny than in the parents, and heterozygosity levels were generally slightly higher in progeny that in parents (Appendix 1).

CH and overall outcrossing rate and F_{is} calculations with MLTR

CH outcrossing rate did not significantly differ between populations ML, MS and DL (mean value between 0.22 and 0.25 with overlapping 95% confidence intervals, Table A.3). The lower precision of the CH outcrossing rate estimates in populations DS and MS was probably due to the smaller sample size and the fewer loci used for the estimates compared to the large populations, and in population DS in particular, the lower genetic diversity per locus. When the four loci that amplified poorly were included in population MS, the CH outcrossing rate estimate did not change significantly, but its standard deviation was lower compared to the estimation with only nine loci (mean ± sd: 0.228 ± 0.074).

Parental inbreeding coefficient (F_{is}) estimates were high for all four populations, ranging from 0.73 in population DS to 0.97 in population DL (Table A.3). Estimation precision of F_{is} was also high except for population DS. Biparental inbreeding was close to zero in all four populations (Table A.3), meaning that mating between related individuals was infrequent. The MLTR software could not calculate paternity correlation for populations MS and DS, and the value calculated for population DL was lower than that for population ML. The sampling pattern is similar in the two populations (mean ± se number of flowers sampled for seeds per individual were 7.263 ± 0.964 and 7.077 ± 0.976; mean ± se number of seeds genotyped per family were 5.77 ± 0.57 and 5.42 ± 0.52 in DL and ML respectively), meaning that the average number of pollen donors was actually higher in population DL than in population ML. The overall outcrossing rate ($t_{overall}$) estimates all ranged below 0.1, being particularly low in population DS (Table A.4).

Table A. 3. Estimates of parental F_{is}, CH outcrossing rate, biparental inbreeding and paternity correlation using Ritland's progeny array approach. Parental F_{is} was estimated using molecular marker data from the maternal parents. CH outcrossing rate, biparental inbreeding and paternity correlation were estimated using the molecular marker data from the maternal parents and the progeny.

Population	ML	MS	DL	DS
Parental F_{is}	0.739 ± 0.069	0.895 ± 0.082	0.975 ± 0.014	0.728 ± 0.449
CH outcrossing rate	0.254 ± 0.051	0.246 ± 0.207	0.221 ± 0.045	0.037 ± 0.152
Biparental inbreeding	0.034 ± 0.024	0.001 ± 0.037	-0.021 ± 0.022	0 ± 0.001
Paternity correlation	0.585 ± 0.121	-	0.274 ± 0.117	-

Table A. 4. Estimates of the outcrossing rate in the CH flowers and the whole individual. Progeny G_{CL} and G_{CH} – probability of seed set estimates in CL and CH flowers respectively, $t_{overall}$ – overall outcrossing rate taking into account the CH proportion and the seed set probability for a CH flower's ovule.

Population	G_{CL}	G_{CH}	$\frac{P_{CH}G_{CH}}{P_{CH}G_{CH} + P_{CL}G_{CL}}$	*Overall t*
ML	0.778	0.754	0.192	0.049
MS	0.718	0.796	0.347	0.085
DL	0.738	0.641	0.172	0.038
DS	0.757	0.897	0.359	0.013

Discussion

In this study we show that the total production of flowers and the CH proportion varies among the four populations of *Lamium amplexicaule* we monitored. This variation is significant between habitat types, with less flowers and higher CH proportion in unfavourable than in favourable habitats, but not between geographic origins. The CH outcrossing rate is approximately 0.24 for three of four populations studied, and lower, but not significantly different in the fourth population. The overall outcrossing rate estimates, which take into account the CH outcrossing rate and the CH proportion is below 0.1 for all four populations. Overall, the outcrossing rate estimates are very low, which is consistent with previous classifications of *L. amplexicaule* as a highly selfing species (Fryxell, 1957).

Effect of the habitat type on plant growth and CH proportion

There is a significant decrease in plant size in the unfavourable habitats for *Lamium amplexicaule*, as shown by the reduced total number of flowers. Though no precise measures were made in the scope of this paper, these results are consistent with different levels of interspecific competition across habitats. *L. amplexicaule* is a ruderal species, found generally in ploughed fields, roadsides and vineyards, before the habitats are colonized by other species (Tutin et al., 1993). Populations originating from unfavourable habitats such as populations MS and DS in this study are less common. Moreover, it has been shown experimentally that interspecific competition decreases *L. amplexicaule* above ground biomass for up to 42% (Baude et al., 2011). Unfavourable habitats in this study were highly colonized by other species and in general the plant density was very high, while favourable habitats bore virtually no other vegetation than *L. amplexicaule* with low density, meaning that even intraspecific competition should be very low in the latter. However we cannot exclude the influence of other environmental factors on *L. amplexicaule* growth such as nutrients availability, light exposition or physical qualities of the soil (i.e. vineyards are regularly ploughed rendering the soil less compact than in lawns) or a combined action of all them.

CH proportion also varied between populations and this variation was due to the habitat in which the populations are found rather than to their geographical origin. Unfavourable habitat populations have higher CH proportions than favourable habitat populations. Survey data of the four populations in this study and six new populations in the spring of 2011 (totalling three unfavourable and two favourable habitat populations in each region) confirm the pattern observed here (data not shown). This result is somewhat surprising since in general CH flowers are considered more costly to produce because they have a larger corolla and more pollen than CL flowers (Lord, 1980b), and they also produce nectar. CH proportion should therefore increase in favourable habitats (reviewed in Oakley et al., 2007). There are, however, several perennial species in which the reverse pattern is observed – CL flowers are produced

in conditions enhancing plant growth, and CH flowers are produced when plant growth is slowed down (reviewed in Oakley et al., 2007). This has been explained by the fact that perennials can adjust the proportions of selfing and outcrossing by using stored resources, thus reducing the impact of environmental stress (Berg and Redbo-Torstensson, 1998). If this is the case then other factors, such as pollinator abundance, could be an important driver of the CH proportion. In a growth chamber experiment using *Lamium amplexicaule*, plants exposed to long day conditions have higher CH proportions. The photoperiod effect is even more pronounced when coupled with higher temperatures and higher light intensity (Lord, 1982). It has been further shown that the production of CH flowers is determined early in the development of the flower bud, since a single external application of *gibberellins* (plant hormones whose production is induced by long photoperiods) enhances the production of CH flowers for up to two weeks (Lord and Mayers, 1982). Thus the CH proportion and to some extent the outcrossing potential of *L. amplexicaule* could be partially determined in advance, anticipating the mating environment. In our study, the photoperiod is about one hour longer in Dijon than in Montpellier because of the phenology delay in Dijon, but this difference does not affect the overall production of CH flowers.

Does cleistogamy variation translate into selfing variation?

CH outcrossing rate estimates were roughly 0.24 in populations ML, DL and MS, and 0.037 in population DS, though because of the large standard deviation estimates in the latter, the difference between DS and the other three populations is not significant. The high standard deviations in population DS are due to the fact that the only two polymorphic loci in this population have low levels of polymorphism. Population MS also had high standard deviation estimates but the estimates precision was considerably improved when the four loci with null alleles were included in the analysis. Low standard deviations are found in populations ML and DL. The precision of the outcrossing rate estimates (sd < 0.1 for three out of four populations) is due to the particular genetic structure of the populations we studied: parents are

highly homozygous because of low overall outcrossing rates. Since only CH progeny (which is more outcrossed than the overall outcrossing rate) are analysed, outcrossing events can be easily identified because they significantly increase heterozygosity over multiple loci in the CH progeny. We were also able to calculate the overall outcrossing rate at the population level. The overall outcrossing rate is the lowest in population DS because of the low CH outcrossing rate estimates, it is higher in populations ML and DL and the highest in population MS because of the high CH proportion.

CH outcrossing rate estimates for other cleistogamous species found in the literature cover a wide range of values, with significant variation between species, populations of the same species, and between different years for the same population. In *Viola pubescence*, CH outcrossing rate estimates for the same population differ significantly in two consecutive years (0.4 and 0.93, Culley, 2002). In *Impatiens capensis* outcrossing rate in CH flowers varies from 0.97 to 0.3 in different populations, according to two different studies (Waller and Knight, 1989, Mitchell-Olds and Waller, 1985). CH proportions were not reported in these studies, but the high variation of CH outcrossing rates between years and between populations suggest that the connection between CH proportion and the overall outcrossing rate is complex. In *Impatiens pallida* estimates of the CH outcrossing rate in three consecutive years for the same population did not differ significantly (around 0.55, Stewart, 1994), but the link between CH proportion and overall outcrossing rate was not explored either. In our study, the variation of the CH outcrossing rate is not significant among populations. Thus, the main component determining the variation of the overall outcrossing rate among populations is the CH proportion variation across habitats.

Hypotheses for the CH proportion patterns as an adaptive mixed mating system

Our results showed that plants in unfavorable habitats have higher CH proportions, which does not fit in the resource adaptation hypothesis. On the other hand, increasing the CH proportion increases in turn the overall outcrossing rate in the populations surveyed. In this section, we discuss the possibility that

the observed pattern results from classical forces involved in the evolution of the selfing rate, namely avoidance of inbreeding depression and pollination environment.

Environmental variation of inbreeding depression as well as its importance for the maintenance of mixed mating systems have been well documented (Cheptou and Donohue, 2011). It is generally admitted that inbreeding depression is higher in stressful that in benign or favorable environments, which may favor an increase of the outcrossing rate in the former. In our study, small population habitats are less favorable to plant development than large population habitats. Under this hypothesis, if inbreeding depression is higher in competitive, unfavourable habitats, higher CH proportions will be adaptive there. Consistent with this scenario, Cheptou et al. (2000) have shown that outcrossing rates increase with competition in the weed *Crepis sancta*, in parallel to inbreeding depression magnitude. Admittedly, this hypothesis needs that *L. amplexicaule* shows some inbreeding depression in spite of being a highly selfing species (Lande and Schemske, 1985, see Winn et al., 2011 for empirical data). However, because CL flowers exhibit total pollen discounting (Lloyd, 1992) CH flowers can be selected even if inbreeding depression is low.

Another classical explanation for the variation of the CH proportion in cleistogamous species is the adaptation to the variation in pollinator activity. Lord (1982) observed that long photoperiods and warm temperatures increase the production of CH flowers in *L. amplexicaule*. These environmental conditions correspond to the spring season, when pollinators are abundant and CH flowers can be easily pollinated. When the photoperiod is shorter and the temperatures are colder (i.e., in autumn) pollinators are scarce, and plants favor the production of closed flowers that are obligately selfed. CH seed set relative to CL seed set estimated here is higher in unfavorable than in favorable habitats (Fig. A.1.C) indicating that CH flowers in unfavorable habitats are more efficiently pollinated than those in favorable habitats.

Therefore, increasing the CH proportion in unfavorable habitats is consistent with an adaptation to the pollination environment.

A change in the CH proportion according to environmental variation of inbreeding depression levels or pollinators abundance could be due to local adaptation, i.e., genetic differentiation, or a plastic response. While plastic variation of the CH proportion has been demonstrated in this species (Lord, 1979b, Lord, 1982, Lord and Mayers, 1982), we cannot formally rule out that part of the observed variation in the four populations studied could be due to genetic differentiation.

By measuring jointly CH outcrossing rates and CH proportions, our study indicates that variation of the CH proportion translates into variation of the overall outcrossing rate. While the overall outcrossing rate variation is low in the populations examined here, the evolutionary significance of among habitat variation in CH rate deserves to be studied. Our study suggests that the variation of cleistogamous traits in *L. amplexicaule* could be considered in the context of classical forces affecting mating system evolution, such as pollinator abundance and inbreeding depression. In order to further explore the relationship between inbreeding depression and CH proportion, experimental crosses of *L. amplexicaule* aiming to estimate inbreeding depression and its potential variation across seasons are in progress. While plasticity of the CH proportion in *L. amplexicaule* has been experimentally demonstrated in common garden experiments, further studies should be made to determine whether the variation observed in our study is due to plasticity or genetic differentiation among populations.

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CHAPTER 3. Adaptive plasticity across seasons in the annual cleistogamous plant *Lamium amplexicaule*

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Introduction

Plasticity is the ability of a given genotype to modify its phenotype in response to environmental variation (Bradshaw, 1965). Plasticity can be adaptive, meaning it provides better fitness for a plastic genotype compared to a non plastic one across variable environments (Sultan, 1995). Nevertheless, phenotypic change that does not increase fitness can also be observed if an organism is unable to respond suitably to environmental changes, or if it is unable to maintain a constant phenotype in a changing environment (canalization, Debat and David, 2001).

For plasticity to be adaptive, several conditions need to be fulfilled. First, plastic individuals need to be subject to environment dependent selection (Via and Lande, 1985) – each of the phenotypes expressed by plastic genotypes should confer higher fitness in a particular environment which induces its expression, and lower fitness in the other environments. This could be achieved if stabilizing selection, with different optimal phenotypes operates in each environment (Lande and Arnold, 1983, Via and Lande, 1985). Second, the selective advantage conferred by an adaptive phenotype must be higher than the costs associated to the maintenance and expression of plasticity (DeWitt et al., 1998). Costs of plasticity can be detected by comparing a plastic and a fixed genotype in the same environment – if both produce the same phenotype with the fitness of the plastic genotype being lower than that of the fixed phenotype, then this indicates a cost of plasticity. Third, the phenotypic response of a plastic genotype needs to be appropriate to the environmental change, i.e. the organism must be able to reliably predict environmental variation and respond rapidly to this change (DeWitt et al., 1998). If the time necessary to establish a plastic response is longer than the length of the environmental change, of if environmental variation is stochastic, then a fixed phenotype would be more appropriate (Roff, 2002). Inversely, if environmental change takes place over a longer period of time and is easily anticipated, then a plastic response is more suitable. An easy way to capture environmental variation is to rely on regular periodical changes, such as changes brought by seasonal variation or photoperiod.

Plasticity in plants has been well documented for morphological traits such as individual size or leaves forms (Sultan, 2000). Mating system plasticity has been less studied though it is confirmed by empirical evidence. For instance, sex expression in some species has been found to depend on environmental conditions with favourable conditions generally increasing femaleness (Korpelainen, 1998). General knowledge about plasticity described above applies also to plastic mating systems – if relative values of different reproductive modes depend on environmental conditions, then a plastic reproductive system should be favoured in variable environments. For instance, selfing strategies should be favoured in environments where pollinators are scarce, or outcrossing strategies should be favoured in environments with high levels of inbreeding depression (Lloyd, 1979). However, the evolution of plastic reproductive traits is limited by an additional constraint compared to plastic non-reproductive traits, which is the frequency dependent selection (Ernande and Dieckmann, 2004). This problem could be easily illustrated in dioecious species. Producing high proportion of females in a given environment will automatically increase reproductive success of males. Besides, in such situations pollen limitation creates a second mechanism of negative frequency dependence selection on sexual phenotypes. Frequency dependent selection implies that there are no environment dependent optimal phenotypes and thus constrains the evolution of plasticity in reproductive traits (see also Shaw and Mohler, 1953).

A certain type of frequency dependence is also found in hermaphroditic mating systems. In hermaphroditic populations, individuals capable of selfing leave more copies of their genes because they can use their pollen to fertilize their own ovules as well as the ovules of other plants. The magnitude of this advantage – called automatic advantage of selfing (or the cost of outcrossing, Fisher, 1941, Lloyd, 1979) – depends on the opportunity to sire ovules devoted to outcrossing and thus on the frequency of outcrossers in the population. Contrary to selection in dioecious populations, selfing and outcrossing exclude each other in this type of selection and only complete selfing or complete outcrossing are stable strategies depending on the magnitude of inbreeding depression (Lloyd, 1979). Different forms of

selection, taking into account for instance pollen discounting or reproductive assurance, have however been proposed to account for stable mixed mating (reviewed in Goodwillie et al., 2005).

Cleistogamy is a particular case of mixed mating, in which closed flowers (cleistogamous, CL) which are obligately self-pollinated, and open flowers (chasmogamous, CH) which are potentially outcrossed, are found within the same individual or within different individuals of the same species (Lord, 1981, Culley and Klooster, 2007). Because of their particular morphology, CL flowers are unable to export their pollen (complete pollen discounting, Holsinger, 1991). For many cleistogamous species the proportion of CH flowers can vary according to environmental conditions such as photoperiod, temperature, light intensity, nutrient and water availability, competition, herbivory or a combination of these factors (Lecorff, 1993, Lord, 1982, Berg and Redbo-Torstensson, 1998, Cortes-Palomec and Ballard, 2006, Schemske, 1978, Steets et al., 2006, Bell and Quinn, 1987). The variation of CH proportion across environments can be due to the different production costs of the two floral types (CH flowers being in general more costly than CL flowers, e.g. Schemske, 1978), the difference in fruiting success or seed set, differences in flowering phenology (Redbo-Torstensson and Berg, 1995, Winn and Moriuchi, 2009), different seed dispersal strategies (Berg and Redbo-Torstensson, 1998), adaptation to pollinator abundance (Masuda et al., 2004) or inbreeding depression (Stewart, 1994). Plasticity of CH proportion translates to plasticity of the outcrossing rate if CH flowers substantially outcross. Therefore if CH proportion plasticity is adaptive, then cleistogamous species are capable of maintaining plastic mixed mating throughout variable environments.

Lamium amplexicaule L. is an annual cleistogamous species that produces CH flowers in variable proportions. The variation of the CH proportion in this species is influenced by environmental cues such as photoperiod and temperature (Lord, 1982), with long photoperiods and warm temperatures increasing the CH proportion. In temperate climates, *L. amplexicaule* can have one generation in spring

and one in autumn. Seasonal change corresponds to environmental variation that is periodical and regular, associated with reliable environmental cues such as photoperiod or light intensity, it is thus a variation pattern that is easy to predict and respond to with an appropriate phenotype. It is therefore plausible that populations of *L. amplexicaule* experiencing two different seasons should have developed adaptive plastic mechanisms in response to seasonal variation.

In order to test the adaptive plasticity of cleistogamy to seasonal changes in *Lamium amplexicaule*, we studied individuals coming from four French natural populations in spring and in autumn in a common garden experiment. Two populations came from Northern France, and two from Southern France. For each population studied we measured several morphology, phenology and cleistogamy related traits. Our goals were to 1) estimate plasticity across seasons of these traits; 2) characterize the form of selection operating on the measured traits within seasons (directional and or/stabilizing); and 3) test for the adaptive character of plastic cleistogamy across seasons as well as compare adaptive plastic responses among regions.

Materials and Methods

Model plant

Lamium amplexicaule is a weedy annual of the mint family (*Lamiaceae*) native to Europe and Asia and documented as an invasive species in all other continents (USDA-NRCS, 2002). It is known as both winter annual (seeds dormant through autumn and winter, flowering in spring) and summer annual (seeds dormant through spring and summer, flowering in autumn) (Baskin and Baskin, 1981). *L. amplexicaule* can have up to two generations per year (Baskin and Baskin, 1981), provided that environmental conditions are appropriate for its germination and development. Flowering in this species is ascending within whorls – in the beginning of the flowering season CL flowers are produced on the lowest whorls (true cleistogamy, sensu Lord, 1981), followed by CH flowers in variable proportions in subsequent

whorls (Lord, 1980b). The percentage of CH flowers produced during the flowering season can vary from 0 to 50% in response to environmental cues such as photoperiod and temperature (Lord, 1982), with long photoperiods and high temperatures increasing the CH proportion, and short photoperiods and cold temperatures decreasing the CH proportion. These experimental conditions correspond to natural conditions in spring (long day, warm temperatures) and autumn (short days and cold temperatures). The average outcrossing rate of CH flowers in four French populations (described below) was estimated at 25% for the spring season (Stojanova et al, unpublished data).

Study sites and seed sampling in natural populations

In spring 2010, four French populations of *L. amplexicaule* were monitored during the flowering season – two populations in Northern France, near Dijon, and two populations in Southern France, near Montpellier. In each geographical region, one population was characterized as "small" – with less than 40 individuals (population DS in Dijon, GPS coordinates 47°16'37.14"N, 5°03'43.37"E and population MS in Montpellier, 43°46'16.36"N, 3°47'28.03"E), and the other was characterized as "large" – with several hundred individuals (populations DL in Dijon, 47°15'58.88"N, 4°59'11.51"E, and ML in Montpellier, 43°44'56.28"N, 3°51'06.49"E). Flowering phenology has a delay of about two weeks in Dijon compared to Montpellier (mean flowering peak in Montpellier – 5th of April, mean flowering peak in Dijon 18th of April, personal observation). Large populations are found in vast, regularly ploughed vineyards (early successional vegetation cover) with low vegetation cover density, while small population habitats are non cultivated/undisturbed (such as lawns), with dense vegetation cover. Plants in large populations are visually bigger than plants in small populations – number of flowers and number of secondary axes per plant are higher in the former (pers.obs.). Populations in Dijon are known to have spring and autumn generations, whereas those in Montpellier have only a spring generation (pers. obs.) because of the dry summer climate which prevents germination of the autumn cohort seeds. Therefore, Dijon populations are exposed to a more variable environment, with environmental changes taking place on a timescale

that is longer than the lifespan of the plants. We checked all population sites in late summer/early fall for recently emerged plants. Population DL is the only one that had grown in the autumn 2010, and the same growth pattern was observed in 2011 (data not shown).

In each of the four populations monitored, twenty individuals were tagged with colored tape on the principal axis. Several calices bearing CH flowers of each tagged individual were marked with acrylic paint. After corollas fell out, calices were closed using acrylic paint. At the end of the flowering season seeds from marked calices were collected and stored in a dry place at room temperature. Seeds were sampled from CH flowers in order to maximize genetic variability of our experimental design.

F1 common garden generation

To control for maternal effects, a common garden F1 generation was produced from the seeds collected in the field. In February 2011, a maximum of 12 CH seeds per family were randomly selected and sown in a sterile Petri dish with two pieces of regularly humidified Whatman paper. Petri dishes were placed in growth chambers with 12h light exposure at 25°C, and 12h in the dark at 13°C. Successfully germinated seeds were planted in 14 x 10 well plates until they produced at least 4 leaves. One plant per family was then transplanted directly in the soil of a ploughed plot on the experimental field of CEFE-CNRS, resulting with 18, 13, 16 and 15 plants for populations ML, MS, DL and DS respectively, that were considered as the maternal parents of the F2 generation.

Experimental design for plasticity measurements on the F2 generation

In order to avoid among populations gene flow, only CL flower seeds were collected from the plants of the F1 generation for the common garden study. In May 2011, several CL flowers calices were marked on each F1 plant. At the end of each flowering season, seeds in marked CL calices were collected and stored at room temperature resulting in 18, 13, 16 and 13 full-sib families that constituted the F2 descendants for populations ML, MS, DL and DS respectively.

For each family, seeds were randomly divided in two lots in order to measure trait values in autumn and spring. Up to 8 seeds per family were germinated under the same conditions as described above on the 15th of August 2011 for the autumn treatment and on the 15th of February 2012 for the spring treatment. Germination timing was chosen in order to maximize photoperiod magnitude during the flowering period in both seasons, ranging from 13 to 15 hours of daylight in spring and from 11 to 9 hours in autumn. When most of the plants had at least four developed leaves, a maximum of 5 seedlings were transplanted in 450 cm^3 pots and placed outdoors, in CEFE experimental field. To facilitate manipulations, pots were assembled in blocks of 20. The position of the pots within blocks as well as the position of the blocks was regularly randomized. Because some of the families did not germinate at all, the number of families available was 13(15), 10 (11), 13(13) and 10(10) for populations ML, MS, DL, and DS in autumn (respectively in spring), with a total of 223 plants in autumn and 224 plants in spring. Pots were watered *ad libitum*.

Data measurements

Phenological traits – Plants were checked for flowers every other day in order to record the date of the first CL flower (d_{CL}) and the date of the first CH flower (d_{CH}). For the purpose of statistical analysis, the data was encoded as number of days counted from the beginning of the experiment in each season (15th of August and 15th of February in autumn and spring respectively).

Flower type counts and cleistogamy – Flower counts were made on the principal axis only. Number of calices and CH proportion on the principal axis are highly correlated with total number of flowers and total CH proportion respectively (number of flowers Spearman's rho = 0.78, p-value < 0.0001, and CH proportion Spearman's rho = 0.89, p-value < 0.0001 in a sample of 98 individuals). For each plant, we counted the number of empty CL-bearing calices when the first CH flower was observed, which correspond to constitutive cleistogamy flowers, N_{CLC}. After the first CH flower was noted, plants were checked every four days for new CH flowers. Since CH flowers wilt in about four days only recent fresh

flowers were recorded during each monitoring date, thus avoiding recounting the same flower. The sum of all CH flowers counted on the principal axis during the flowering period was named total number of CH flowers, N_{CH}. At the end of the flowering season, we counted the calices on the principal axis in order to estimate the total number of flowers produced, N_{cal}. The total number of CL flowers on a plant, N_{CL}, was estimated as $N_{CL} = N_{cal} - N_{CH}$.

Cleistogamy traits – Three different flower ratios related to cleistogamy proportions were calculated. Global CH proportion (on the principal axis) was estimated as $p_{CH} = N_{CH}/N_{cal}$. Constitutive cleistogamy proportion, which corresponds to the portion of flowers produced at the beginning of the flowering period that are obligately CL was estimated as $p_{CLC} = N_{CLC}/N_{cal}$. p_{CLC} was set to 1 for the plants that produced no CH flowers (most of which were observed in autumn) and 0 for the few plants that produced no CL flowers in spring. Induced CH proportion, corresponding to the fraction of CH to CL flowers produced after the constitutive CL flowering, was estimated as: $p_{CHpl} = N_{CH}/(N_{cal} - N_{CLC})$. Induced CH proportion data were not estimated for plants that produced no CH flowers.

Pollination success estimates – During the flowering season several CH and CL calices were marked on each individual using acrylic paint. After corollas fell out, calices were closed with a small drop of acrylic paint. At the end of the flowering season seeds produced in each marked calyx were counted. Since the maximal number of seeds per flower is invariably four, the proportion of fertilized CH and CL seeds (G_{CH} and G_{CL} respectively) per individual was estimated as the number of seeds counted in all marked CH or CL flowers divided by four times the number of marked CH or CL calices respectively. G_{CH} and G_{CL} are estimators of the pollination success in CH and CL flowers respectively.

Data analysis

Plasticity of traits across seasons - All plasticity analysis were performed with type III General Linear Mixed Models, (PROC GLM procedure, SAS). For each model, family (nested in population) was fitted as

random factor, while season, population and their interaction were fitted as fixed factors. Data were normalized by square-root-transformation of count data (N_{cal}, d_{CH}, d_{CL}), and arcsinus-square-root-transformation of proportion data (p_{CH}, p_{CLC}, p_{CHpl}, G_{CH}, G_{CL}). The significance of fixed effects was tested using F tests. Visual examination of the residuals showed that some models do not fulfill the heteroscedasticity requirements, but their conclusion did not seem to deviate significantly from the patterns observed in raw data.

Estimates of fitness in cleistogamous plants – The fitness of a hermaphrodite is the sum of its maternal and paternal contribution to the offspring (Morgan and Schoen, 1997): each individual passes on two copies per selfed progeny, one copy per outcrossed progeny and one copy through pollen export, in the absence of pollen discounting (Lloyd, 1979). The contribution of each of these portions to parental fitness is weighted by the relative fitness of the offspring types, which could be affected by various factors such as inbreeding depression in selfed progeny (Lloyd, 1979), or, in cleistogamous species, flower type from which the progeny is issued (see Oakley et al., 2007). We then estimate individual fitness of a cleistogamous plant as twice the contribution of CL progeny and selfed CH progeny, to which is added the contribution of successfully pollinated outcrossed ovules and successful pollen export competing with the outcross pollen in the population (see Lloyd, 1979):

Parent fitness	CL seeds	CH seeds		Pollen export
		Outcrossed	Selfed	
$w =$	$N_{CL}G_{CL}2w_{CL} +$	$N_{CH}G_{CH}(tw_{CHO} + 2(1-t)w_{CHS}) +$		$N_{CH}G_{CH}\frac{Total_{CH}\overline{G_{CH}}tw_{CH}}{Total_{CH}\overline{G_{CH}}}$

with w_{CL}, w_{CHO}, w_{CHS} as viability of CL progeny, outcrossed CH progeny and selfed CH progeny; t as the outcrossing rate of CH flowers, $\overline{G_{CH}}$ as the average pollination success and $Total_{CH}$ as the total number of flowers available for pollination in the population. Note that because of the complete pollen discounting in CL flowers, the quantity of pollen exported is directly proportional to the number of CH flowers on the individual. This equation simplifies to:

$$w = N_{CL} G_{CL} 2w_{CL} + N_{CH} G_{CH} 2(tw_{CHO} + (1-t)w_{CHS})$$……Eq[1]

As a consequence, and contrary to the classic single flower morph mating systems theory, the fitness of a cleistogamous individual does not depend on the mating system traits in the population, i.e. frequency dependence is cancelled in cleistogamous populations. Thus, individual fitness (through male and female fitness components) can directly be estimated by counting seeds produced by each flower type on an individual plant.

Estimates of the selection coefficients within seasons – We tested directional and stabilizing selection effects in spring and in autumn on morphology (number of calices, N_{cal}), phenology (date of first CL and first CH flower, d_{CL} and d_{CH} respectively), and cleistogamy-related traits (global CH proportion, p_{CH}, constitutive cleistogamy proportion, p_{CLC}, and induced CH proportion, p_{CHpl}). To cope with zero-inflated distributions of the proportion variables (p_{CH}, p_{CLC}, and p_{CHpl}), we calculated means per family for all traits (morphology and phenology included). Mean fitness per family was first estimated assuming no viability differences between CL and CH progeny (i.e. overall seed production). Since the number of ovules per flower in *L. amplexicaule* is invariably four, overall seed production for each individual was estimated as: $G = G_{CL} * (N_{cal} - N_{CH}) * 4 + G_{CH} * N_{CH} * 4$. For the purpose of statistical analysis, G was divided by its mean value in the sample studied, and for each trait a Generalized Quadratic Model was fitted using the lm function in software R 2.15.1 as follows: G = Population * (trait + trait^2). Significance levels of each variable were assessed using the *drop1* function (type II model testing), and whenever possible, the model was simplified by dropping out the non significant interaction terms. The regression coefficients for the linear and quadratic terms of the GQL correspond to directional (linear coefficients) and stabilizing (negative quadratic coefficients) selection in each season.

Testing the adaptive character of global CH proportion plasticity - In order to test whether plastic CH proportion is adaptive, we analyzed the relationship between individual fitness and global CH

proportion. If plasticity of p_{CH} is adaptive, we expect the fitness associated with mean p_{CH} value expressed in each season to be close to the maximal fitness value predicted by the selection curve. We estimated optimal global CH proportion, p_{CH}*, in spring and in autumn as the x-value that corresponds to the maximum fitness value estimated by the GQM of fitness on p_{CH} described in the previous section. In order to facilitate comparison between observed values and optimal estimates, the data were not transformed for this analysis. Since fitness maximum is *a priori* sensitive to viability of each progeny type (w_{CL}, w_{CHO} and w_{CHS}, see equation [1]) which was not estimated in this study, we built different plausible scenarios to test for the robustness of our predictions to survival of the different progeny:

(1) General scenario, with no difference in survival between the three seed types ($w_{CL} = w_{CHO} = w_{CHS} = 1$). The fitness calculated this way corresponds to the overall seed production, *G*.

(2) Inbreeding depression scenario in which inbred progeny, regardless the type of flower they are issued from, have lower survival rates than outbred progeny ($w_{CL} = w_{CHS} = 0.6$ or 0.8, $w_{CHO} = 1$)

(3) Outbreeding depression scenario, with inbred progeny having a higher survival rate than outbred progeny ($w_{CL} = w_{CHS} = 1.2$, $w_{CHO} = 1$)

(4) Flower type scenario, with higher survival rates for CL progeny ($w_{CL} = 1$, $w_{CHO} = w_{CHS} = 0.5$) or lower survival rates for CL progeny ($w_{CL} = 0.5$, $w_{CHO} = w_{CHS} = 1$).

We used the CH outcrossing rate estimated for these populations in a previous study i.e. $t = 0.25$ (Stojanova et al, unpublished data).

Table P. 1. Analysis of the between season variation of morphology (total number of flowers, N_{cal}), phenology (date of the first CL and first CH flower: d_{CL} and d_{CH} respectively), cleistogamy (CH proportion, CL by constitution and induced CH proportion: p_{CH}, p_{CLC} and p_{CHpl} respectively) and pollination related traits (CL and CH seed set respectively: G_{CL} and G_{CH}). Explanatory variables in italics are declared as random. Analyzed variables were transformed (squared root transformation for count data, asin-squared root transformation for proportion data) in order to follow a normal distribution. Results are based on type III sum of squares.

	N_{cal}			d_{CL}			d_{CH}			p_{CH}			p_{CLC}			p_{CHpl}			G_{CL}			G_{CH}		
Variable	**DF**	**F**	**Pr > F**	**DF**	**F**	**Pr > F**	**DF**	**F**	**Pr > F**	**DF**	**F**	**Pr > F**	**DF**	**F**	**Pr > F**	**DF**	**F**	**Pr > F**	**DF**	**F**	**Pr > F**	**DF**	**F**	**Pr > F**
Season	1	808	**<0,001**	1	35,15	**<0,001**	1	15,56	**<0,001**	1	272,5	**<0,001**	1	191,7	**<0,001**	1	24,84	**<0,001**	1	0,59	0,444	1	6,76	**0,01**
Pop	3	66,95	**<0,001**	3	26,54	**<0,001**	3	38,39	**<0,001**	3	2,79	0,051	3	2,2	0,099	3	2,78	**0,046**	3	14,24	**<0,001**	3	5,28	**0,002**
Family $_{Pop}$	46	2,81	**<0,001**	46	3,78	**<0,001**	45	1,19	0,205	46	2,06	**<0,001**	46	1,54	**0,018**	45	1,03	0,433	46	1,4	0,051	45	1,77	**0,004**
Season x Pop	3	10,94	**<0,001**	3	2,13	0,097	3	1,46	0,226	3	2,61	0,052	3	2,09	0,101	3	0,98	0,405	3	10,19	**<0,001**	3	16,88	**<0,001**

Results

Traits variation across seasons

Total number of flowers – Season has significant effect on the total production of flowers on the principal axis (Table P.1), with more than a twofold N_{cal} increase in spring compared to autumn (mean ± standard error for populations ML, MS, DL and DS respectively in autumn: 19.49 ± 1.41; 6.43 ± 0.60; 21.39 ± 1.08 and 19.07±1.20; and in spring: 48.68 ± 1.62; 17.05 ± 0.93; 50.12 ± 1.28 and 52.55 ± 1.71). Population MS produces significantly less flowers and is less plastic than the other three populations – excluding it from the analysis renders the Population and interaction terms non significant (data not shown).

Flowering dates – CL flowering of the plants begins earlier in spring than in autumn (Figure P.1.A, Table P.1). Population MS begins flowering with almost a one-month delay in both seasons compared to the other three populations which are rather synchronized (Figure P.1.A). The interaction term is not significant. The inverse seasonal pattern is observed for CH flowering – all populations produce CH flowers significantly later in spring than in autumn (Figure P.1.B, Table P.1). Population MS has a significant delay of 10-15 days compared to the other three populations.

Comparing d_{CL} and d_{CH} values for each population shows that in autumn, CH flowering starts before CL flowering for population MS and almost at the same time as CL flowering for population ML. Though this may be true on the population level, individual plants always produce CL flowers first (constitutive cleistogamy) followed by the production of CH and CL flowers in variable proportions. The results observed here could be explained by the delayed flowering onset of plants that produce only CL flowers, which could lead to a higher estimate of d_{CL} in autumn. Excluding plants that produced only CL flowers from the mean estimates does not affect noticeably d_{CH} in both seasons, nor that of d_{CL} in spring. However, d_{CL} values in autumn are considerably lower for plants that produced both CL and CH flowers

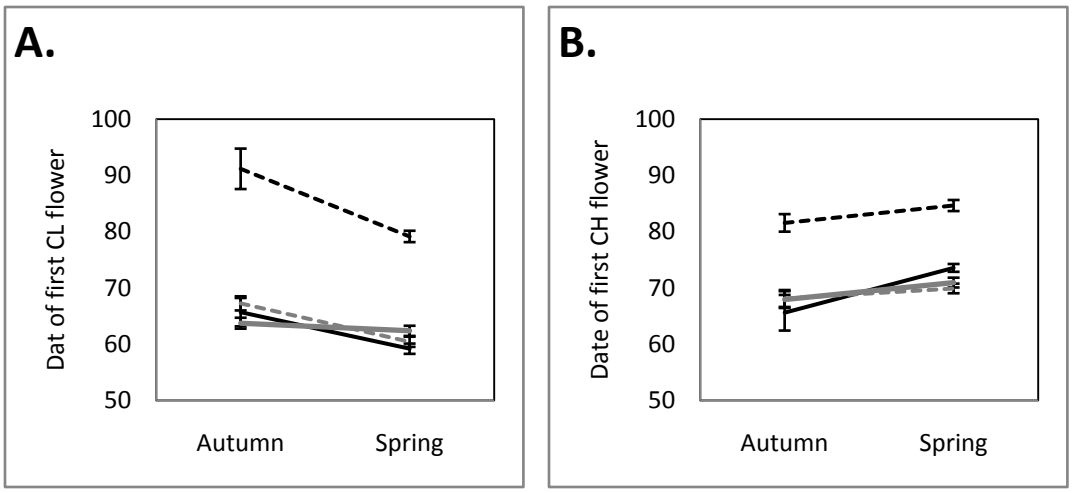

Figure P. 1. Average flowering onset for CL (A) and CH flowers (B). Black lines: Montpellier populations, gray lines: Dijon populations. Dotted lines: small populations (from unfavorable habitats), full lines: large populations (from favorable habitats). Vertical bars present standard error estimates.

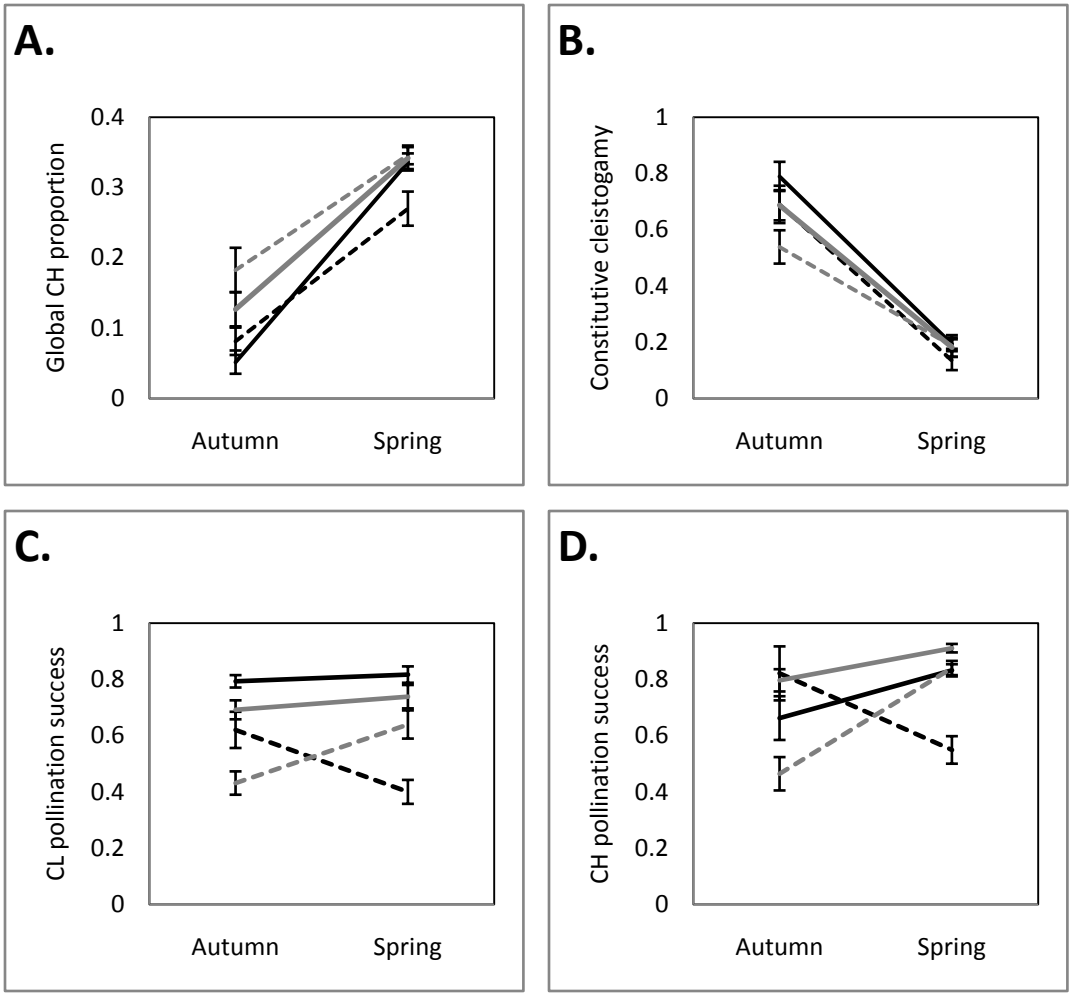

Figure P. 2. Cleistogamy and pollination success average values in four French populations. A. global CH proportion, B. constitutive cleistogamy proportion, C. pollination success of CL flowers, and D. pollination success of CH flowers. Black lines: Montpellier populations, gray lines: Dijon populations. Dotted lines: small populations (from unfavorable habitats), full lines: large populations (from favorable habitats). Vertical bars present standard error estimates.

(mean ± standard deviation: 53.33 ± 4.90, 73.89 ± 6.15, 59.59 ± 3.73, and 62.20 ± 4.26 for populations ML, MS, DL and DS respectively, compare with Figure P.1.A). This shows that on individual level CL flowering occurs earlier than CH flowering, and that plants which do not produce CH flowers, almost all of which were in autumn, tend to start flowering later than plants that produce CL and CH flowers.

Open flowers proportion – Overall CH rate (p_{CH}) is significantly higher in spring that in autumn for all four populations (Figure P.2.A, Table P.1). Neither Population, nor Season x Population have significant effect on p_{CH}. Family is highly significant, because several families produced no CH flowers in autumn. Constitutive cleistogamy (p_{CLC}) is significantly lower in spring than in autumn (Figure P.2.B, Table P.1). The Population and the interaction term have no significant effect on constitutive cleistogamy proportion, but Family is significant, again because of the high variation of p_{CLC} in certain families in autumn (data not shown). Induced CH proportion (p_{CHp}) could be calculated only for individuals that produced at least one CH flower. Therefore, there are fewer data for the analysis of this trait, especially in autumn, compared to other traits presented in this study as shown by the standard error estimates (mean ± standard error for populations ML, MS, DL and DS respectively in autumn: 0.249 ± 0.05, 0.259 ±0.04, 0.312 ± 0.04 and 0.366 ± 0.05 and in spring: 0.409 ± 0.01, 0.303 ± 0.02, 0.401 ± 0.02 and 0.395 ± 0.01). p_{CHp} is less variable across seasons than the other two cleistogamy-related traits, but significant increase of p_{CHp} in spring compared to autumn is observed (Table P.1). Population is marginally significant, and family and the interaction term are not significant.

Seed production – Significant Population x Season interaction is detected for the probability of a CL ovule to develop into seed (CL pollination success, Figure P.2.C, Table P.1). This is due to the fact that variation pattern of G_{CL} in population MS is differs from the other three populations. Mean CL pollination success is higher in autumn than in spring for population MS, while it varies little across seasons for the other three populations (Figure P.2.C). Population has a significant effect, with plants from small populations

producing less seed than plants from large populations. All explanatory variables are significant for CH pollination success estimates (Table P.1, Figure P.2.D). The interaction season x population is highly significant probably because of population MS, which is the only one to produce less seeds in spring than in autumn.

The overall seed production, *G*, which corresponds to individual fitness estimate according to the general scenario, is two to five times higher in spring than in autumn (mean ± sd overall seed production for populations ML, MS, DL and DS in autumn: 61.41 ± 28.07, 18.86 ± 17.56, 61.45 ± 25.83, 31.65 ± 18.63; and in spring: 169.40 ± 59.54, 30.87 ± 20.34, 159.89 ± 49.11, 152.75 ± 65.34).

Selection on plasticity

Positive directional selection acts on total number of flowers in both seasons and in all populations, as shown by the significant linear selection coefficients in Table P.2. This result is not surprising, since increasing the total number of flowers or the number of flower bearing axes also increases the number of seeds produced in a plant.

Linear selection coefficients for CL flowering date are negative and significant in both seasons, meaning that early flowering should increase individual fitness. We also observe significant positive quadratic selection coefficient for this trait that could indicate disruptive selection on the flowering date (plants that flower early or late are favored, but not plants with intermediate flowering date). This result is due to two observations (one family in population ML and one in population MS) that have relatively high fitness in spite of their late flowering. Excluding these two points from our analysis renders the quadratic term of the regression non significant (data not shown). Selection does not seem to affect the beginning of CH flowering in autumn, since neither linear, nor quadratic selection coefficients are significant. In spring, however, our analysis shows that there is a significant quadratic selection coefficient which is positive in population DS and negative in the other three populations. Again,

Table P. 2. Table P. 3. Selection coefficients for morphology (total number of flowers, N_{cal}), phenology (date of the first CL and first CH flower: d_{CL} and d_{CH} respectively), cleistogamy (CH proportion, CL by constitution and induced CH proportion: p_{CH}, p_{CLC} and p_{CHpl} respectively) related traits. β_1: linear coefficient (directional selection), β_2: quadratic coefficient (stabilizing selection). Bold numbers represent statistically significant selection coefficients (p-value<0.05).

	Autumn								Spring							
	ML		MS		DL		DS		ML		MS		DL		DS	
	β_1	β_2	β_1	β_2	β_1	β_2	β_1	β_2	β_1	β_2	β_1	β_2	β_1	β_2	β_1	β_2
N_{cal}	**0,409**	-0,078	**0,409**	-0,078	**0,409**	-0,078	**0,409**	-0,078	**0,572**	0,019	**0,572**	0,019	**0,572**	0,019	**0,572**	0,019
p_{CH}	**0,222**	**-0,101**	**0,222**	**-0,101**	**0,222**	**-0,101**	**0,222**	**-0,101**	0,702	**-0,812**	-0,057	-0,061	0,000	**-0,527**	0,037	**-0,688**
p_{CLC}	**-0,198**	**-0,123**	**-0,198**	**-0,123**	**-0,198**	**-0,123**	**-0,198**	**-0,123**	**0,110**	**-0,085**	**0,110**	**-0,085**	**0,110**	**-0,085**	**0,110**	**-0,085**
p_{CHpl}	0,050	-0,030	0,050	-0,030	0,050	-0,030	0,050	-0,030	1,144	**-0,864**	-0,096	**-0,056**	-0,024	**0,150**	-0,049	**-0,709**
d_{CL}	**-0,489**	**0,103**	**-0,489**	**0,103**	**-0,489**	**0,103**	**-0,489**	**0,103**	**-0,392**	0,074	**-0,392**	0,074	**-0,392**	0,074	**-0,392**	0,074
d_{CH}	-0,144	-0,041	-0,144	-0,041	-0,144	-0,041	-0,144	-0,041	0,644	**-0,496**	0,039	**-0,012**	0,099	**-0,213**	-1,712	**1,247**

removing two aberrant points – one in population DS and one in DL – renders the quadratic selection coefficient non significant for all populations (data not shown).

Considering the plasticity of cleistogamy-related traits, patterns of stabilizing selection (significant negative quadratic regression coefficients) are observed for global CH proportion and constitutive cleistogamy in both seasons, as well as for induced CH proportion in spring. Optimal p_{CH} proportion (p_{CH}*) is lower in autumn than in spring in all populations (Table P.3, Figure P.3). The stabilizing selection coefficient on p_{CH} is not significant for population MS in spring. Inversely, optimal p_{CLC} rates are higher in autumn (estimated p_{CLC}* in autumn: 0.47 for all populations) than in spring (estimated p_{CLC}* in spring: 0.34 for all four populations). Stabilizing selection coefficients are not significant for induced CH proportion (p_{CHpl}) in autumn, probably because of the lower size of the analyzed sample. Plants that produced only CL flowers, which are mainly in autumn, are not taken into account for the calculations of p_{CHpl}, thus excluding entire families from the analysis of p_{CHpl} in autumn and lowering the power of the statistical analysis. Nevertheless, optimal induced CH proportion is lower in autumn (p_{CHpl}* estimates in autumn: 0.33 for all populations) than in spring (p_{CHpl}* estimates in spring: 0.54, 0.42, 0.38, 0.43 for populations ML, MS, DL and DS respectively).

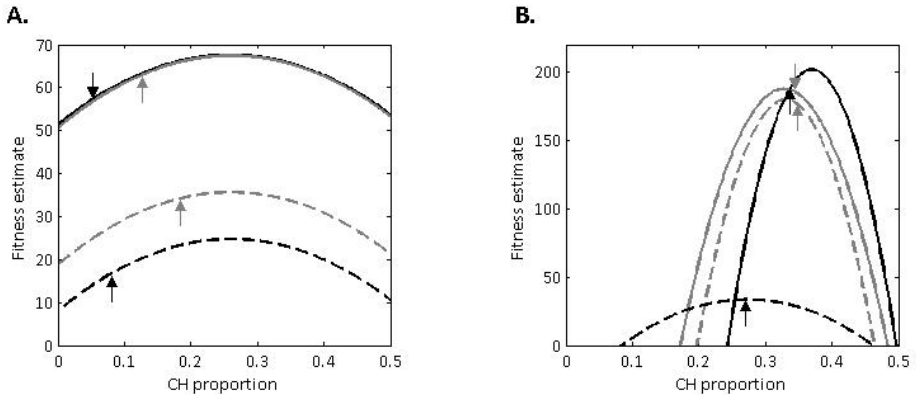

Figure P. 3. Selection gradients on global CH proportion in A. autumn and B. spring. Red: population ML, orange: population MS, blue: population DL, green: population DS. Curves were traced using the linear and quadratic selection coefficients estimated by the general quadratic regressions of non transformed fitness on non transformed trait values. Arrows indicate the average CH proportion observed for each population in the given season.

Adaptive character of CH proportion plasticity

Optimal CH proportion estimates (p_{CH}*) for each population are higher in spring than in autumn in all progeny fitness scenarios, though the difference between seasons in population MS is very small (Table P.3, Figure P.4). Optimal CH proportion estimates (p_{CH}*) do not vary noticeably in different progeny viability scenarios. In the flower type scenarios in autumn, lower survival of CL progeny resulted with more noticeable increase of p_{CH}*compared to the general scenario, meaning that plants need to increase the production of CH flowers in order to compensate for the important loss of CL progeny. Inversely, higher survival of CL progeny relative to CH progeny resulted in equivalent decrease of p_{CH}*. Overall, average p_{CH} values observed in each population resembled closely the estimated optimal values within their corresponding seasons. Observed means in autumn were slightly lower than the optimal estimates, and this difference was more pronounced for Montpellier populations.

Discussion

In this paper we study the plasticity and the selection on different phenological, morphological and cleistogamy traits in four natural populations coming from contrasted geographical regions and habitats in a common garden in spring and in autumn. We show that season has significant effect on all traits, except pollination success of CL flowers that does not vary across seasons for populations ML, DL and MS. We also observe significant among populations differences for plant size, flowering dates and pollination success. Most of the traits studied here are under selection – positive directional selection for plant size, negative directional selection for the CL flowering date and stabilizing selection for global CH proportion and constitutive cleistogamy proportion. The optimal CH proportion values within a season are lower in autumn than in spring, and the observed CH proportion means per population in each season are closer to the optimal value of the corresponding season than to the optimal value of the opposite season.

Plastic variation of traits across seasons and population differentiation

Plants in spring produce more flowers and their overall seed production is higher than in autumn in all four populations. Flowering date after germination is significantly delayed in autumn compared to spring. This is consistent with the fact that short lived species can invest more in their vegetative stage without compromising their reproductive success in environments favorable to their growth (Chapin et al., 1993). Also, plants tend to produce CH flowers earlier than CL flowers in autumn than in spring on the population level, because the only CH flowers producing plants in autumn start flowering earlier than the average flowering date in the population. A remarkable proportion of the plants in autumn do not produce CH flowers at all. This result fits the general pattern seen in other cleistogamous species in which favorable environments enhance the production of CH flowers (Schemske, 1978). Plants that produce only CL flowers in autumn start flowering later than the average flowering date. The lack of CH flowers in autumn could be either due to the fact that late flowering plants are very small and thus unable to invest resources in showy, CH flowers, or because the environmental conditions become inappropriate for the production of CH flowers in late autumn (e.g. photoperiod is too short). Indeed, CH proportion in *L. amplexicaule* could be less than 1%, if photoperiod is shorter than 10h and day – night temperatures are 21 - 10°C (Lord, 1982). All these observations (smaller size and lower seed production, delayed CL flowering and absence of CH flowers for some individuals in autumn) suggest that environmental conditions during spring are more favorable to the development of *L. amplexicaule,* thus increasing the production of chasmogamous flowers in this season.

Interestingly, morphological and phenological characteristics (late flowering) of population MS differ from the other three populations. This pattern could be explained by a particular phenology encountered in this population. Indeed, it has been shown that certain populations of *L. amplexicaule* (USDA-NRCS, 2002) germinate in late autumn, pass the winter in a vegetative stage and then flower in spring simultaneously with the spring germinating populations. Observations made on the site of

population MS in November 2012 revealed plants in vegetative stage. The delay of CL and CH flowering in population MS could therefore be a constraint of our experimental design in which seeds of population MS have germinated in late winter of late autumn. Another consequence of this phenology delay is that flowering occurred during unfavorable environmental conditions (higher temperatures) in late May compared to natural conditions, which could have affected late vegetative growth and floral development. The phenological delay may explain why pollination efficiency of CL and CH flowers is lower in population MS in spring compared to the other three populations, and why this population does not follow the same seasonal variation pattern (i.e. no variation for the CL pollination success, and higher pollination success in spring for CH flowers). Unfavorable environmental conditions may also explain the smaller plant size in population MS. However, the particular plant architecture of plants in population MS (more lateral axes, less whorls per axis with smaller internode lengths), and the reduced size of plants could also be due to population differentiation. In line with this, microsatellite estimates of the genetic structure of these four populations showed that the number of private alleles was the highest in population MS (Stojanova et al., 2013b), supporting the population differentiation hypothesis.

In spite of the morphological and phenological differences between the populations studied, or whether they have experienced one or two seasons in their natural habitat, the plasticity pattern of cleistogamy-related traits across seasons does not differ between populations – global CH proportion is around 0.3 in spring and around 0.1 in autumn. The increase of CH production in conditions that favor plant growth is commonly observed for annual cleistogamous species (reviewed in Oakley et al., 2007). Moreover, in several other studies of cleistogamy plasticity patterns similar to ours were observed – CH proportion variation depends exclusively on the environmental conditions, with no population differentiation for this trait, though morphological or phenological differences can be observed across populations. For instance, CH proportion did not change for three different populations of *Impatiens noli-tangere* grown under two different light intensity conditions though population differentiation was observed for

flowering time (Masuda et al., 2004). In reciprocal transplant experiments of individuals coming from one *Calathea micans* population, CH production was influenced by light intensity and nutrient availability, but it was not influenced by plant size (Lecorff, 1993). Light intensity is a reliable environmental cue which indicates habitat quality – in general, low light intensity indicates high vegetation density, which could in turn reflect a set of ecological conditions such as increased interspecific competition levels (Donohue et al., 2000). In our study, environmental variation corresponds to seasonal changes which are generally easy to predict because they are associated with a set of cues that always change in the same manner, such as photoperiod that is increasing in spring and decreasing in autumn. The fine tuning of cleistogamy related traits in response to predictable environmental cues suggests an adaptation to environmental variation, provided that each phenotype is advantaged in the environment that induces its production (Via and Lande, 1985).

Selection on plasticity

According to our estimates of selection coefficients, larger plants (with more flowers) and early CL flowering are favored in spring and in autumn. This result is not surprising – larger plants produce more flowers and seeds (higher fitness) and selection for early flowering is common in short lived species (Munguia-Rosas et al., 2011). Interestingly, cleistogamy related traits (CH proportion and constitutive cleistogamy proportion) were under stabilizing selection for all populations in autumn and for three out of four populations in spring. The optimal values (i.e. phenotype that maximize individual fitness) differed among seasons, with optimal CH proportion being lower in autumn than in spring. Thus producing higher CL proportions is adaptive in autumn, and producing higher CH proportions is adaptive in spring, as suggested by Lord (1982). Average CH proportions in each season were close to the optimal estimates for these traits, confirming the adaptive character of plastic cleistogamy to seasonal variation in the four *L. amplexicaule* populations studied here. Admittedly, for plasticity to be adaptive the costs of maintaining a plastic genotype need to be lower than the benefits provided by plasticity (DeWitt et

al., 1998). We were not able to estimate the costs of plasticity in our study because of the small sample sizes, but the fact that plasticity is maintained in populations that do not normally experience seasonal variation (i.e. MS, ML and DS) suggests that maintaining plasticity on cleistogamy is not too costly (DeWitt et al., 1998). However, it is worth noticing that there is more pronounced divergence between the observed average CH proportion and the optimal estimates for Montpellier populations in autumn compared to the Dijon populations in autumn or any population in spring. This result is consistent with the fact that populations from southern France experience only the spring season: though they have maintained plasticity for cleistogamy, their response to autumn conditions is not fully adaptive. Population DS, which also has one flowering season, maintains a more appropriate average CH proportion in autumn probably because of the low population differentiation on the regional scale. The absence of stabilizing selection in spring for population MS is probably due to the highly unfavorable environmental conditions experienced by this population in late spring which resulted in substantial decrease of seed production for this population.

Adaptive scenario for the maintenance of plastic cleistogamy

Our results show that plasticity of cleistogamy is an adaptation to seasonal variation in *L. amplexicaule* and thus each flower type needs to have some selective advantage in the environment that favors its production compared to the non induced flower type. One possible explanation for the adaptive character of plastic cleistogamy is the differential cost production of CH and CL flowers. Since plants are bigger in spring than in autumn, they may exploit more resources to produce CH flowers, which are supposedly more costly (Schemske, 1978). If this is the case, smaller plants should produce less CH flowers. Though this is true for the variation of CH proportion across seasons, within a given season each population maintains the same CH proportion regardless of the plant size, suggesting that plastic cleistogamy of *L. amplexicaule* is not entirely an adaptation to the differential costs of the two flower types. Our results suggest on the contrary that CH flowers are not too costly (see above). Besides, our

results suggest that CH variation could be a response to variable pollination environments. The pollination success of CL flowers is globally independent of seasonal variation and does not vary in three out of four populations studied. Being autonomous for their pollination, CL flowers could provide reproductive assurance in environments where pollinators are scarce (Waller, 1980, Schoen and Lloyd, 1984). On the other hand, CH pollination success is higher in spring than in autumn for three out of four populations studied, which is consistent with the prediction made by Lord – CH flowers are more successfully pollinated in spring, when pollinators are more abundant (Lord, 1982). Thus regulating the CH proportion across seasons results with a more efficient pollination strategy compared to that encountered in single morph flower species.

By measuring the plasticity on CH proportion and the selection acting on this trait in different seasons, we show that plastic cleistogamy is an adaptation to seasonal variation in *L. amplexicaule*. The adaptive character of plasticity is maintained even for populations that do not experience seasonal variation. Our findings about the pollination success of CL flowers (which is maintained stable across seasons) and that of CH flowers (which is higher in spring than in autumn) are consistent with the idea that plastic cleistogamy is an adaptation to the variable pollination environments across seasons.

CHAPTER 4. Can environment dependent inbreeding depression account for the maintenance of cleistogamy in variable environments? An experimental study.

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Introduction

Inbreeding depression is a central force shaping mating systems evolution. Indeed, most of the theory for the evolution of mating systems includes the deleterious effects of inbreeding (reviewed in Goodwillie et al., 2005). In its basic formulation, inbreeding depression opposes to the automatic advantage of selfing (cost of outcrossing, Fisher, 1941) by reducing the survival of selfed progeny and leads to complete outcrossing (or respectively complete selfing) if the fitness of inbred progeny is lower (respectively higher) than half the fitness of outbred progeny (Lande and Schemske, 1985). In addition, high magnitudes of inbreeding depression also reduce the benefits of reproductive assurance conferred by selfing (Herlihy and Eckert, 2002, Goodwillie and Knight, 2006, Schoen and Brown, 1991a).

In evolutionary models, inbreeding depression is assumed either constant (Lloyd, 1979) or evolving through time via the purging of recessive lethal or semi lethal alleles (Lande and Schemske, 1985). However, recent accumulation of empirical data suggests that the magnitude of inbreeding depression can vary with environmental conditions (Armbruster and Reed, 2005). The account of environmental dependence of inbreeding depression in more recent theory of mating systems showed that mixed mating could be a stable reproductive strategy under certain specific conditions. For instance, Cheptou and Mathias (2001) used an adaptive dynamics approach to study the evolution of mixed mating of an annual species that can experience two environments with inbreeding depression varying either in space or in time, or both. They showed that temporal and spatio-temporal variation in inbreeding depression can maintain stable mixed mating systems by increasing the (geometric) average fitness in time of a given phenotype, compared to the fitness of a pure outcrosser or a pure selfer that experiences the same environmental conditions.

Though evidence for the evolutionary importance of the dependency of inbreeding depression with environment have accumulated in the past few years, most studies including variable and evolving inbreeding depression were based on experimental observations which are not fully representative of

the environmental conditions encountered in natural populations. In experimental studies the environmental variation is simulated by experimental conditions often assimilated to stressful versus benign environments (Armbruster and Reed, 2005, Fox and Reed, 2011), which do not reliably represent the actual environmental variation in natural populations. Moreover, most of the experimental designs focus on one or only a few factors, and can probably overlook the importance of other factors or interactions between factors in natural conditions. Thus, the estimates of inbreeding depression in controlled conditions are useful to analyze its variation across environments, but the ecological relevance of these estimates remains unclear (Fox and Reed, 2011, Enders and Nunney, 2012). Some species, however, experience regular and predictable environmental changes and thus can be easily studied in semi-natural conditions. For instance, seasonal variation is a periodical change of a set of parameters that are easy to predict, since often associated with reliable cues such as photoperiod, temperatures and rainfall. The precise factors that would influence individual fitness cannot be distinguished in a semi-natural study including variation across seasons, but the estimates of inbreeding depression in such conditions should provide ecologically meaningful information. Controlled condition studies can further be performed to identify the exact environmental factors that determine individual fitness in a given environment (Enders and Nunney, 2012).

Cleistogamy is a particular type of mixed mating in which two floral morphs are encountered within the same individual or the same population – closed flowers that are obligately selfed (cleistogamous, CL) and open flowers that can potentially outcross (chasmogamous, CH). Therefore cleistogamy is considered as a particular type of mixed mating. In many cleistogamous species, CH proportion can vary with predictable environmental conditions, such as photoperiod, light intensity, temperature, nutrient and water availability or interspecific competition levels, or even different combinations of those (Lord, 1982, Bell and Quinn, 1987, Schemske, 1978, Schmitt et al., 1987, Cortes-Palomec and Ballard, 2006). These environmental cues are easily predictable because their variation is regular (photoperiod) or

almost regular (light intensity, temperature) thus providing direct information about the environmental impact on the mating system, or because their effect on plant vegetative development indirectly indicates the quality of the reproductive environment (nutrient and water availability). In both cases plants can anticipate and adjust their phenology (e.g. flowering). Moreover, observations in natural populations show that the production of CL and CH flowers often varies across seasons (Winn and Moriuchi, 2009, Masuda et al., 2004, Cortes-Palomec and Ballard, 2006). Classical explanations for the maintenance of cleistogamy often focus on the morphological and functional differences of the two floral types, such as the differences in production costs of CL and CH flowers (Schemske, 1978), differences in the pollination reliability and success (Lord, 1982, Culley, 2002), or different (non genetic) properties of the two seed types (CL- and CH-issued seeds) – seed size (Trapp and Hendrix, 1988), or different dispersal strategies (Berg and Redbo-Torstensson, 1998). However, since each floral type is devoted to a different mating type, plastic cleistogamy is potentially an adaptive adjustment of the mating system in response to environmentally dependent inbreeding depression (Winn and Moriuchi, 2009, Winn et al., 2011). Yet only a few studies measured inbreeding depression in cleistogamous species (Culley, 2000, Oakley and Winn, 2008, Trapp and Hendrix, 1988), one of which measured the performance of CH outcrossed versus CH selfed progeny in two different environments (Oakley and Winn, 2008). The hypothesis of plastic cleistogamy as an adaptation to environmentally dependent inbreeding depression has never been tested before. In this context, outcrossed progeny of CH flowers should be favored in environments with high inbreeding depression, while selfed progeny of CL flowers should be favored when inbreeding depression is low. Since pollen discounting is complete in CL flowers, CL progeny do not benefit the automatic advantage of selfing (Holsinger, 1991). As a consequence, in absence of pollen limitation, CH flowers should be advantageous whenever inbreeding depression is positive. However, the hypothesis of adaptation to environmentally dependent inbreeding depression does not exclude the effects of floral type differences that may influence progeny fitness.

Lamium amplexicaule L. is a cleistogamous annual species from the mint family. This species has a winter or a summer annual cycle (Baskin and Baskin, 1981). Winter annuals germinate at the end of the winter, flower at the beginning of spring until mid spring and their seeds are dormant throughout the rest of the year. Summer annuals cycle is less defined, but plants usually start germinating in late summer to early autumn, when water levels are sufficient for seed germination, then flower until mid to late autumn, depending on their germination date. Thus in temperate climates, *L. amplexicaule* populations are exposed to temporal variation in their environment. CH proportion in *L. amplexicaule* is influenced by environmental cues that are associated with seasonal variation such as photoperiod and temperature with long, warm days (i.e. spring) enhancing CH production, and short, cold days (i.e. autumn) enhancing CL production (Lord, 1982). Though the physiological adjustment of the CH proportion has been well studied for this species (Lord, 1979b, Lord, 1982, Lord and Mayers, 1982) as well as the lineage differentiation for "CH rich" and "CH poor" genotypes (Correns, 1930), little is known about the CH proportion and fitness variation of this species across seasons (but see Lord, 1979b). The goal of this study was to assess seasonal fitness variation in *L. amplexicaule*. We estimated surrogates of fitness in spring and in autumn for three types of control-pollinated progeny of *L. amplexicaule* – outcrossing of CH flowers, selfing of CH flowers and selfing of CL flowers – in both seasons. We measured CH proportion, fitness related traits (total number of flowers and seeds) and phenology traits (flowering dates). Our goal was also to determine whether seasonal variation of CH proportion is consistent with an adaptive response of *L. amplexicaule* mating system to environmentally dependent inbreeding depression if evidence for the latter were found.

Materials and methods

Controlled crosses

In the autumn 2009, plants bearing mature seeds were collected from a *L. amplexicaule* population in Dijon. This population is large, growing in a favorable habitat (Stojanova et al. submitted), and it has two generations per year. A seed mixture obtained from this sample containing seeds of CL and CH flowers of several plants was sown in a common tray containing equal quantities of soil, sand and loam. After emergence, 60 seedlings were randomly chosen from the common tray and transplanted in 10x10x10 *cm* individual pots that were kept in an isolated greenhouse. From the 5th to the 19th of February 2010, when the majority of the plants were at their CH flowering peak, 20 plants were randomly chosen for performing controlled pollination. CH flowers on these plants were hand pollinated with self or outcross pollen and several CL flowers whose seeds are obligately selfed were also marked. To avoid uncontrolled self-pollination, CH flowers used for the hand pollinations were emasculated a day before their anthesis by removing with tweezers the upper labia and the four non dehisced anthers. The day after, when the stigma of the emasculated flowers was receptive, it was pollinated with self or outcross pollen. Hand pollinations were made by gently brushing receptive stigmas with mature anthers. We used mature anthers collected from other flowers of the same plant for the self-pollination treatment and mature anthers collected from CH flowers of another plant chosen randomly among the 60 transplanted individuals for the outcross pollination treatment. *L. amplexicaule*'s pollen is bright orange, thus it can be easily distinguished on the stigmas that are white, and thus ensuring successful hand pollination. To rule out any possibility of uncontrolled pollination during the controlled crosses and until all corollas fell out, emasculated CH flowers without additional pollination were used as negative controls. None of the control flowers produced seeds. The calyx base of the hand pollinated CH flowers (or the selected CL flowers) were marked with water-based paint, and after the corollas fell out, calices were closed using a drop of paint to contain the seeds until full maturity. Mature seeds were collected from the 20th of

February to the 10th of April. On each plant we had four to seven flowers of each cross type (CH outcrossed seed – CH_{out}, CH selfed seed – CH_{self}, and CL selfed seed – CL_{self}). The three cross types can be assigned to two different factors: inbreeding type (selfed – CH_{self} and CL_{self} against outcrossed: CH_{out}) and flower type (open for CH_{out} and CH_{self} against closed for CL_{self}). Mean seed set per flower was relatively high and similar among all crosses (mean ± se for CH_{out}, CH_{self} and CL_{self} respectively: 3.54 ± 0.11; 3.55 ± 0.10; 3.70 ± 0.08), but some mother plants produced as few as two or three seeds when including all flowers. Seeds were stored in 96 well plates in a dark place at room temperature until the beginning of the experiment. Each well corresponded to all seeds produced by a single flower. In May 2011, one random seed from each well was weighed.

Experimental design

Seeds obtained from the control crosses of each mother plant were randomly divided in two lots. Up to 12 seeds of each cross type per mother plant were germinated in spring and in autumn. Because *L. amplexicaule* seeds can be dormant (Baskin and Baskin, 1981), more seeds were sown in the autumn part of the experiment, which started four months after the seeds were collected. The number of seeds planted in autumn was Ceiling [(n/2) + 1], with n being the number of seeds obtained from a mother plant for a given cross type, and the remaining seeds were planted in spring. On the 19th of August 2010 (for the autumn part of the experiment) and the 4th of February 2011 (for the spring part of the experiment), seeds were sown in sterile Petri dishes with two pieces of regularly humidified Whatman paper. Petri dishes were placed in growth chambers with 12h light exposure at 25°C, and 12h in the dark at 13°C. Successfully germinated seeds were planted in 14x10 well plates. When the majority of seedlings produced at least four leaves, seedlings were transplanted in individual 10x10x10 *cm* pots containing a mixture of soil, sand and humus in equal proportions. The soil mixture used in this experiment was also separated in two lots and sterilized in each season before transplantation manipulations. Pots were placed outdoors, in CEFE experimental field, assembled in blocks of 16 to

facilitate manipulations. The position of pots within blocks as well as the position of the blocks on the tables was regularly and randomly changed. For some families, the germination rate of a particular cross type was zero, but overall the germination rate was high in both seasons, being systematically higher in autumn (mean ± se for CH_{out}, CH_{self} and CL_{self} respectively: 0.86 ± 0.055; 1 ± 0.0; 0.91 ± 0.030) than in spring (mean ± se for CH_{out}, CH_{self} and CL_{self} respectively: 0.80 ± 0.064; 0.87 ± 0.034; 0.76 ± 0.055). The mean number of plants used in the experiment (after some loss mainly due to transplanting) for CH_{out}, CH_{self} and CL_{self} was respectively (mean ± se): 5.35 ± 0.59; 6.4 ± 0.68; 7 ± 0.72 in autumn and 5.60 ± 0.58; 6.40 ± 0.34; 7.05 ± 0.54 in spring. Pots were watered *ad libitum*.

Data measurements

Phenology traits – After being placed outdoors, plants were checked for flowers every other day to be able to record the date of the first CL flower (d_{CL}) and the date of the first CH flower (d_{CH}) on the principal axis, which is the first axis to be produced and therefore the first to flower. For the purpose of statistical analysis, the data was encoded as the number of days from the beginning of the experiment in each season (19th of August and 4th of February in autumn and spring respectively).

Flower counts – After the first CH flower was observed, and for each season, plants were checked every four days for new CH flowers on the principal axis. Since CH flowers wilt in about four days, only recent, fresh flowers were recorded during each monitoring date, thus avoiding recounting the same flower. These data were used to calculate N_{CH} – the total number of CH flowers recorded during the flowering season. At the end of the flowering season, we counted the total number of calices on the principal axis to estimate N_{cal}, the total number of flowers produced. The total number of CL flowers on a plant, N_{CL}, was calculated as $N_{CL} = N_{cal} - N_{CH}$ and CH proportion, p_{CH}, as $p_{CH} = N_{CH}/N_{cal}$. In addition, to estimate the correlation between the measures at the level of the principal axis and the whole plant, one plant was randomly chosen in each family and each cross type, for which N_{CH} and N_{cal} were counted on all axes (thus enabling us to calculate total CH proportion and total number of flowers).

Morphological traits – At the end of the flowering season, when signs of advanced senescence were visible (i.e. no flowers produced for at least seven days, the majority of the plant's axes wilted), the height of the principal axis, the number of secondary axes, and the number of whorls were recorded for each plant. The aerial part of plants was then collected in paper bags and dried in a stove at 65°C for about 72 hours. Dried plants were then weighed to obtain estimates for their above ground dry mass, *M*. Dried mass was highly and significantly correlated with the number of calices produced on the principal axis (Spearman's rho = 0.74, p-value < 0.0001), thus analyses of plant size were made using the total number of calices on the principal axis.

Pollination success estimates – During the flowering season five to six CH and CL calices on the principal axis were marked on each plant using acrylic paint. After corollas fell out, the marked calices were closed with a small drop of acrylic paint. At the end of the flowering season the seeds produced in each marked calyx were counted. Since the maximal number of seeds per flower is invariably four, the proportions of CH and CL fertilized ovules (G_{CH} and G_{CL} respectively) for each individual was estimated as the number of seeds counted in all marked CH or CL flowers divided by four times the number of marked CH or CL calices respectively. G_{CH} and G_{CL} are estimators of the pollination success in CH and CL flowers respectively. Overall seed set, *G,* was estimated as $G = 4*G_{CL}*N_{CL} + 4*G_{CH}*N_{CH}$.

Statistical analysis

Seed weight variation from controlled crosses was analyzed using a general mixed model including the number of seeds per cross type as covariable, and mother plant, cross type and the interaction cross type x mother plant as random explanatory variables. Analysis was performed using the PROC GLM function in SAS software.

Traits measured on the principal axis (p_{CH}, N_{cal}, G_{CL}, G_{CH}, G, d_{CL} and d_{CH}) were analyzed by fitting a general mixed model of a given trait with season, cross type, family (mother plant) and all the second

and third degree terms as explanatory variables. Family and interaction terms containing family were considered as random explanatory variables, whereas season, cross type and season x cross type were considered as fixed factors. The proportion data (p_{CH}, g_{CL}, g_{CH}) were normalized by arcsin-square-root transformation (Zar, 1999), d_{CL} and d_{CH} were square-root transformed, while a visual examination of the distribution of N_{cal} and G showed that they follow a normal distribution. To assess the influence of the flower type and the inbreeding magnitude on each of the traits measured, similar general mixed models were fitted on the above traits, but cross type was changed for either the inbreeding type (with two levels – inbred or outbred) in the first model or for flower type (with two levels – open or closed) in the second model. The analyses of general mixed models were made in SAS software with the PROC GLM function. The goodness of fit of the models (ratio of the sum of squares imputed to the model and the total sum of squares) was calculated for each of the three general mixed models (cross type, flower type and inbreeding degree) to compare the different explanatory variables between them.

Results

Seed weight is slightly lower for selfed progeny of CH flowers, but the cross type variable is not significant (mean ± sd in *mg* for CH_{out}, CH_{self} and CL_{self} respectively: 0.78 ± 0.10; 0.74 ± 0.10; 0.80 ± 0.12, Table D.1). The only significant factor explaining the variation of seed weight is the number of seeds per calyx but its regression coefficient was very low (-0.02, Table D.1).

Table D. 1. Analysis of the mean weight of seeds produced from hand controlled pollinations. Cross holds for the three types of hand controlled pollination (CH_{out}, CH_{self}, CL_{self}). Explanatory variables in italics are declared as random; results are based on the type III sums of squares.

Source	DF	F Value	p-value
Cross	2	2.61	0.0772
Family	19	0.91	0.5742
Number	1	8.72	**0.0037**
Cross x Family	38	1.17	0.2566

Table D. 2. Analysis of plasticity across seasons of CH proportion, number of calices, CL and CH pollination success, total seed set and onset of CL and CH flowering. Cross holds for the three types of hand controlled pollination (CH_{out}, CH_{self}, CL_{self}). Explanatory variables in italics are declared as random; results are based on the type III sums of squares.

Explanatory variables	CH proportion			Calices			CL fertilization success			CH fertilization success			Total seed set			Date of first CL flower			Date of first CH flower		
	DF	F Value	Pr > F	DF	F Value	Pr > F	DF	F Value	Pr > F	DF	F Value	Pr > F	DF	F Value	Pr > F	DF	F Value	Pr > F	DF	F Value	Pr > F
Season	**1**	**315,78**	**<0,001**	**1**	**156,78**	**<0,001**	**1**	**3,88**	**0,049**	**1**	**7,01**	**0,008**	**1**	**102,87**	**<0,001**	**1**	**77.63**	**<0.001**	1	1.07	0.3019
Cross	2	1,27	0,281	2	1,44	0,238	2	0,76	0,468	**2**	**5,26**	**0,006**	2	0,31	0,730	**2**	**30.23**	**<0.001**	**2**	**6.01**	**0.0026**
Family	19	0,81	0,696	**19**	**2,95**	**<0,001**	**19**	**1,93**	**0,011**	**19**	**1,7**	**0,033**	**19**	**2,41**	**0,001**	19	1.59	0.0551	19	1.19	0.2580
Season x Cross	**2**	**9,84**	**<0,001**	**2**	**23,3**	**<0,001**	2	2,85	0,059	2	1,36	0,257	**2**	**19**	**<0,001**	**2**	**36.98**	**<0.001**	**2**	**19.50**	**<0.001**
Season x Family	19	1,25	0,215	19	1,09	0,352	**19**	**2,96**	**<0,001**	19	1,63	0,046	19	1,08	0,371	19	1.12	0.3296	**19**	**2.06**	**0.0056**
Cross x Family	38	0,71	0,903	**38**	**2,09**	**0,000**	**38**	**2,48**	**<0,001**	38	0,68	0,927	**38**	**2,13**	**0,000**	38	1.16	0.2422	38	1.10	0.3160
Season x Cross x Family	35	0,6	0,969	**35**	**2,26**	**<0,001**	**36**	**2,23**	**<0,001**	**35**	**1,59**	**0,020**	**35**	**1,87**	**0,002**	36	0.79	0.8031	36	1.21	0.1877
Model Sum of Squares			13.56			94045			5.26			15.52			1419199			43.37			38.95
Total Sum of Squares			25.92			182579			18.42			54.78			3147197			87.76			118.94

The correlation between the principal axis estimators of N_{cal} and p_{CH} and the total N_{cal} and p_{CH} per plant is highly significant and close to one for both traits (Spearman's rho for number of calices: 0.783, p-value < 0.0001, and Spearman's rho for CH proportion: 0.893, p-value < 0.0001, Figure D.1). Thus, measures on the principal axis can be considered as representative of those of whole plants.

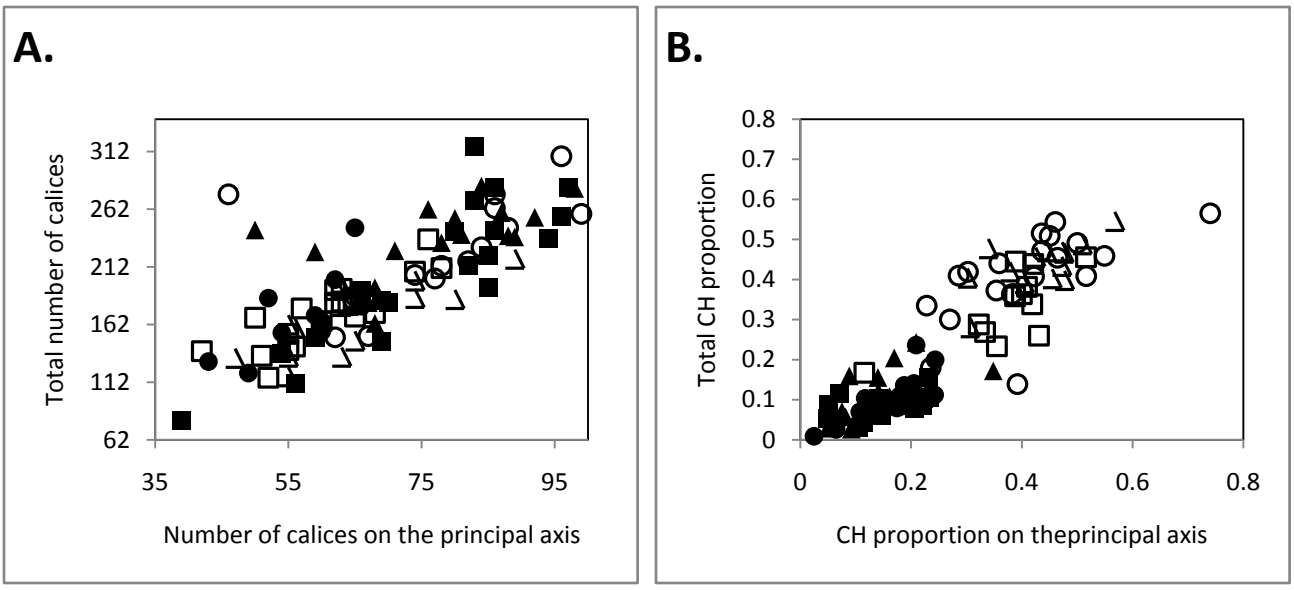

Figure D. 1. Total number of calices explained by the number of calices on the principal axis; B. Total CH proportion explained by the CH proportion on the principal axis. Full symbols hold for autumn, while empty ones for spring; triangles represent CH_{out} progeny, squares CH_{self} progeny, and circles – CL_{self} progeny.

Almost all traits studied vary significantly across seasons, except the beginning of CH flowering (Table D.2). Cross type has a significant effect on CH pollination success and the dates of CH and CL flowering, but it is not significant for the remaining traits (CH proportion, plant size, total seed set and CL pollination success, Table D.2). The interaction season x cross type is significant for all traits except pollination success (both CH and CL). There is significant variation among families for the number of flowers, pollination success and total seed set as shown by the significant family term in the corresponding models. For certain traits, some of the interactions terms with family are also significant, meaning that the response to seasonal variation or the effect of cross type is not the same for all of the families studied.

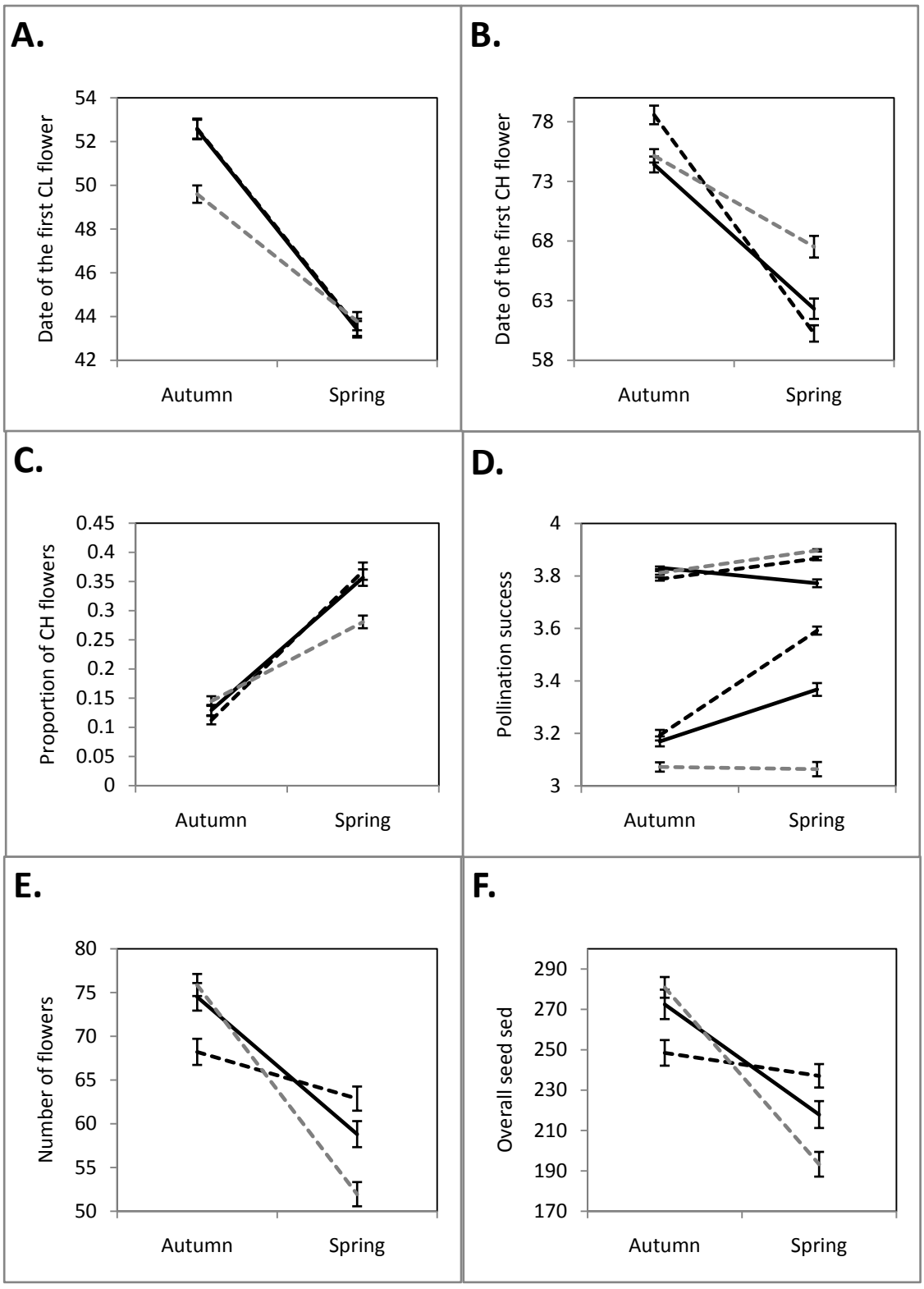

Figure D. 2. Life-history traits and fitness components of CL and CH flowers : onset of CL (A) and CH (B) flowering across seasons; proportion of CH flowers (C); pollination success of CL (upper lines) and CL (lower lines, D), total number of flowers (E) and seeds (F) produced. Solid black lines hold for CH outcrossed progeny, dotted black lines for CH selfed progeny, dotted gray lines for CL selfed progeny, and vertical bars represent two standard errors.

Since *L. amplexicaule* flowering starts with the production of CL flowers only (constitutive cleistogamy), CL flowering begins 15 to 25 days earlier than CH flowering, depending on season and cross type. The beginning of flowering, whether CL or CH, is earlier in spring than in autumn (Figure D.2.A and B), however, only CL flowering start date varies significantly across seasons (Table D.2). The lack of significant effect of season on the beginning of CH flowering could be due to the lower sample size in autumn – some plants do not produce CH flowers at all in autumn, thus reducing the power of the statistical analysis of the season effect. CH proportion is higher in spring than in autumn (Figure D.2.C). The variation pattern is similar for the CH_{self} and CH_{out} progeny, which have an almost three-fold increase in spring compared to the values observed in autumn. CL_{self} progeny CH proportion, p_{CH}, is higher in autumn compared to the other two cross types, but they are also less plastic, with lower p_{CH} in spring compared to the other two cross types. For all cross types and in all seasons, CL flowers are more successfully pollinated than CH flowers, as shown by the higher pollination success of CL ovules compared to CH ovules (one-tailed Student's t = -94.1373, df = 1185, p-value <0.0001, Figure D.2.D). CL pollination success varies very little across seasons and cross types: season has only a marginally significant effect on G_{CL} while cross type or season x cross type are not significant at all (Table D.2). CH fertilization success, on the other hand, increased significantly in spring for the CH_{out} and CH_{self} progeny, but does not vary across seasons for CL_{self} progeny (Figure D.2.D). This observation could explain the significant cross type effect for G_{CH}. The pollination success of CH flowers is relatively high, with three or more ovules developing into seed regardless of the season and the cross type. The number of calices is higher in autumn than in spring (Figure D.2.E). CL_{self} progeny are the largest in autumn and the inverse pattern is observed in spring – CL_{self} progeny are the smallest, bearing the fewest calices out of the three cross types. The total seed set follows a variation pattern similar to number of flowers, as it is higher in autumn than in spring, with the CL_{self} progeny having the highest *G* in autumn, and inversely, the lowest *G* in spring (Figure D.2.E) and thus resulting in significant season x cross type interaction.

Table D. 3. Analysis of plasticity across seasons of life-history traits. Inbreeding holds for inbreeding type (outcrossed or selfed). Explanatory variables in italics are declared as random; results are based on the type III sums of squares.

Explanatory variables	CH proportion			Calices			CL pollination success			CH pollination success			Total seed set			Date of first CL flower			Date of first CH flower		
	DF	F Value	Pr > F	DF	F Value	Pr > F	DF	F Value	Pr > F	DF	F Value	Pr > F	DF	F Value	Pr > F	DF	F Value	Pr > F	DF	F Value	Pr > F
Season	**1**	**274,24**	**<0,001**	**1**	**112,23**	**<0,001**	1	2,87	0,091	1	3,51	0,062	**1**	**80,7**	**<0,001**	**1**	**47.61**	**<0.001**	1	1.66	0.1986
Inbreeding	1	1,3	0,254	1	2,22	0,137	1	1,78	0,183	1	0,3	0,586	1	0,44	0,507	1	0.08	0.7722	**1**	**6.73**	**0.0098**
Family	19	0,71	0,809	**19**	**2,93**	**<0,001**	19	1,08	0,367	**19**	**1,67**	**0,038**	**19**	**2,56**	**0,000**	19	1.03	0.4267	19	1.00	0.4631
Season x Inbreeding	1	1,85	0,175	1	0	0,950	1	2,04	0,154	1	0,67	0,415	1	0,27	0,603	1	0.10	0.7471	1	0.02	0.8866
Season x Family	19	1,37	0,136	19	0,91	0,576	19	1,46	0,093	**19**	**1,8**	**0,020**	19	0,95	0,515	19	0.50	0.9643	**19**	**2.41**	**0.0008**
Inbreeding x Family	19	0,81	0,695	**19**	**2,15**	**0,003**	19	1,55	0,064	19	0,56	0,934	**19**	**2,42**	**0,001**	19	0.86	0.6306	19	0.96	0.5054
Season x Inbreeding x Family	17	0,39	0,988	17	1,4	0,133	17	1,23	0,234	17	1,3	0,186	17	1,36	0,152	17	0.51	0.9480	17	1.44	0.1104
Model Sum of Squares		12.39			69707			3.02			10.40			1027588			17.85			24.16	
Total Sum of Squares		25.92			182579			18.42			54.78			3147197			87.76			118.94	

Table D. 4. Analysis of plasticity across seasons of life-history traits. Flower type holds for flower morph from which the analyzed individuals are issued (open or closed). Explanatory variables in italics are declared as random; results are based on the type III sums of squares.

Explanatory variables	CH proportion			Calices			CL pollination success			CH pollination success			Total seed set			Date of first CL flower			Date of first CH flower		
	DF	F Value	Pr > F	DF	F Value	Pr > F	DF	F Value	Pr > F	DF	F Value	Pr > F	DF	F Value	Pr > F	DF	F Value	Pr > F	DF	F Value	Pr > F
Season	**1**	**286,89**	**<0,001**	**1**	**177,98**	**<0,001**	1	2,56	0,110	**1**	**8,39**	**0,004**	**1**	**110,29**	**<0,001**	**1**	**104.15**	**<.0001**	**1**	**6.45**	**0.0114**
Flower type	1	2,84	0,092	1	0,87	0,351	1	0,23	0,634	**1**	**9,12**	**0,003**	1	0,17	0,681	**1**	**35.03**	**<.0001**	**1**	**11.59**	**0.0007**
Family	19	0,93	0,541	**19**	**2,41**	**0,001**	**19**	**2,86**	**<0,001**	19	1,52	0,073	**19**	**1,87**	**0,014**	19	1.24	0.2160	19	1.30	0.1770
Season x Flower type	**1**	**21,14**	**<0,001**	**1**	**36,16**	**<0,001**	1	2,01	0,157	1	1,07	0,302	**1**	**29,04**	**<0,001**	**1**	**53.00**	**<.0001**	**1**	**27.99**	**<.0001**
Season x Family	19	1,56	0,062	19	1,2	0,255	**19**	**4,36**	**<0,001**	**19**	**1,7**	**0,033**	19	1,3	0,178	19	1.07	0.3782	**19**	**2.13**	**0.0036**
Flower type x Family	19	0,75	0,764	19	1,35	0,147	**19**	**4,33**	**<0,001**	19	0,64	0,880	19	1,04	0,410	19	0.86	0.6349	19	1.34	0.1522
Season x Flower type x Family	18	0,71	0,803	**18**	**2,46**	**0,001**	**19**	**3,83**	**<0,001**	**18**	**1,81**	**0,022**	**18**	**1,84**	**0,018**	19	0.50	0.9622	19	0.82	0.6870
Model Sum of Squares		13.00			75695			4.57			12.17			1050820			32.09			28.20	
Total Sum of Squares		25.92			182579			18.42			54.78			3147197			87.76			118.94	

Changing cross type for inbreeding degree in the statistical models does not change the significant effect of season on the plasticity of the traits studied, except for the season effect on $G_{CH,}$ which becomes non significant. However, inbreeding degree does not have a significant effect on its own (except on d_{CH}), and the interaction season x inbreeding degree is significant for none of the traits studied (Table D.3). No major changes are observed in the significance levels when cross type is changed for flower type in the statistical analyses for all of the traits, except for some of the interaction terms containing the family factor (Table D.4). Season remains significant for all of the traits studied except G_{CL}; flower type, likewise cross type, is significant for G_{CH}, d_{CL} and d_{CH}; and the interaction season x flower type, likewise cross type, is significant for all of the traits except G_{CL} and G_{CH}. General mixed models with flower type as an explanatory variable explain more of the total variation than models with inbreeding degree as an explanatory variable, as shown by the goodness of fits of the models. In general, models including flower type have a 3% higher sum of squares than other models, and up to 16% for the date of the first CL flower, Table D.5).

Table D. 5. Percentage of type III sum of squares explained by the three General Linear Mixed Models (model with cross type, inbreeding degree or flower type). The values were calculated as the ratio between the Model Sum of squares over the Total Sum of squares.

Model	Cross type	Inbreeding	Flower type
CH proportion	52.32	47.82	50.15
Number of flowers	51.51	38.18	41.46
CL fertilization success	28.55	16.41	24.83
CH fertilization success	28.34	18.99	22.22
Total seed set	45.09	32.65	33.39
Date of first CL flower	49.42	20.34	36.56
Date of first CH flower	32.75	20.31	23.71

Discussion

All traits in this study vary significantly across seasons, except CL pollination success. CH proportion is higher in spring than in autumn, which is consistent with the literature about the cleistogamy variation in *L. amplexicaule*. In our experiment, autumn is the more favorable season for the development of *L. amplexicaule*: plants produce more flowers and more seeds in autumn than in spring, a pattern that differs from *in situ* observations made for another experiment in autumn 2011 and spring 2012 and from observations made in natural populations in 2010 and 2011. Cross type is not significant for all the traits except CH pollination success. Importantly, there is a significant season x cross type interaction meaning that the effect of cross type changes across seasons. Finally, the variation among different types of progeny is better explained by the flower type than the inbreeding degree of a given individual.

Fitness components variation across seasons for different cross types

Beyond the seasonal effect, the important results of this study is the cross type x season interaction on components of fitness. Thus when measuring the relative fitness of inbred and outbred individuals in *L. amplexicaule*, the effect of the environment has to be taken into account. Despite recent accumulation of strong evidence of the importance of environmental variation for the fitness of inbred and outbred individuals (Armbruster and Reed, 2005), such comparisons have rarely been made for cleistogamous species. One exception is a study of *Viola septemloba*, in which fitness performances in the field and in greenhouse conditions were compared between selfed and outcrossed progeny of CH flowers. This study shows that the probability of flowering and the individual fecundity were lower in the field, which is considered as the more stressful environment (Oakley and Winn, 2008); however no comparisons were made between CL and CH progeny.

Inbreeding depression in cleistogamous species is generally expected to be low because these species have generally high selfing rates, and thus deleterious alleles are easily purged (Oakley et al., 2007). This is probably the case in *L. amplexicaule*, for which previous estimates of outcrossing rates have revealed

very high levels of selfing (Stojanova et al., unpublished). Our results also show that the components of fitness of *L. amplexicaule* are more dependent on flower type than on inbreeding degree. Although comparisons of inbred and outbred individuals in cleistogamous species studies are rare, comparisons of CL and CH progeny are very common, and most of the studies show that CL progeny has generally higher fitness (reviewed in Oakley et al., 2007), suggesting that there are other advantages, besides inbreeding depression, of CL over CH flowers. In *Viola Canadensis*, Culley (2000) studied the effect of inbreeding depression and flower type by comparing the same three cross types as in our study (CH_{out}, CH_{self} and CL_{self}) in a common garden. CH_{self} individuals were as good as or even better than CH_{out} progeny for most fitness measures, suggesting that inbreeding degree had little effect on the fitness measured in the experimental environment, except for some late life history traits. CL_{self} and CH_{self} individuals did not show significant difference for most traits studied individually, except higher CH flowers production for CL issued individuals. However, cumulative fitness was greater for CL_{self} individuals, meaning that the flower type was also the major determinant of individual fitness in that particular experimental environment.

In some cleistogamous species, the fitness difference between CL and CH progeny arises from the morphological or physiological differences between the two seed types. For instance, substantial differences in fruit size, seed number or seed size can be found in some cleistogamous species (see Plitmann, 1995b, and Oakley et al., 2007 for a review). In species that invest more resources in CL seeds, the effects of inbreeding could be completely cancelled out by the flower type effect due to the seed size advantage of CL seeds. This is the case with the hog peanut (*Amphicarpaea bracteata*), which produces aerial CH and CL flowers containing one to three dry seeds, and subterranean CL flowers that have only one large and fleshy seed. A study comparing subterranean CL progeny, aerial CL progeny, hand selfed CH progeny, and naturally pollinated and thus potentially outcrossed CH progeny showed that subterranean seeds resulted in larger plants that produce more flowers and have higher CH

proportions, but there was no difference between the three different progeny types of aerial seeds regardless of their cross type (Trapp and Hendrix, 1988).

Because seed weight in our study does not differ between CL and CH seed, we can exclude that variation in the overall quantities of nutrients between different seed types accounts for fitness variation. However, other and maybe more subtle differences could change the properties of CL and CH progeny. There can be a difference in the quality of the albumen between the two seed types, resulting in better resources available in CL seed for an equivalent seed weight. In a recent study, it has been shown that epigenetic processes can interfere with the effect of inbreeding on individual fitness. In the perennial outbreeding plant *Scabiosa columbaria*, inbred progeny fitness components (dry mass, number of leaves and photosynthetic activity) are lower than those of outbred progeny, and its DNA methylation levels are higher for inbred individuals. Further, demethylating inbred individuals with a chemical agent increases their fitness, thus cancelling out the fitness difference between the two progeny types (Vergeer et al., 2012). DNA methylation is dependent on both genetic and environmental conditions (Bossdorf et al., 2008). Hence, epigenetic processes appear as a plausible mechanism to account for the flower type effect observed in our study.

CH proportion variation as an adaptation – different scenarios with regard to our fitness estimates

The CH proportion in our study is ~ 0.1 in autumn and 0.3 in spring, which is consistent with the results obtained for four other populations of *L. amplexicaule* studied in the same conditions of seasonal variation (Stojanova et al., unpublished). Interestingly, in our study we observe high CH proportions in the less favorable habitat, which is not consistent with the "differential cost of flowers" hypothesis (Schemske, 1978, Waller, 1979). Under this hypothesis CH flowers in *L. amplexicaule* should be produced when environmental conditions are favorable for the plant development because they are more costly to produce (they produce more pollen and nectar, and have bigger corollas; Baude et al.,

2011, Lord, 1979a, Lord, 1980b), while CL flowers, which are cheaper to produce, should be favored in more stressful environments. Numerous cleistogamous species follow the pattern predicted by this hypothesis, with higher CH proportions being associated with larger plants (Oakley et al., 2007). The inverse pattern has been observed in several cleistogamous species, most of which are perennial (Oakley et al., 2007). Perennials are able to store resources that can be used in the following flowering season, thus compensating the potential negative impact of a lack of resources. As a consequence, perennials could adjust their CH proportion in response to other environmental factors, such as pollinator abundance (Jasieniuk and Lechowicz, 1987, Berg and Redbo-Torstensson, 1998).

One possible explanation for the observed variation in CH proportion here is the adaptation to the pollination environment. Since CL flowers are independent of pollinator activity they should be favored when pollinators are scarce (reproductive assurance hyopthesis, Albert et al., 2011, Redbo-Torstensson and Berg, 1995). In our study CH pollination success was higher in spring than in autumn for progeny from CH flowers, which coincided with higher CH proportions. These results are consistent with the pollinator abundance hypothesis for *L. amplexicaule* according to Lord (1982): in spring, when pollinators are abundant, plants tend to produce more CH flowers that could be easily pollinated, and inversely, in autumn, when pollinators are scarce, plants tend to produce CL flowers that are autonomous for their pollination.

Another possible explanation could include the dormancy properties of *L. amplexicaule* seeds. The dormancy in this species has already been documented (Baskin and Baskin, 1981). Moreover, our observations in natural populations show that *L. amplexicaule* does not systematically have two generations per year. In Southern France, for instance, the species behaves as a spring annual only, as well as in closed and unfavorable habitats in Northern France. If the dormancy of seeds produced in autumn prevents them to germinate in spring, then these seeds, which are mainly issued from CL

flowers, would germinate the following autumn, when they have the highest expected fitness. The same holds for seeds produced in spring, which are mostly issued from CH flowers, and which would have the highest expected fitness if they would develop into seedlings the following spring. The dormancy mechanisms in *L. amplexicaule* can be innate, meaning that seeds are incapable of germinating for a certain period of time after they are produced, or could be conditional, with ripening induced by appropriate ecological conditions (Baskin and Baskin, 1998). For this hypothesis to be tested, we need to evaluate the effect of the two dormancy types on seeds issued from spring and autumn plants, and complete our study on fitness components across seasons for individuals issued from the same cross types on maternal plants coming from spring populations.

Finally, one may ask if CL plasticity is a way to adapt to environment dependent inbreeding depression. Testing the adaptation to inbreeding depression of *L. amplexicaule* requires taking account of the contribution of different progeny types to the parental fitness i.e. their performance measured in their focal environment. CH flowers in *L. amplexicaule* outcross at a 25 % rate (Stojanova et al., submitted), thus the progeny produced from a CH flower in a natural population is a mixture of selfed and outcrossed individuals. If we average the fitness contribution of CH progeny between CH_{self} and CH_{out}, we can deduce that plants produced from CL flowers will be more adapted to the autumn season, when they produce more flowers and more seeds, and plants produced from CH flowers will be more adapted to the spring season. The flowering pattern observed in this study is contradictory with this conclusion: plants produce many CL flowers in autumn, whose progeny could germinate in spring when they have the lowest fitness, suggesting that CH proportion variation pattern observed here is not adaptive to environmentally dependent inbreeding depression. This could be partly due to the unusually high spring season temperatures that could have resulted in an unexpected fitness decrease in the spring season. Independent data from two natural populations near Dijon that were surveyed in 2010 and 2011 showed that the number of flowers was significantly lower in 2011, which had been warmer than 2010,

though there was no difference in number of flowers between seasons (Stojanova et al., unpublished). If the actual fitness values in natural populations in spring are similar to those we observed in our study in autumn then adaptation to environmentally dependent inbreeding depression could be a plausible explanation for variation in CH proportion observed in this study.

Inbreeding depression is a central force in the evolution of mating systems that could maintain mixed mating if it is environmentally dependent. However, this study shows that when mixed mating is provided by highly specialized structures, such as the two floral morphs in cleistogamous species, inbreeding depression cannot explain the adaptive character of mixed mating on its own.This is probably due to the joint evolution of inbreeding depression and other characteristics inherent to the differences between CL and CH types and that could have antagonistic effects on individual fitness. Further studies are required to distinguish the particular effect of each of these factors as well as the effect of their interaction on the adaptive character of plastic cleistogamy, and to some extent, plastic mixed mating.

Discussion

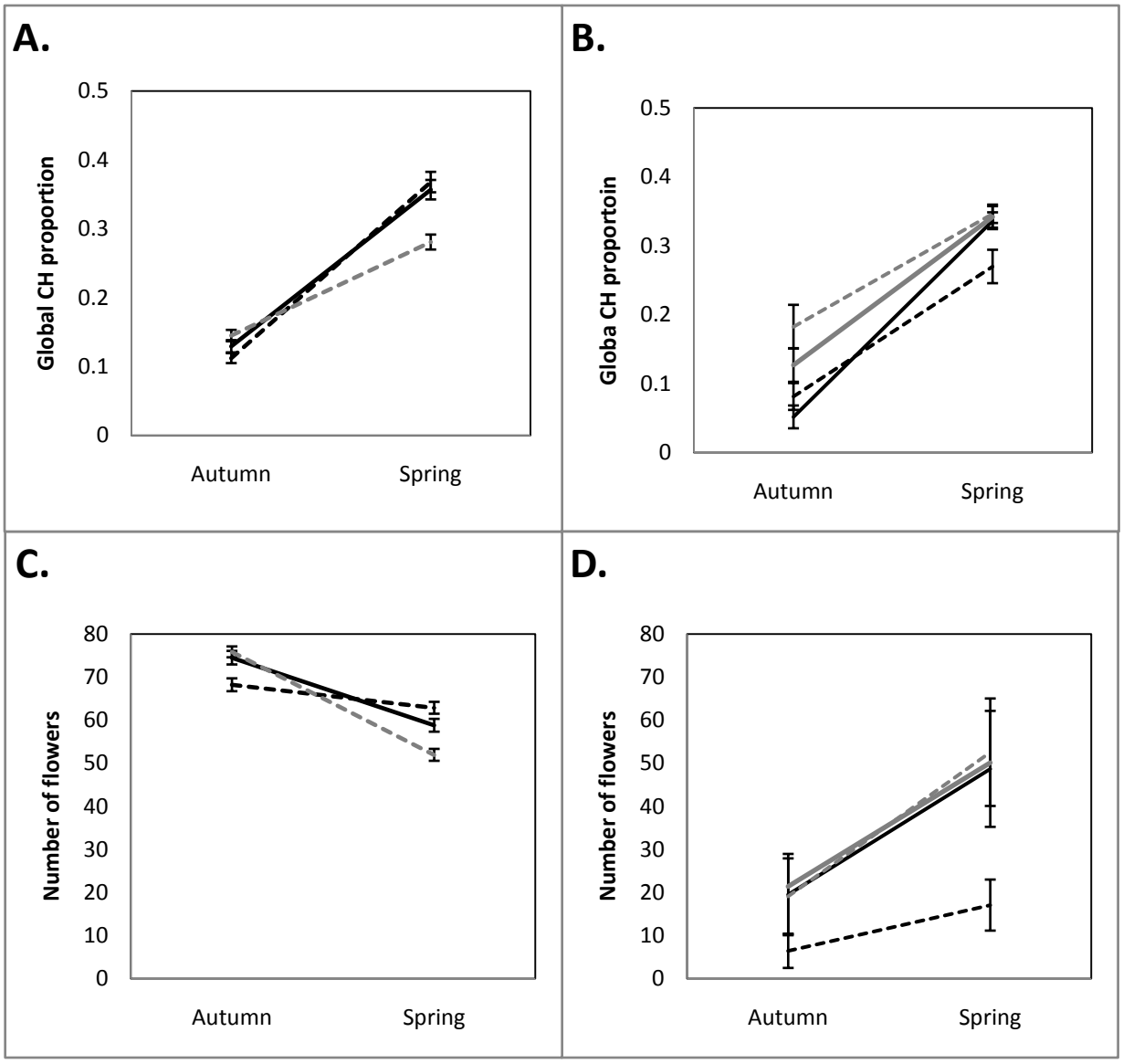

Figure C. 1. Variation of CH proportion (A and B) and total number of flowers (C and D) across seasons in two common garden experiments. A. and C. Average values for individuals issued from different cross types in one autumn population in Dijon. Solid black line – CH outcrossed, dashed black line – CH selfed, dashed gray line – CL selfed progeny. B. and D. Average values for individuals from four different populations. Solid lines – populations from Montpellier, dashed Lines – populations from Dijon. Black lines – populations from favorable habitats, grey lines – populations from unfavorable habitats. Vertical lines present standard errors. The important differences in the number of flowers between the two experiments (panels C and D) are due to the smaller size of the pots in which plants from D. were cultivated.

Patterns of plasticity on cleistogamy in *L. amplexicaule*

Is variation of chasmogamy proportion plastic? – Cleistogamy in natural populations of *L. amplexicaule* varies in time and in space. We observe temporal variation in response to standard seasonal changes and to randomly changing factors (e.g. temperature fluctuations within a given season or year). We also observed spatial variation. Spatial variation of CH proportion was tested on two levels – across two distinct geographical regions and across different population sites – but was not observed across geographical regions. As expected, we show that CH proportion is generally higher in spring than in autumn. More surprising is the observation that populations that were 500 *km* apart differed less if they were found in similar habitats than populations from different habitat types within a small geographical region (less than 10 *km* apart). Temporal variation suggests that variation of CH proportion is plastic, but we cannot formally rule out the possibility of population differentiation regarding our observations of spatial patterns. We further studied the variation of CH proportion in common garden experiments, in which we tested the effect of season, region, habitat, and controlled cross type and showed that in all cases, variation of CH proportion is mainly plastic.

Adaptive character of plastic cleistogamy – The results of our semi-natural common garden experiments show that seasonal variation of the CH proportion is finely tuned. Indeed, regardless of the plant size which varied in the two common garden experiments, CH proportion did not vary much within seasons, but it significantly varied between seasons (Figure C.1). Seasonal variation is predictable, thus it is easy to respond to it by producing adequate phenotypes. CH proportions observed in each season are close to the optima of stabilizing selection gradients in each season, though populations that have only a spring generation (near Montpellier) show more discrepancy between the optimum and the average observed phenotype in autumn than populations that have both a spring and an autumn seasons (near Dijon). Plastic cleistogamy could have evolved as an adaptation to environmental variation because i) each phenotype provides higher fitness in the environment that induces its production (Via and Lande,

1985), ii) plasticity is not costly because it is maintained even in individuals that do not experience environmental variation (DeWitt et al., 1998), and iii) accurate prediction of environmental variation is possible because the plant relies on reliable environmental cues (Karban et al., 1999).

What is the cleistogamous mating type? – Another important result of this thesis is that CH proportion variation can translate into outcrossing variation for some of the populations studied in spring. Our estimates of the CH outcrossing rate show that this trait does not differ among four natural populations coming from contrasted habitats and regions. Thus assessing the outcrossing rate of a cleistogamous mating system resumes to a simple count of the two floral types. Admittedly, our estimates are not informative about the outcrossing rate in autumn, which could be different than those in spring because of differences in pollinators between the two seasons. Our results suggest a strong relationship between the mating type (selfing and outcrossing) and the CH proportion in some of the environmental conditions encountered by *L. amplexicaule*. Since CH proportion variation across seasons seems to be adaptive, one might ask what evolutionary forces maintain cleistogamy, and whether they are similar to those operating in mating systems for plants with a single floral type.

How do we explain adaptive plasticity of cleistogamy

According to our results in common garden experiments, the effect of the season on the variation of CH proportion is not related to plant size, contrary to observations for other cleistogamous species (Schemske, 1978, Trapp and Hendrix, 1988, Berg and Redbo-Torstensson, 1998). To some extent, our observations exclude the hypothesis that plastic cleistogamy is an adaptation to different production costs of CL and CH flowers in response to resources available for plant growth. Though this is true for plasticity across seasons, our experimental observations reveal another pattern within seasons. Late flowering plants in autumn produce fewer flowers than early flowering plants, and in general they produce no CH flowers. It is thus plausible that there are two distinct mechanisms that govern the CH

production: the onset of CH flowering, which requires a minimal size threshold, and plastic CH proportion, which is independent of plant development. This is consistent with Lord's observation of constitutive cleistogamy in *L. amplexicaule*, and Oakley et al. (2007) observation that CL production is favored over CH production in most cleistogamous species.

When CH production is not dependent on resources, other environmental factors should favor plastic cleistogamy. The effect of pollination ecology on CL and CH production is one of the rare hypotheses that have been theoretically (Masuda et al., 2001, Schoen and Lloyd, 1984) and experimentally (Masuda et al., 2004, Berg and Redbo-Torstensson, 1998, Redbo-Torstensson and Berg, 1995, Culley, 2002) explored in CL species. The most common hypothesis about pollination ecology in cleistogamous species is the adaptation to pollinators abundance (Schoen and Lloyd, 1984): when pollinators are scarce, CL flowers are produced because they are capable of autonomous selfing, and when pollinators are abundant, CH flowers, which are potentially outcrossed, should be favored. A similar verbal model was suggested by Lord (1982) for *L. amplexicaule*, in which it was supposed that pollinators are more frequent in spring than in autumn, hence the higher CH proportion in spring. CH pollination success estimates in both of our common garden experiments are higher in spring than in autumn, whereas CL pollination success does not vary across seasons, which is consistent with this hypothesis. However, CL seed set is generally higher than CH seed set in both seasons, thus the contribution of the CH flowers to the progeny count is not sufficient to explain the maintenance of plastic cleistogamy on its own in the scope of this hypothesis. Since CL reproduction is highly advantageous, in order for CH reproduction to be maintained it needs to confer some substantial advantage to individual fitness.

Another hypothesis related to pollination ecology is the avoidance of geitonogamy (Masuda et al., 2001), stating that cleistogamy should be maintained because producing CL flowers allows the plant to decrease geitonogamy rates without decreasing its seed set. As a result, CH proportion should decrease

when total number of flowers increases (Masuda et al., 2001, Masuda et al., 2004). However, our experimental studies do not confirm this pattern (see above). Furthermore, this hypothesis considers CL flowers as a fail-safe mechanism while CH flowers are the basic mating type, whereas from our observations it is clear that CL flowers are the basic production in *L. amplexicaule*.

Pollination ecology could not account for the sustainability of plastic cleistogamy on its own; we therefore explored the differences in the progeny produced by the two floral types. Our results exclude the possibility of differences in the albumen of the seeds (energetic reserves), since CL and CH seeds have the same weight, and also because plant development was not dependent solely on the flower type they were issued from, but rather on the interaction between the flower type and the season.

In monomorphic flower species environmentally dependent inbreeding depression can account for the maintenance of mixed mating (Cheptou and Mathias, 2001, Cheptou and Schoen, 2002). In cleistogamous species CL progeny could also suffer from inbreeding depression, and plastic variation of the CH proportion could be an adaptation to predictable changes in the magnitude of inbreeding depression. Whenever inbreeding depression is high, CH progeny should be favored, and in environments with low inbreeding depression CL reproduction may be more advantageous because of the low costs of CL flowers. However, our fitness measures of CH outcrossed, CH selfed and CL selfed progeny did not reveal inbreeding depression effects *per se*, because the flower type (CH or CL) has more effect on progeny fitness than the inbreeding degree (inbred or outbred). Since overall selfing rates in *L. amplexicaule* are very high, it is likely that we failed to detect inbreeding depression because of the efficient purging of deleterious alleles (Crnokrak and Barrett, 2002). Comparisons between CL and CH progeny in several other cleistogamous species have shown that the flower type from which the plants are issued has an important effect on the fitness estimates (Oakley et al., 2007), but in most of these studies CL progeny have higher fitness than CH progeny, thus confirming the idea that inbreeding

depression has been purged, and that other non-genetic differences account for the fitness variation between the two progeny types. In our study individual fitness of a given cross type depends on the season in which it is measured, meaning that there is an environmental dependent determinant.

One hypothesis that we did not test was Lu's model that confronts purging through CL flowers and heterosis through CH flowers (Lu, 2002). Genetic diversity and heterozygosity levels in each of the four populations studied on the molecular level were rather low, thus heterosis should not increase much within population outcrosses. We observe some genetic differences between the populations (i.e. private alleles were observed in all four populations). Moreover, the geographical distances between populations in this study are rather small, meaning that pollen transfer between populations is plausible. In order to test heterosis of between population progeny, we need to compare the fitness of within population controlled crosses progeny to that of between populations controlled crosses progeny.

None of the hypotheses discussed above can account for the maintenance of plastic cleistogamy in *L. amplexicaule* on its own, but they do account for different aspects of cleistogamy evolution in this species. Based on our results, we conclude that i) the onset of CH production in *L. amplexicaule* is favored only in environments that provide enough resources for the full development of the CH phenotype but the plasticity of CH producing individuals depends solely on environmental conditions; ii) plastic cleistogamy can be an adaptation to variation in pollinator activity across seasons, and iii) the different progeny types produced can be more or less advantageous according to the environment in which they establish. We have primarily focused on the effect of the environment on the CH proportion and fitness, but individual fitness depends also on the survival of the progeny produced. To obtain more

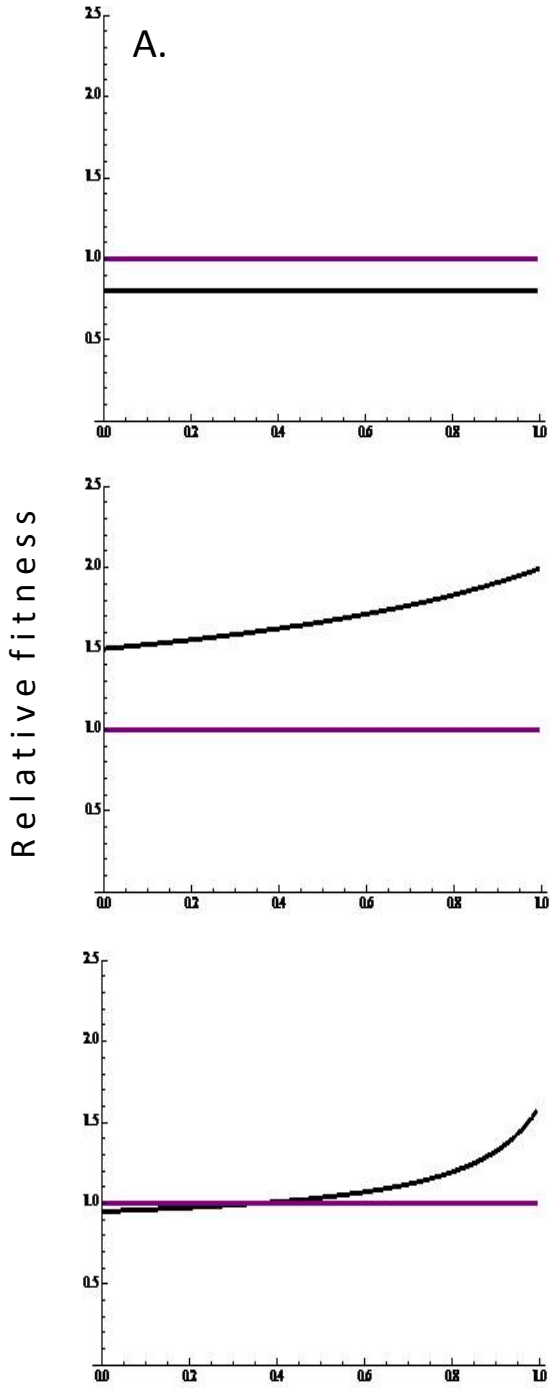

Figure. Relative fitness of selfed (black) and outcrossed (purple lines) individuals to that of outcrossed individuals as a function of their frequency. A. no inbreeding depression, no pollen discounting. B. total pollen discounting, inbreeding depression = 0.2. C. pollen discounting = 0.7, inbreeding depression = 0.2

Box.1. The effects of pollen discounting on the evolution of mixed mating.

Selfing individuals in an outcrossing population transfer two copies of their genes through selfed progeny and one copy through outcrossed progeny, whereas complete outcrossers transfer only two copies (one trough outcrossed ovules and one through outcrossing pollen). If the population consists exclusively of selfing individuals, the relative fitness conferred by selfing to that of outcrossing will be even higher, because in the absence of mating partners, outcrossed individuals could leave only one copy (through the exported pollen) while selfed individuals could still leave two copies (though selfed seed). Thus when the frequency of selfing increases, and provided that there is no inbreeding depression or pollen discounting, the fitness advantage of a selfing individual increases as well until only complete selfing persist. The exactly inverse pattern (increasing the fitness conferred by outcrossing when frequency of outcrossing increases) is observed when inbreeding depression is complete (i.e. no selfed individuals survive). This type of frequency dependent selection prevents the maintenance of a stable mixed mating strategy in the population (Figure A).

If we suppose complete pollen discounting, the automatic advantage of selfing no longer holds, because selfers cannot export pollen for outcrossing. With no additional evolutionary forces, selfing and outcrossing confer the same relative fitness – they both leave one copy of their genes through ovules and one through pollen. In this case, outcrossing will be favored for any positive value of inbreeding depression (Figure B). Finally, when pollen discounting has intermediary values and inbreeding depression is non null, each mating type is advantaged in some circumstances (Figure C), which is one of the conditions necessary for the emergence of mixed mating. It is important to notice however that these two parameters by themselves do not stabilize mixed mating.

accurate fitness estimates, potential differences in the recruitment of the two progeny types according to environmental variation should be assessed too.

Can cleistogamy of *L. amplexicaule* contribute to a better understanding of plastic mixed mating evolution?

According to our results, most of the non genetic differences between the two floral types (flower costs, seed energy reserves, seed dispersal), that are generally used to explain the evolution of cleistogamy cannot account for the maintenance of plastic cleistogamy in *L. amplexicaule*. In addition we show that CH proportion translates into outcrossing rate, meaning that plastic cleistogamy is a particular type of plastic mixed mating. In this section we discuss how can cleistogamy contribute to models of evolution of plastic mixed mating systems through its particular features.

Previously in this thesis it was stated that the evolution of plasticity can be constrained if frequency dependent selection operates on the trait(s) considered (Ernande and Dieckmann, 2004). In monomorphic flower mixed mating, the automatic advantage results in a frequency dependent selection that leads the mating system towards complete selfing (if inbreeding depression is low) or complete outcrossing (if inbreeding depression is high) (Lande and Schemske, 1985, Lloyd, 1979, Lloyd, 1992, Box 1). In cleistogamous species this type of frequency dependence no longer holds because of the complete pollen discounting associated with selfing in CL flowers which cancels the automatic advantage (Box 1). The absence of frequency dependence leaves a possibility for the emergence of plastic mixed mating strategies. However, the lack of frequency dependence does not sustain mixed mating on its own, and additional factors are necessary to enable the evolution of plastic mixed mating.

In the past decade, evidence about the existence of environment dependent inbreeding depression has accumulated for many different organisms, including hermaphroditic plants (Armbruster and Reed, 2005, Fox and Reed, 2011). The estimates of inbreeding depression in these studies need to be

considered with caution, since in general they are obtained in experimental environments, which do not necessarily reflect the behavior of natural populations. Most of these studies test for the effect of stressful versus benign environments, but the definition of environmental stress is a rather obscure term. Moreover, contrary to the general assumption made in these studies, it has been shown that environmental stress does not necessarily increase inbreeding depression magnitude (Cheptou and Donohue, 2011, Ronce et al., 2009). Our estimates of environment dependent inbreeding depression are ecologically relevant, since they were made in semi-natural conditions that reflect closely the seasonal variation encountered in natural populations (Enders and Nunney, 2012). Of particular interest in our results is the observation that the values of inbreeding depression observed are positive in spring and negative in autumn (outbreeding depression, Lynch, 1991). This observation is not consistent with the hypothesis that inbreeding depression is mainly due to the accumulation of mildly deleterious recessive alleles, which would only decrease the fitness of inbred individuals though its magnitude may vary. Rather, the fitness decrease we observe in some environments could be partly due to genes that are deleterious in this particular environment but at the same time confer fitness advantage in other environments (antagonistic pleiotropy, see Cheptou and Donohue, 2011). Such shifts can be made through epigenetic modifications of the genes. Indeed, the effects of epigenetics on gene expression is one possible mechanism for plasticity (Richards et al., 2013), but epigenetic effects on inbreeding depression and mating system have only recently been demonstrated (Vergeer et al., 2012). Vergeer et al. (2012) showed that DNA methylation levels depend on the inbreeding degree, being higher in inbred individuals, and also that demethylating inbred individuals with a chemical agent restores the fitness of inbred individuals to that of outbred individuals. In cleistogamous species, the differences of the two floral types could account for epigenetic differences between the two progeny types in addition to the genetic differences due to the different mating strategies. If this is the case, then cleistogamy offers interesting new perspectives for the study of the joint evolution of inbreeding depression and plastic

mixed mating systems. If epigenetic modifications do account for the differences in the magnitude of inbreeding depression observed in our model, or if there are other genetic mechanisms that change the deleterious effects of a given genotype with environmental variation, then the long term evolution of inbreeding depression will differ form that predicted by simple mutation-selection models (i.e. purging). Additional investigations in this field could help explaining the discrepancy between the observed and predicted values for purging of inbreeding depression, with observations lower than expected under the model of mutation-selection balance of deleterious alleles (Kelly and Willis, 2001).

Perspectives

Estimates of CH outcrossing rate – The outcrossing proportion of CH flowers in autumn remains unknown. If pollination community structure or pollinator abundance varies across seasons, this could also change the outcrossing rate. Knowing the outcrossing rate in autumn could help to strengthen the view that cleistogamy is a mixed mating system. For instance, if the outcrossing rate does not vary across seasons, then CH proportion variation is truly a manner of adjusting the outcrossing rate in response to environmental cues prior to flowering, and if the outcrossing rate decreases in autumn, then cleistogamy could be a reliable way to ensure reproduction at smaller costs. In line with this, gathering information about the pollinators that visit *L. amplexicaule* and their potential variation across seasons could help better understanding the pollination ecology of the species and the adaptation of cleistogamy to pollinator activity.

Testing heterosis – Another hypothesis that was not tested here was the fitness increase due to heterosis in crosses between different lineages of *L. amplexicaule*, which could be easily done by comparing progeny from within population and between population crosses. Within population heterosis is less likely because of the low genetic diversity at the population level, but one could nevertheless select the most diversified genotypes for this comparison. Between populations heterosis assumes that pollen can be transferred between populations, which is a plausible scenario given the

small geographic distances between populations. Previous between population crosses showed that different lineages of *L. amplexicaule* are not always compatible (no viable seeds produced, Bernstrom, 1952). However, the lineages studied by Bernstrom came from very distinct geographical regions and were potentially different varieties. This is probably not the case in the populations we studied, though population MS may be an exception.

Spatial variation - The experimental studies in this thesis focus mainly on the temporal seasonal variation effect on cleistogamy in *L. amplexicaule*. However, field observations show that there is also a significant spatial variation, mostly across habitats. Reciprocal transplant experiments, potentially combined with seasonal variation could help elucidating the ecological significance and the adaptive character of plastic cleistogamy in response to habitat or regional variation.

Molecular mechanisms of cleistogamy – The molecular characteristics of cleistogamy have rarely been studied except for some cultivated species such as barley, rice and soybean (Turuspekov et al., 2004, Khan et al., 2008, Maeng et al., 2006). These studies show that there are different ways to control the production of the two floral types and the CH proportion (loci with different alleles, regulating genes, quantitative trait loci). In *L. amplexicaule*, the only data available about the genetics of cleistogamy are from controlled crosses of CH rich and CH poor lineages which show Mendelian segregation of the CH proportion character, which is also influenced by environmental variation. Today's sophisticated techniques of molecular biology can help identify the precise genes and molecular mechanisms that influence the CH production, as well as the environmental (epigenetic) modifications of these genes that account for plastic cleistogamy.

APPENDIX 1. Isolation and Characterization of microsatellite markers for the cleistogamous species *L. amplexicaule*.

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Primer Note

Isolation and characterization of microsatellite markers for the cleistogamous species *Lamium amplexicaule* (Lamiaceae)[1]

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- *Premise of the study: Lamium amplexicaule* is a cleistogamous plant that produces both closed flowers (obligately self-pollinated) and open flowers (potentially outcrossed). The conditions for the maintenance of such a mating system depend on the outcrossing rate of the open flowers, which can be estimated using neutral microsatellite markers.
- *Methods and Results:* Forty primer pairs corresponding to microsatellite motifs obtained by coupling multiplex microsatellite enrichment and next-generation sequencing were tested. Thirteen primers amplified with satisfying results. The polymorphism of these markers was studied in four French populations. Allele number varied from one to eight per locus and per population. Heterozygosity levels were significantly lower than those expected under Hardy–Weinberg equilibrium.
- *Conclusions:* Our results are consistent with a partial self-fertilization pattern. These markers will be used to estimate the outcrossing rate as well as population differentiation in *L. amplexicaule*.

Key words: cleistogamy; Lamiaceae; *Lamium amplexicaule*; microsatellite markers; outcrossing rate.

Cleistogamy is the coexistence of both closed flowers that are obligately self-pollinating and open flowers that are potentially outcrossing on the same individual. It has been generally considered as an example of a mixed mating system, and its evolutionary stability is still enigmatic (Goodwillie et al., 2005). *Lamium amplexicaule* L. is an annual cleistogamous weed of the mint family (Lamiaceae) native to Europe and Asia. The species has been introduced to all other continents where it has become invasive (USDA–ARS, 2003). It has been documented as both a winter annual (seeds dormant through autumn and winter, flowering in spring) and a summer annual (seeds dormant through spring and summer, flowering in autumn [Baskin and Baskin, 1981]). Even though nectar production and pollinators' visits of open flowers have been documented for this species (Orueta and Viejo, 1999), the species is considered as predominantly self-pollinating (Fryxell, 1957). The percentage of open flowers produced during the flowering season never exceeds 50% and can vary in response to environmental cues such as photoperiod and temperature, being as low as zero when plants are exposed to short, cold days (Lord, 1982).

Lord (1982) suggested that such a plastic production of open flowers is an adaptation to long, warm days, i.e., spring season, when pollinators are abundant. Likewise, when pollinators are scarce in the short, cold, autumn days, the production of closed flowers providing autonomous self-fertilization is favored. Such an adaptation assumes that open flowers are able to outcross substantially, thus increasing individual fitness by avoiding the deleterious effects of inbreeding (Charlesworth and Willis, 2009). To analyze the mating system of this species, we developed 13 microsatellite markers that will be used to estimate the outcrossing rate of *L. amplexicaule*.

METHODS AND RESULTS

Plant DNA was extracted from fresh plant tissue using the DNeasy Plant Mini Kit (QIAGEN, Courtaboeuf, France) following the manufacturer's protocol. An enriched DNA library was obtained by Genoscreen (Lille, France) by coupling multiplex microsatellite enrichment and next-generation sequencing on 454 GS-FLX Titanium platforms according to the method described in Malausa et al. (2011). Noncompound sequences containing microsatellite motifs longer than five repeats were retained. A total of 211 markers were returned, out of which the 40 markers with the longest repeat sequences were further tested for amplification. PCR amplification was performed in a final volume of 10 µL containing 5 µL of Multiplex PCR Master Mix (QIAGEN), 2 µM of each primer (Eurogentec, Angers, France), 2 µL of pure water, and 1 µL of DNA extraction solution (approximate concentration 50 ng/µL). PCR conditions were as follows: 15 min activation of the HotStart *Taq* DNA polymerase at 95°C, 30 cycles including 60 s initial denaturation at 94°C, 90 s at annealing temperature (Table 1), and 60 s extension at 72°C, followed by 30 min final extension at 60°C. Amplification of a DNA fragment of the expected size was

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Table 1. General characteristics of the amplified loci for *Lamium amplexicaule*.

Locus	Primer sequences (5′–3′)	Repeat motif	Size range (bp)	T_a (°C)	Dye	Multiplex	GenBank accession no.
LA-Di02	F: CATATAACCTACATACCAAACCCTC R: GCCGGAGAGGTATTTTTGGT	$(CA)_{13}$	200–204	58	PET	1	JX050158
LA-Di05	F: ATTCAATTTTAGGGGGTCGG R: GTGATTCATTCCTTACAACTTTACC	$(GT)_{13}$	105–118	58	PET	1	JX050160
LA-He02	F: AGTTTCTCCACCGCAAACC R: ATCCCATCCACATCCATCAC	$(CCATCT)_7$	94–124	58	NED	1	JX050161
LA-He03	F: CGAAAGATGGACTGTTGTTCTG R: GGAGGCTAACAATTGCCATT	$(AAGAGG)_8$	89–131	58	FAM	1	JX050162
LA-Tri02	F: AGACAGAAGGCAAAGCTGGA R: ATTCCCTCGTATTCCAACCC	$(CTT)_{11}$	154–172	58	VIC	1	JX050166
LA-Tri07	F: CTGGGGGTGAAGGAATGAAT R: TCAATCTCATCCACAAGGCA	$(CTT)_{17}$	139–203	58	FAM	1	JX050168
LA-Te04	F: TGAGAACAATGTAATGCCAGAAA R: GGCACTTCTCCGACAAACTC	$(ATGT)_8$	155–204	58	FAM	2	JX050163
LA-Te05	F: GGGTTTTTCCCGATCTGAAT R: CTCTGTCCCATAAAAATATGTTCAGA	$(TACA)_9$	96–134	58	NED	2	JX050164
LA-Tri05	F: GAGTGGCGGCTCTAACTCAG R: TCTGCGAATTCACCCTTTCT	$(CTT)_{12}$	137–163	58	VIC	2	JX050167
LA-Te07	F: CTAATTGGGGATGTGAGATAAA R: CTCAACATTCGTTTCACCCA	$(ATAC)_{11}$	175–199	55	VIC	3	JX050165
LA-Tri08	F: AAGCAAGAAGTGGCCAAGTTA R: TGGTCTTAAATAGATTTCTTGT	$(TGT)_{19}$	243–278	55	VIC	3	JX050169
LA-Tri11	F: CAAAATCTACATAAACCCGAGA R: AGGAAGGATGCATACCATGC	$(TGA)_{23}$	85–123	55	FAM	3	JX050170
LA-Di03	F: TTAGTCGTCTGACCTTGGG R: AGTTGAGAGTTAAAACACTTAGTAAG	$(GA)_{13}$	162–189	52	PET	3 post PCR	JX050159

Note: T_a = annealing temperature.

obtained for 14 primer pairs. The forward primers of the 14 loci were labeled with a FAM, VIC, PET, or NED fluorescent dye (Applied Biosystems, Life Technologies SAS, Courtaboeuf, France). Using the same PCR protocol as above, each primer pair was tested on five individuals originating from five different locations in France. Out of the five individuals tested, three came from the populations M, C, and E used for the detailed polymorphism study below (GPS coordinates in Table 2), and two came from populations that were not used for further analysis (individual A1, coordinates: 43°38′19.86″N, 3°51′52.84″E; and individual B1, coordinates: 49°19′03.28″N, 05°04′24.34″E). PCR products were then diluted in water. Dilution varied from 1/50 to 1/200 according to the concentration of the PCR product. Three microliters of diluted PCR product were pooled in 15 µL of deionized formamide (Applied Biosystems) and 0.2 µL of 500 LIZ GeneScan size standard (Applied Biosystems). PCR products prepared this way were sized using an ABI PRISM 3100 sequencer (Applied Biosystems) and the software GeneMapper version 4.1. Thirteen primer pairs out of the 14 tested were polymorphic on the five individuals and had easily readable chromatograms (Table 1). These primer pairs were combined into three multiplexes for further PCR: mix 1 contained LA-Di02, LA-Di05, LA-He02, LA-He03, LA-Tri02, and LA-Tri07; mix 2 contained LA-Te04, LA-Te05, and LA-Tri05; and mix 3 contained LA-Te07, La-Tri08, LA-Tri11, and LA-Di03. Locus LA-Di03 was added in mix 3 after PCR amplification (Table 1).

A more detailed study of polymorphism was performed on individuals from four different populations of *L. amplexicaule* in France—two large populations with several hundred individuals each (population E around Montpellier, and population M around Dijon) and two small populations with fewer than 40 individuals

Table 2. Results of initial primer screening in four French populations[a] of *Lamium amplexicaule*.

	Population C (N = 19)				Population E (N = 40)				Population M (N = 36)				Population P (N = 19)			
Locus	A	H_o	H_e	A_p	A	H_o	H_e	A_p	A	H_o	H_e	A_p	A	H_o	H_e	A_p
Di02	1	—	—		2	0.051	0.097*	1	1	—	—		1	—	—	1
Di05	1	—	—		3	0.026	0.370*	1	2	0.000	0.475*		2	0.333	0.500	
He02	1	—	—		2	0.000	0.142*	2	2	0.000	0.056*	1	1	—	—	
He03	1	—	—		3	0.075	0.388*	2	3	0.028	0.344*	1	1	—	—	1
Tri02	1	—	—		2	0.026	0.204*	1	2	0.000	0.375*	1	1	—	—	1
Tri07	3	0.056	0.156*	2	7	0.231	0.681*	4	9	0.029	0.741*	4	1	—	—	
Te04	1	—	—		2	0.053	0.051	1	1	—	—		2	0.053	0.051	2
Te05	1	—	—		3	0.200	0.301*	3	1	—	—		1	—	—	
Tri05	1	—	—		3	0.075	0.119*	1	1	—	—		1	—	—	
Te07	1	—	—		2	0.077	0.163*		2	0.000	0.490*		2	0.000	0.198*	
Tri08	1	—	—		3	0.075	0.423*	2	1	—	—		0	NA	NA	
Tri11	2	0.059	0.057*	1	4	0.077	0.334*		2	0.000	0.424*	1	0	NA	NA	
Di03	1	—	—		2	0.086	0.082		2	0.000	0.312*	1	0	NA	NA	

Note: A = number of alleles; A_p = number of private alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; N = number of plants sampled; NA = no amplification.

[a] Populations: C = Dijon, small population (47°16′37″N, 5°03′43″E); E = Montpellier, large population (43°44′56″N, 3°51′06″E); M = Dijon, large population (47°15′58″N, 4°59′11″E); P = Montpellier, small population (43°46′16″N, 3°47′28″E).

* Significant deviation from Hardy–Weinberg equilibrium after Bonferonni correction.

(population P around Montpellier, and population C around Dijon). GPS coordinates of the four populations are in Table 2. For each of the four populations a voucher has been deposited in the Botanical Institute of Montpellier herbarium collection (voucher accession numbers: population C, MPU000543; population E, MPU000544; population M, MPU000545; and population P, MPU000546). Nineteen to 40 individuals were sampled per population (Table 2). Total DNA was extracted from dried plant tissue using the DNeasy96 Plant Kit (QIAGEN) following the manufacturer's protocol for dry plant tissue, with an additional lysis step of incubation at 65°C for 90 min. Multiplex PCR amplification was performed with the same protocol as cited above.

The proportion of amplified individuals was high for most of the loci. In population P, loci LA-Di05, LA-Di03, LA-Tri08, and LA-Tri11 amplified poorly or not at all probably because of a substitution in the primer sequence. In the other three populations, the missing results were found mainly in one or two individuals that did not amplify throughout most of the loci. When the poorly amplified loci in population P were excluded, the mean proportion of individuals successfully amplified was 0.9 or higher for all populations. Expected heterozygosity and Hardy–Weinberg equilibrium tests were performed using GenAlEx (Peakall and Smouse, 2006). Number of alleles for each locus in the four populations, heterozygosity levels, and private alleles are shown in Table 2.

CONCLUSIONS

These newly developed microsatellite markers showed high amplification success even though their polymorphism levels within populations are not very high, especially in the small populations. Expected heterozygosity for polymorphic loci was generally low, and most of the polymorphic loci showed a significant deficit in observed heterozygotes. On the other hand, there are important differences in allelic patterns between populations. These observations are consistent with partially self-pollinating populations. Markers developed in this study can thus be used to (1) study structure and differentiation within populations and (2) calculate estimates of the outcrossing rate of open flowers in the large populations of *L. amplexicaule*. Estimating the outcrossing rate of open flowers will allow the relationship between cleistogamy rate and individual outcrossing rate to be determined and will thus relate the plasticity of cleistogamy to mating system plasticity.

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ABSTRACT
Cleistogamy differs from classical mixed mating systems, for which species with single floral morph self-fertilize at intermediate rates: cleistogamous plants produce both closed cleistogamous flowers (CL) that are obligately selfed and open chasmogamous flowers (CH) that are potentially outcrossed. Because CL flowers cannot export pollen (total pollen discounting), cleistogamous species do not benefit from the automatic advantage of selfing. Furthermore, costs for producing the two floral types are different, and the two types of progeny they produce (CL and CH) have different properties that go beyond the differences between selfed and outcrossed progeny. The proportion of individual CH flowers is often plastic, suggesting this trait is an adaptation of the outcrossing rate to environmental variation. Here, we studied an annual cleistogamous species, *Lamium amplexicaule*, that has both spring and autumn generations each year, and whose CH proportion correlates with variation in seasonal cues. We combined data from field surveys, semi-natural experimental studies in spring and autumn, genetic analyses of neutral markers, and some theoretical modeling to i) assess the variation in CH proportion and its plasticity, ii) assess the outcrossing rate of CH flowers and its relation to the CH proportion, iii) test the adaptive character of plastic cleistogamy, and iv) test evolutionary scenarios that could explain the maintenance of plastic cleistogamy in *L. amplexicaule*. We show that cleistogamy in *L. amplexicaule* is plastic and adaptive to seasonal variation, and that CH proportion variation translates into variation of the overall outcrossing rate. Classical explanations for cleistogamy evolution relying on resource allocation to CL and CH flowers do not fit our data; we instead propose that the adaptive character of plastic cleistogamy could be due to environmentally dependent variation in fitness of CL and CH progeny and pollinator abundance. More studies of the evolution of cleistogamy need to account for the combined effect of classical evolutionary forces that operate on the reproductive systems of monomorphic flower species and the effect of floral specialization to different mating types.