

Using a cost–benefit approach to understand the evolution of self-fertilization in plants: the perplexing case of *Aquilegia canadensis* (Ranunculaceae)

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Abstract

Plants exhibit tremendous variation in whether mating occurs via self-fertilization versus outcrossing, and many species practice a mixture of both. In theory, the evolution of selfing is influenced by a few large costs and benefits, and the benefits of selfing should be balanced by its costs at equilibrium. We have attempted a cost–benefit analysis of selfing, using population genetic surveys, experimental manipulations and quantitative genetics to a spring-flowering, short-lived perennial, *Aquilegia canadensis*. This species invests heavily in large, nectar-rich flowers but achieves full seed set in the absence of pollinators by autonomous autogamy, and selfs 76% of its seed in natural populations. Floral emasculations reveal that 75% of selfing occurs via autogamy, which increases per-flower seed production (reproductive assurance), but pre-empted ovules and resources that could be used to make outcrossed seed (seed discounting). This is costly because outcrossed progeny appear to survive to maturity 10-fold more often than selfed progeny. Almost all the apparent nonautogamous selfing involves cross-pollination between relatives (biparental inbreeding). This could facilitate the evolution of mixed mating, but not when inbreeding is so strong. Though selfing seems strongly disadvantageous, *A. canadensis* exhibits striking heritable variation in the separation between anthers and stigmas within flowers (herkogamy), which correlates negatively with selfing. This suggests that the mating system is at equilibrium. There is no applicable theoretical explanation for high selfing in the face of strong inbreeding depression, which occurs in a variety of plants. Understanding the evolution of these enigmatic mating systems remains a major challenge.

Keywords: *Aquilegia*, biparental inbreeding, reproductive assurance, seed discounting, self-fertilization.

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Introduction

If a given trait is the product of natural selection then the benefits of that trait should be balanced by its costs in populations at equilibrium. Testing this prediction requires natural or experimentally induced trait variation across which costs and benefits in terms of fitness can be compared. Successful application of this cost–benefit

approach should also recognize that: (i) the trait occurs in the developmental–genetic context of the whole phenotype so that its evolution may be constrained by trade-offs with other traits; (ii) comprehensive fitness costs and benefits have to be evaluated in a life history framework; and (iii) the trait probably evolved in an ecological milieu that varies in time and space (Gould & Lewontin 1979; Pigliucci & Kaplan 2000). This paper provides an overview of how we have used manipulative experiments and population genetic surveys to quantify some of the most important costs and benefits of inbreeding, and discusses some of the challenges we have encountered in trying to explain the evolutionary maintenance

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of high levels of self-fertilization in natural populations of a columbine, *Aquilegia canadensis* L. (Ranunculaceae, Fig. 1).

Inbreeding is a major component of the mating system in many plants and animals and can profoundly influence a variety of important ecological and evolutionary processes (Hamrick & Godt 1990; Charlesworth 1992; Jarne & Charlesworth 1993; Thornhill 1993). Inbreeding via self-fertilization is particularly prevalent in plants because most species are hermaphroditic and many are self-compatible to some extent (Richards 1986; Lloyd & Schoen 1992). Moreover, the extent to which individuals self-fertilize and the floral traits that influence the mating system vary widely, even among closely related species. Thus, the transition between outcrossing and selfing mating systems appears to be one of the most common evolutionary pathways in plants (Stebbins 1974), and understanding the genetic and ecological factors involved has been a major goal of plant evolutionary biology since Darwin (1876).

Cost–benefit approach to the evolution of self-fertilization

Theory suggests that the direction and magnitude of natural selection on the relative amount of self- versus cross-fertilization is determined by a relatively small set of potentially large costs and benefits summarized in Table 1. This framework simplifies extensive theoretical

Aquilegia canadensis L. (Ranunculaceae)



- Short-lived perennial Occurs on rock outcrops Eastern North America¹
- Flowers in late spring²
- Each flower has ~125 ovules and makes ~60 seeds^{2,3}
- Flowers provide abundant, dilute nectar^{4,5}
- Visited by bumble bees & hummingbirds⁶
- Self-compatible⁷
- Not dichogamous⁵
- High seed set when excluded from pollinators^{7,8} (highly autofertile)

Fig. 1 The study species, *Aquilegia canadensis*, as portrayed by Agnes Kliber. Superscripts refer to supporting published work. 1, Whittemore (1997); 2, Kliber and Eckert (2004); 3, Mavraganis and Eckert (2001); 4, Macior (1978); 5, Griffin *et al.* (2000); 6, Macior (1966); 7, Routley *et al.* (1999); 8, Eckert and Schaefer (1998).

Table 1 Selective costs and benefits of inbreeding and how they vary among the different modes of inbreeding

Selective factors	Effect of inbreeding	Mode of inbreeding		
		Autogamy	Geitonogamy	Biparental inbreeding
Benefits				
Gene transmission advantage	Increases male fitness by siring own seeds	Yes	Yes	No
Reproductive assurance	Increases seed production when outcross pollination is limited by scarcity of pollinators and/or potential pollen donors	Often	Rarely	No
Costs				
Inbreeding depression	Reduces survival and/or reproductive success of progeny	Yes	Yes	Some
Pollen discounting	Reduces outcross siring success	Maybe	Probably	Yes
Seed discounting	Reduces production of outcrossed seed by using ovules and/or resources	Maybe	Usually	Yes

Autogamy is self-pollination between anthers and stigmas within flowers; geitonogamy is self-pollination between different flowers on the same plant; and biparental inbreeding is cross-pollination between related individuals. Autogamy might occur autonomously without pollinator visitation, whereas geitonogamy and biparental inbreeding are mediated by pollinators.

work over the past 25 years (detailed reviews in Barrett & Eckert 1990; Jarne & Charlesworth 1993; Uyenoyama *et al.* 1993; Waller 1993; Holsinger 1996; Charlesworth & Charlesworth 1998). Briefly, early theoretical models pitted the benefit of avoiding the genetic cost of outcrossing (also called the transmission advantage of selfing) against the cost of inbreeding depression and, in general, predicted only two stable evolutionary equilibria: predominant outcrossing associated with strong inbreeding depression or predominant selfing associated with weak inbreeding depression (e.g. Lande & Schemske 1985; Charlesworth & Charlesworth 1987). In contrast, a burgeoning collection of marker-gene-based estimates of selfing and outcrossing from natural populations of a wide variety of plants clearly indicate that a substantial proportion of species, particularly animal-pollinated species, engage in a broad mixture of both selfing and outcrossing (Schemske & Lande 1985; Aide 1986; Barrett & Eckert 1990; Barrett *et al.* 1996; Vogler & Kalisz 2001).

Subsequent theoretical models made four major advances. First, they critically examined the benefit of reproductive assurance (Lloyd 1992; Schoen *et al.* 1996; Morgan *et al.* 1997; Johnston 1998; Sakai & Ishii 1999; Tsitrone *et al.* 2003), which although championed by Darwin (1876) had been largely neglected in both theoretical and empirical studies (but see Cruden & Lyon 1989). Second, they more clearly introduced the costs associated with gamete discounting, when self-fertilization reduces the production of outcrossed seed (seed discounting Lloyd 1992; Morgan *et al.* 1997) or the successful export of pollen to sire outcrossed seed on other individuals (pollen discounting Holsinger 1991, 1992; Lloyd 1992; Harder & Wilson 1998; Johnston 1998). Third, they emphasized that the additional costs and benefits of selfing are likely to depend on the details of pollination ecology that determine how and when self-fertilization occurs (e.g. Lloyd 1992; Lloyd & Schoen 1992; Schoen *et al.* 1996). Finally, some models incorporating these ecological costs and benefits appeared to be able to explain the widespread occurrence of mixed mating systems (e.g. Chang & Rausher 1998; Harder & Wilson 1998; Johnston 1998; Tsitrone *et al.* 2003).

Despite these theoretical advances, a full cost-benefit analysis of selfing has not been attempted for any of the many species that exhibit a broad mixture of selfing and outcrossing. Thus, empirical evidence that these mixed mating systems are adaptive is extremely limited (Barrett 2003).

Costs and benefits of selfing depend on how it occurs

Testing the assumptions and predictions of recent theoretical models starts with estimating how and when selfing

occurs. Table 1 breaks down selfing into the two major components (autogamy and geitonogamy) advocated by Lloyd and Schoen (1992) and contrasts their selective costs and benefits. Table 1 also includes inbreeding via cross-pollination between close relatives (biparental inbreeding) because this form of inbreeding, although very different from selfing in how it occurs and its consequences for fitness, is usually included in marker-gene estimates of self-fertilization (see below). This framework can be used to classify all mating events regardless of whether they occur through male or female sexual function. In practice most empirical work, including our own, has estimated components of female reproductive success (i.e. what proportion of seed is produced through outcrossing vs biparental inbreeding vs selfing). However, it is important to recognize that, in principle, male reproductive success can be partitioned and evaluated in terms of fitness in the same way (Gregorius *et al.* 1987), although measuring and partitioning male fitness is technically challenging.

Both modes of true selfing avoid the genetic cost of outcrossing, but produce offspring that suffer from inbreeding depression. However, Lloyd (1992) argued that the different modes of selfing vary with respect to two main ecological fitness consequences: reproductive assurance and gamete discounting. Autogamy, particularly if it occurs autonomously, might provide reproductive assurance (Cruden & Lyon 1989). It may also occur with little or no reduction in outcrossed seed production (seed discounting, see Fig. 2) or outcrossed siring success (pollen discounting, see Harder & Wilson 1998), particularly if it is delayed until after opportunities for outcrossing. In contrast geitonogamy, like outcrossing, requires

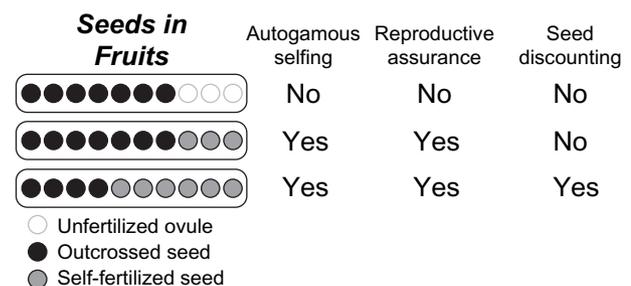


Fig. 2 Illustration of reproductive assurance with and without seed discounting. In these scenarios autogamy is somewhat autonomous (as it is in *Aquilegia canadensis*, see Fig. 1) and it always increases per-flower seed production (see Fig. 4). In one case, this occurs with no loss of outcross seed. In the other case, one outcrossed seed is lost for each extra seed produced through autogamy. Not all possible scenarios are included. For example, it is possible for autogamy to be facilitated by pollinator visitation such that it is not associated with increased seed production and there is a direct trade-off between the number of selfed and outcrossed seeds produced.

the active transport of pollen between flowers and, therefore, provides little or no reproductive assurance. In addition, the ovules and pollen involved in geitonogamous selfing are likely to be discounted from participating in outcrossing. In fact, geitonogamy is usually regarded as an inevitable by-product of cross-pollination in self-compatible plants that bear many open flowers simultaneously, rather than an adaptive mating strategy per se. Likewise, biparental inbreeding is viewed as an incidental result of fine-scale population genetic substructuring combined with localized pollination (Kelly & Willis 2002).

How inbreeding occurs can be estimated by experimentally manipulating opportunities for its various components (Schoen & Lloyd 1992; Kalisz & Vogler 2003). For example, individual flowers can be emasculated (anthers removed) to eliminate the capacity for autogamy; or individual flowers can be left intact on plants where all other flowers have been emasculated to eliminate geitonogamy; or all flowers on a plant can be emasculated to eliminate all true self-fertilization. Of course this approach assumes that manipulation of any one component of the mating system does not have confounding effects on the function of flowers, inflorescences or whole plants that might alter other mating components. For instance, removing anthers from individual flowers to eliminate autogamous self-pollination could also reduce outcross pollination if emasculation: (i) causes damage that impairs fertilization and seed maturation; (ii) elicits a wound response that reduces floral longevity (O'Neill 1997); or (iii) makes flowers less attractive to pollinators, particularly those that seek pollen. The intensity and scale of the manipulation must also be appropriate. For example, emasculating more than one flower at a time on small inflorescences will also reduce opportunities for geitonogamy. Likewise, removing anthers from too many plants in small populations might induce pollen limitation. Very few studies have carefully evaluated the assumptions underlying these types of experimental manipulations (Eckert 2000; Griffin *et al.* 2000; Elle & Carney 2003).

Experimental manipulations of flowers, inflorescences and plants provide alternative phenotypes to compare with naturally occurring resident phenotypes to quantify the fitness costs and benefits of the various modes of inbreeding. In general, theoretical models define reproductive assurance and pollen and seed discounting in terms of a difference in fitness between a resident strategist and some alternative mutant strategist that selfs a small amount more or less than the resident (e.g. Lloyd 1992). The evolutionarily stable mating strategy yields a combination of male and female fitness that cannot be bettered by any alternative phenotype. Considering this, experimentally altered phenotypes will be most useful for testing theoretical predictions if they deviate from the natural phenotypes in ways that could realistically be

achieved by mutation. It may be possible for a radically different phenotype to achieve higher fitness, but it may not be possible for a population to attain that phenotype because intermediate phenotypes yield much lower fitness.

An inferential approach to inbreeding depression

Reduced progeny viability is the fundamental selective cost of self-fertilization that, in turn, determines the magnitude of the other potential costs and benefits. For instance, the relative survival and reproductive success of selfed offspring determines the fitness benefit of reproductive assurance. Because both pollen and seed discounting involve trade-offs between the production of selfed versus outcrossed progeny (through male and female function, respectively), the fitness consequences of this trade-off depend on the relative fitness value of each progeny type.

In one sense, inbreeding depression is the most readily quantified of all the selective factors involved in the evolution of the mating system because, in many species, selfed and outcrossed progeny can be produced via hand-pollination and compared in a formal experiment. This approach was used extensively by Darwin (1876) and has been carried out for dozens of plant species since (reviewed in Charlesworth & Charlesworth 1987; Husband & Schemske 1996; Roff 1997). In contrast, it has been widely confirmed that the expression of inbreeding depression can depend on the environment in which selfed and outcrossed progeny are compared (reviewed in Roff 1997). In addition, the expression of inbreeding depression is expected to vary among traits and among different stages of the life cycle. Yet, the strength of inbreeding depression as expressed in natural populations throughout the life cycle has rarely been estimated (Husband & Schemske 1996). Moreover, the expression of inbreeding depression in nature might covary with environmental conditions among populations and years (Cheptou *et al.* 2002), and such variation can influence the evolution of the mating system (Cheptou & Mathias 2001; Cheptou & Schoen 2002). This is a major problem for cost-benefit analyses of self-fertilization.

We tackled this problem by estimating inbreeding depression using a marker-based approach that compares the degree of inbreeding between life history stages (Ritland 1990). Figure 3 plots the inbreeding coefficients of reproductively mature plants over the estimated levels of selfing for 38 natural populations of *A. canadensis*, many of which were studied in more than 1 year. In almost all cases mature plants were far less inbred than they should be given the level of selfing estimated from progeny array analysis. This would occur if highly inbred individuals suffered greater mortality before reaching reproductive

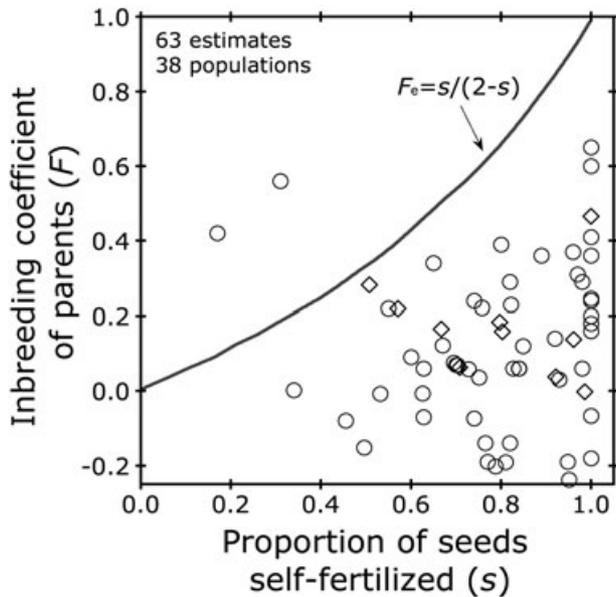


Fig. 3 Observed inbreeding coefficients (F) of reproductively mature plants are almost always less than expected given the level of self-fertilization (s) in natural populations of *Aquilegia canadensis*. The solid line shows F expected at equilibrium when selfed and outcrossed progeny have equal fitness. The points are the observed values from natural populations, each of which is based on approximately 30 arrays of 10 progeny each assayed for two polymorphic allozyme loci. This discrepancy between observed and expected F is consistent with the expression of very strong inbreeding depression. These data are from 38 different populations: 28 in the northern portion of the species' geographic range in Ontario, Canada (○) and 10 in the geographic center of the range in Georgia, North Carolina, Virginia and West Virginia, USA (◇). Nineteen populations were studied in a single year, 15 in two years, three in three years, and one in four years for a total of 63 population \times year estimates. These data are from several studies (Routley *et al.* 1999; Griffin *et al.* 2000; Herlihy & Eckert 2002; Griffin & Eckert 2003; Klüber & Eckert 2004; Herlihy & Eckert in press a, b; C. R. Herlihy, B. Ozimec & C. G. Eckert, unpubl. data, 2004).

maturity. Consequently, estimates of inbreeding depression derived from these data are almost always high and statistically significant (mean $\delta = 0.93$, interquartile range = 0.87–1.01, 1-sample t -test against $\delta = 0$ or $\delta = 0.5$, $P < 0.0001$). These estimates are particularly useful for our purposes because they reflect processes occurring in natural populations and integrate episodes of mortality from seed dispersal through reproductive maturity over several years. Although this approach is based on several assumptions (Ritland 1990; Charlesworth 1991) it is likely that most of these are either met by populations of *A. canadensis* or lead to δ being underestimated (discussed in Routley *et al.* 1999). It appears that in natural populations of *A. canadensis* outcrossed progeny survive to maturity almost 10-fold more frequently than selfed progeny.

Contribution of autogamy to the mating system

The most basic experimental manipulation is to remove anthers from individual flowers to eliminate autogamous self-pollination. If this significantly reduces self-fertilization then autogamous selfing is likely to be an important component of the mating system. If the removal of anthers also reduces the number of seeds produced, then it would appear that reproductive assurance provided by autogamy is beneficial because flowers capable only of allogamous pollination do not set as many seeds. Although many studies have examined the effect of floral emasculation on seed production (discussed below), few have quantified the effect on mating patterns to estimate the level of autogamous self-fertilization (Schoen & Lloyd 1992; Leclerc-Potvin & Ritland 1994; Eckert 2000; Levri 2000). Furthermore, all of these studies involve only one or two populations manipulated in only 1 year. In contrast, we expect that autogamy, particularly autonomous autogamy, may be selected under conditions in which pollinators or the supply of outcross pollen from conspecifics is generally unreliable (Schoen & Brown 1991; Lloyd 1992; Schoen *et al.* 1996). Hence, the prevalence of autogamy and the associated benefit of reproductive assurance might vary substantially among populations and between years within populations (Kalisz & Vogler 2003).

We emasculated individual flowers in seven populations of *A. canadensis* during one flowering season and three populations during two flowering seasons (Herlihy & Eckert 2002; Herlihy & Eckert in press a). All populations engaged in mixed mating with, on average, 76% of seeds being produced through self-fertilization (range = 63–92%). In all populations eliminating autogamous pollination substantially reduced total self-fertilization (Fig. 4), which is indicative of high levels of autogamous self-fertilization (mean estimated $a = 0.56$). As expected there was substantial variation in a among populations (range = 0.37–0.84) and the among-population coefficient of variation in a ($CV = 24\%$) was twofold larger than that for total self-fertilization (12%).

Adaptive significance of autogamy: reproductive assurance versus seed discounting

Although the reproductive assurance hypothesis is the most venerable explanation for self-fertilization (Darwin 1876; Stebbins 1957; Jain 1976; Cruden & Lyon 1989; Holsinger 1996; Schoen *et al.* 1996) it has almost never been rigorously tested. Some general support comes from the dozens of studies that have demonstrated pollen limitation of seed production in natural plant populations by comparing the seed production of naturally pollinated versus hand-crossed flowers (Burd 1994; Larson & Barrett

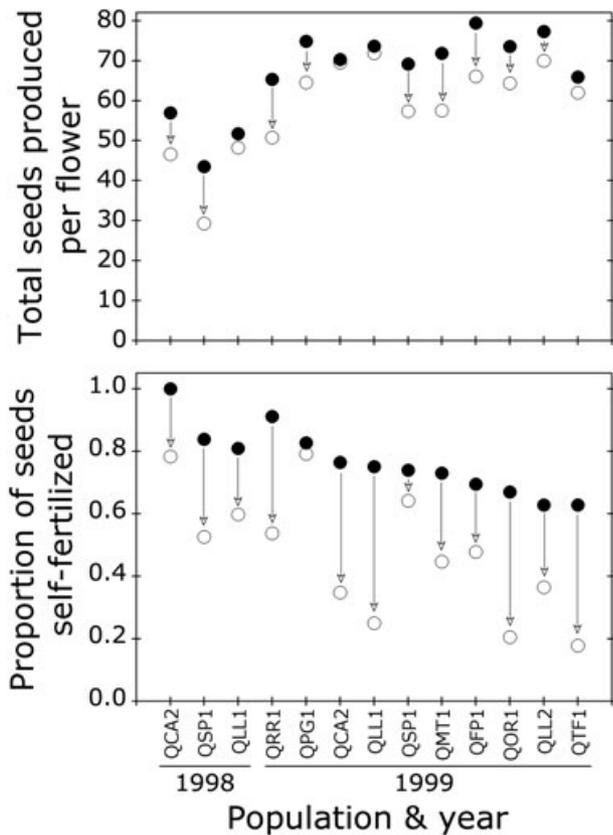


Fig. 4 The effect of eliminating autogamy by floral emasculation on total seed production and self-fertilization in natural populations of *Aquilegia canadensis*. Emasculation reduced seed production in all populations, indicating a per-flower fertility benefit of the reproductive assurance provided by autogamy. However, emasculation caused an even greater reduction in the proportion of seeds self-fertilized, indicating that a substantial portion of seeds are being self-fertilized via autogamy when they could otherwise have been outcrossed (seed discounting, see Fig. 2). Data are from table 1 in Herlihy and Eckert (2002). ●, intact flowers; ○, emasculated flowers.

2000). However, relatively few studies have attempted to quantify the fertility benefit of autogamous self-pollination by comparing intact versus emasculated flowers (reviewed in Cruden & Lyon 1989; Schoen *et al.* 1996; Eckert & Schaefer 1998; Kalisz & Vogler 2003). Even fewer studies have evaluated variation in the fertility benefit of autogamy among populations or across years within populations that might be expected in the pollination environments that select for reproductive assurance (Piper *et al.* 1986; Herrera *et al.* 2001; Elle & Carney 2003; Kalisz & Vogler 2003). Finally, the benefits of increased seed production via autogamy have never been weighed against the cost of seed discounting (Fig. 2).

Reproductive assurance appears to be a likely explanation for high autogamous selfing in *A. canadensis*. Receptive stigmas and dehiscing anthers are in close proximity

within flowers of *A. canadensis* (Griffin *et al.* 2000) and individual flowers can achieve near-maximal seed set when experimentally excluded from pollinators (Eckert & Schaefer 1998; Routley *et al.* 1999). As a result, the seed production of whole plants is not pollen limited in the populations we have studied (Kliber & Eckert 2004). In addition, populations of *A. canadensis* are typically small (<200 flowering plants), vary widely in size between years (Mavraganis & Eckert 2001) and flowering occurs in the spring when pollinator service may be unreliable (Macior 1966, 1978). We have only observed regular visitation to flowers by hummingbirds and bumble bees in large, dense, open populations in which the estimated levels of outcrossing are highest (Herlihy & Eckert in press a).

In terms of total seed production, the reproductive assurance hypothesis appears to be supported in *A. canadensis* (Herlihy & Eckert 2002). Emasculating individual flowers to eliminate autogamy reduced seed production by an average of 13%. Furthermore, the proportional difference in seed set between intact and emasculated flowers varied widely among populations (1–33%) and between years within populations, which would be expected in the variable pollination environments that might select for reproductive assurance (Fig. 4). However, the benefits of reproductive assurance erode once the effects of autogamy on outcrossed seed production are considered. We estimated severe seed discounting associated with autogamy in populations of *A. canadensis* by combining the effect of emasculation on both seed production and self-fertilization. On average, a plant capable of autogamy gains nine seeds per flower via autogamous selfing, but self-fertilizes 16 seeds that could have been outcrossed (Fig. 5). Moreover, the gain in seed production via autogamy is not higher in populations in which a greater proportion of seeds are autogamously selfed, which would be expected if variation in opportunities for outcrossing explained at least some of the variation in selfing among populations (see fig. 2a in Herlihy & Eckert 2002). Because genetic estimates of inbreeding depression are very high in these populations of *A. canadensis* ($\delta = 0.98$) the loss of outcrossed seeds is costly. On average, the fitness of intact flowers relative to emasculated flowers is very low (0.56) and inbreeding depression would have to be much weaker for autogamy to be advantageous (i.e. $\delta < 0.6$, Fig. 6).

The trade-off between selfed and outcrossed seed production may have costs that extend beyond the individual flower because selfing not only uses ovules, but also the resources required to turn those ovules into seeds. As a result, the production of selfed seed may compromise the subsequent production of outcrossed seeds both within and between reproductive seasons (Morgan *et al.* 1997). To date, these broader costs of seed discounting have not been investigated empirically, although we suspect that

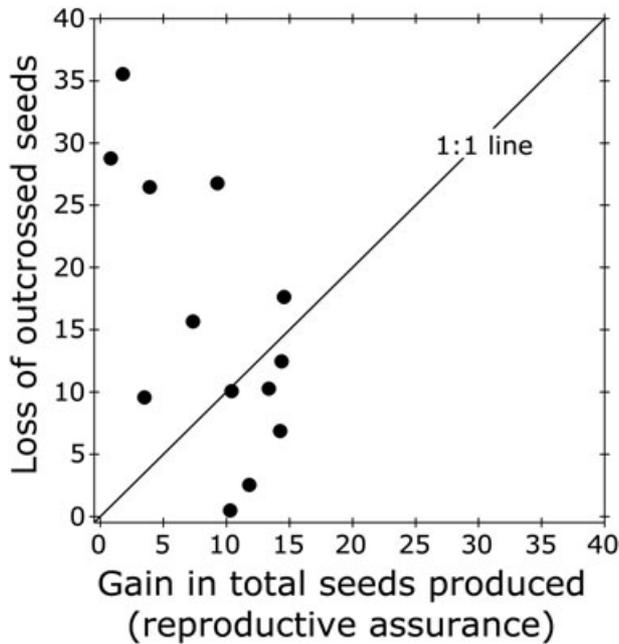


Fig. 5 The gain in total per-flower seed production through autogamous self-fertilization (reproductive assurance) is associated with a greater loss of high-quality outcrossed seed (seed discounting) in natural populations of *Aquilegia canadensis* (redrawn from Herlihy & Eckert 2002). The values presented are calculated from the estimates of selfing and seed production in Fig. 4 (see Herlihy & Eckert 2002 for details of calculations).

they might be substantial in *A. canadensis*. First, there is a dynamic allocation of resources to seeds among flowers within inflorescences (Kliber & Eckert 2004). When the seed production of early flowers is experimentally reduced, the resources saved appear to be allocated directly to increased seed production in later flowers (Fig. 7). Second, approximately 50% of reproductive *A. canadensis* died before the next flowering season in our study populations (C. G. Eckert, unpubl. data, 1999). Work on a wide variety of plants has demonstrated that investment in seed production can reduce survival to, and fertility in, subsequent flowering seasons (reviewed in Thompson & Eckert 2004), thus a demographic cost to reproductive assurance is plausible. Taken together our results suggest that although autogamous selfing provides short-term reproductive assurance in natural populations of *A. canadensis* the overall fitness consequences are likely to be strongly negative.

Contribution of geitonogamy versus biparental inbreeding

In plant populations matings can be fundamentally classified by whether they result from autogamous or allogamous pollination. Autogamy necessarily leads to selfing

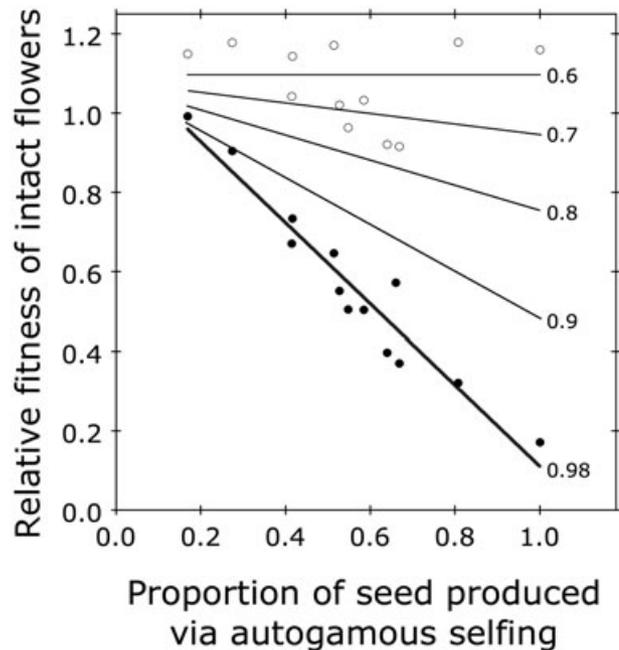


Fig. 6 The genetic cost of reproductive assurance through autogamous self-fertilization in *Aquilegia canadensis*. The relative fitness of intact flowers that are capable of autogamous selfing relative to emasculated flowers not capable of autogamy incorporates the benefits of selfing in terms of avoiding the cost of outcrossing and reproductive assurance, and the costs of selfing associated with seed discounting and inbreeding depression, and is based on the data in Figs 4,5. A strong negative regression among populations between relative fitness and proportion of seeds produced through autogamy is produced by incorporating the level of inbreeding depression estimated for these populations ($\delta = 0.98$, ●), indicating that autogamous selfing is disadvantageous in all populations. We also show the regressions for weaker inbreeding depression ($\delta = 0.9, 0.8, 0.7, 0.6$). If inbreeding depression were much weaker ($\delta = 0.6$, ○) autogamous selfing would be advantageous in all populations.

and at times this mode of selfing can be adaptive (but see above). In contrast, the consequences of allogamy are variable because the composition of allogamous pollen loads depends on interactions between pollinator behavior and plant population structure. Although some allogamous pollinations will produce truly outcrossed progeny, localized pollen transfer may lead to inbreeding through geitonogamous, between-flower self-pollination or through cross-pollination between relatives. Geitonogamy will occur if pollinators visit multiple flowers on a plant in sequence (de Jong *et al.* 1993; Snow *et al.* 1996). Biparental inbreeding will occur if neighboring plants are both genetically related and more likely to exchange pollen (Kelly & Willis 2002). Neither form of allogamous inbreeding is viewed as adaptive, per se, because neither is likely to provide reproductive assurance (but see Hinton 1976) and both should result in strong seed and pollen

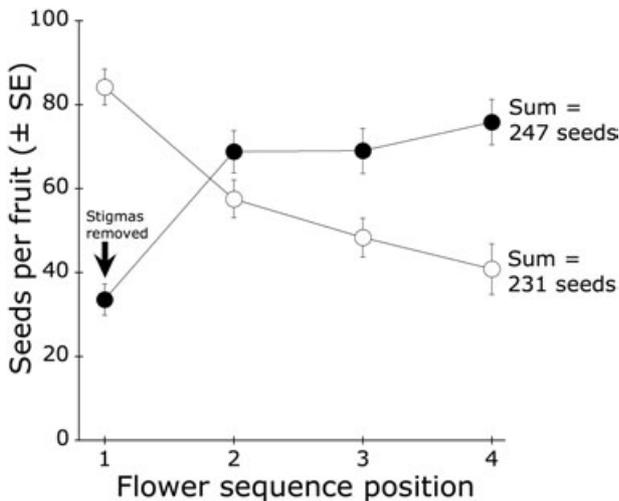


Fig. 7 Experimental demonstration of dynamic resource allocation to seeds within inflorescences of *Aquilegia canadensis* (redrawn from Klüber & Eckert 2004). In unmanipulated inflorescences, seed production declines substantially across the flowering sequence within inflorescences. However, when the seed production of first flowers is decreased by approximately 60% (i.e. approximately the same as the proportion of autogamous self-fertilization) via stigma removal, seed production increases markedly in subsequent flowers to the point where manipulated inflorescences make slightly more seed (247 seeds) than control inflorescences (231 seeds). Points are means based on approximately 40 plants per treatment; error bars are ± 1 SE. These data indicate that the cost of seed discounting is likely to extend to future flowers because selfing uses resources that could have otherwise been invested in outcrossed seed produced by future flowers. ●, seed set of first flower reduced; ○, experimental controls.

discounting (Table 1, see Harder & Barrett 1995). However, geitonogamy combined with strong inbreeding depression may lead to selection of aspects of floral display that influence the probability of geitonogamous pollen transfer (de Jong *et al.* 1992; Harder & Barrett 1996). Likewise, the deleterious effects of biparental inbreeding may favor traits that reduce the temporal and/or spatial proximity of related individuals (e.g. seed dormancy/dispersal) or the transfer of pollen between them (discussed in Griffin & Eckert 2003).

In our populations of *A. canadensis* emasculating of individual flowers reduced, but did not eliminate, apparent selfing. From this residual 'selfing' by emasculated flowers we can estimate that 9–32% of mating events involve allogamous inbreeding (mean = 18%, $n = 9$ populations; Herlihy & Eckert in press a). Again, this component of inbreeding varies much more among populations (CV = 50%) than total apparent selfing (CV = 12%). Partitioning this mating component into geitonogamy (g) versus biparental inbreeding (b) is tricky (Ritland 2002). An estimate of self-fertilization (s) based on single marker loci

(s_s) usually includes some biparental inbreeding (b) because both modes of inbreeding have similar genetic consequences. As more loci are used the multilocus estimate of selfing (s_m) includes progressively less b and, thus, approaches true s . Accordingly, b can be inferred from $s_m - s_s$, and almost all estimates of b in natural populations have been obtained using this approach (Holtsford & Ellstrand 1989; Waller & Knight 1989; Brown 1990). However, computer simulation by Leclerc-Potvin and Ritland (1994) revealed that $s_s - s_m$ will often greatly underestimate b even when many loci are used to estimate s_m and almost no studies have attempted to determine whether the s_m used in these calculations asymptotically approaches the true s (Ritland 2002).

We have attempted to obtain better estimates of b for natural populations of *A. canadensis* using two experimental approaches. First, we compared the frequency of apparent selfing (s_s) between plants rendered incapable of true selfing, because they only presented single emasculated flowers at any given time, and unmanipulated control plants. Apparent selfing by these fully emasculated plants can only involve biparental inbreeding. We applied this approach to six populations of *A. canadensis* (Fig. 8). Based on rather substantial estimates of s_s for fully emasculated plants we estimated that mean $b = 14\%$ (the range among populations was 3.6–24.0%). Thus, almost all allogamous inbreeding in these populations can be accounted for by biparental inbreeding rather than geitonogamous self-fertilization. All estimates of g were low (mean = 4.5%) and not significantly greater than zero in any population. The negligible contribution of geitonogamy to inbreeding is likely to result from the small floral displays typical of this species. Although most plants produced more than one flower throughout a flowering season (mean = 3.0), 63% of all plants bore only one flower at any given time. However, we failed to detect any difference in total selfing between plants bearing only one flower at a time and those with an average of two flowers open simultaneously (Herlihy & Eckert in press a). These results contrast with experimental studies that have detected substantial geitonogamy (Schoen & Lloyd 1992; Eckert 2000), even for species with very small floral displays (e.g. *Mimulus guttatus* with two flowers open simultaneously Leclerc-Potvin & Ritland 1994).

A variant of this emasculating experiment was used by Lu (2000). She estimated the average genotypic relatedness among all non-self mates (m_b) by comparing the inbreeding coefficient (F) of progeny from fully emasculated plants with the F of fully selfed progeny. Her results suggest that in natural populations of *Impatiens capensis* outcrossed mates are as closely related as half-siblings (mean $m_b = 0.25$). Estimates of m_b derived this way for the six populations of *A. canadensis* that we studied are also very high (mean = 0.52) and correlate strongly with esti-

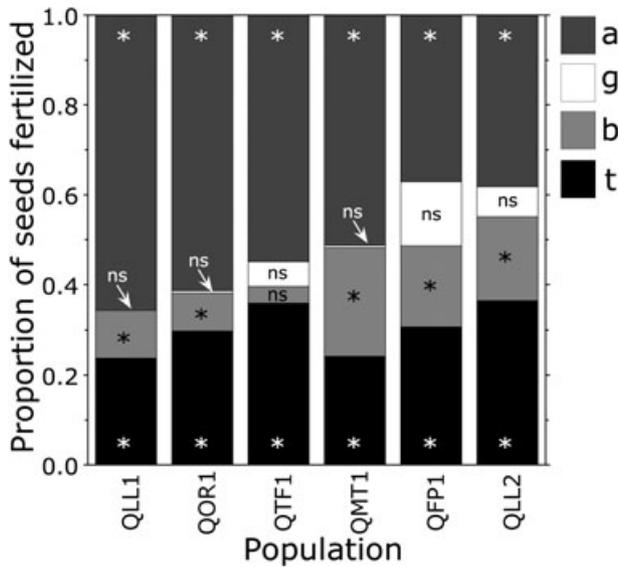


Fig. 8 Estimated components of the mating system for six natural populations of *Aquilegia canadensis* showing the proportion of seeds produced through outcrossing (t), biparental inbreeding (b), geitonogamous self-fertilization (g) and autogamous self-fertilization (a). Autogamous selfing was estimated by comparing the level of selfing in emasculated flowers (not capable of autogamy) with intact control flowers. The contribution of biparental inbreeding (b) to non-autogamous inbreeding ($g + b$) was estimated from the level of apparent selfing by fully emasculated plants incapable of any true selfing. Asterisks indicate estimates of individual components that are significantly greater than zero (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, based on 1000 bootstraps with the progeny array as the unit of resampling). Redrawn from C. R. Herlihy & C. G. Eckert (in press a).

mates of b based on apparent selfing by emasculated plants ($r = +0.78$).

Based on the premise that biparental inbreeding occurs as a consequence of fine-scale spatial genetic structure of reproductive plants combined with localized pollen transfer, b can also be estimated by comparing s_s between plants transplanted to random locations within populations (presumably away from close relatives) and control plants that have been dug up but replanted into their original locations. Griffin and Eckert (2003) applied this approach to two populations of *A. canadensis* and found that plants that had been moved engaged in substantially less apparent selfing than controls, indicating that, on average, 28% of progeny are the product of biparental inbreeding. This approach was also used by Kelly and Willis (2002) who did not detect any biparental inbreeding in two natural populations of *Mimulus guttatus*. Their results corroborate genetic data from the same populations showing that neighboring plants are not more related than expected by chance (Sweigart *et al.* 1999). Direct estimates of fine-scale genetic structure are not

available for natural populations of *A. canadensis*. However, using spatial genetic studies to corroborate the results of transplant studies assumes that genetic structure is sampled at the scale over which matings occur, which can be very difficult to verify. The elegance of the transplant approach is that it uses the plants themselves as probes for relevant genetic structure.

The concordance between emasculating and transplant experiments strongly suggests that biparental inbreeding is a substantial component of the mating system in populations of *A. canadensis*. By modifying both the costs and benefits of selfing, biparental inbreeding can, under some conditions, lead to the evolution of mixed mating systems (Uyenoyama 1986; Yahara 1992). On one hand, relatedness among mates reduces the genetic cost of outcrossing ($c_{out} = [1 - m_b]/2$) and, thus, the transmission advantage of selfing. In contrast, biparental inbreeding results in the expression of inbreeding depression among progeny produced via allogamous pollination, thereby reducing the selective advantage of cross-pollination (Waller 1993). All else being equal selfing is selected for when $c_{out} > \delta$ and is selected against when $c_{out} < \delta$ (Yahara 1992). Hence, biparental inbreeding should have the greatest influence on the evolution of selfing when c_{out} approximates δ . In our study populations of *A. canadensis*, biparental inbreeding substantially reduces c_{out} (mean $c_{out} = 0.24$; range among populations was 0.07–0.39). However, genetic estimates of δ are still very high ($\delta > 0.9$, Fig. 3), thus the cost of selfing greatly outweighs the costs of outcrossing.

Why self-fertilization?

Predominant self-fertilization by *A. canadensis* is an enigma. Based on the species' ecology, reproductive assurance appeared to be a likely explanation and we found evidence that autogamous selfing increases per-flower seed production in most populations. However, if our marker-based estimates of inbreeding depression are roughly accurate (i.e. within $\pm 20\%$ of the true δ , Fig. 6), then reproductive assurance cannot account for such high levels of self-fertilization in *A. canadensis* because the increase in seed production via selfing is associated with a significant loss of high-quality outcrossed seed (Figs 5,6). Thus, selfing appears to be maladaptive in *A. canadensis*. This conclusion is only valid if: (i) inbreeding depression is actually very strong; (ii) our experimental manipulations provide evolutionarily relevant alternative phenotypes; and (iii) the negative consequences of selfing that we estimated for female fitness are not compensated for by increased male fitness. We will now address these issues and then assess the most relevant adaptive explanations for mixed mating systems.

Three caveats

Our evaluation of the fitness consequences of selfing depends largely on the magnitude of inbreeding depression. However, all available data suggest that inbreeding depression is very strong in *A. canadensis*. The inbreeding coefficient of mature plants (F) is much lower than expected given the level of self-fertilization for populations across the geographic range (Fig. 3). For many populations F does not differ from zero, suggesting that selfed offspring rarely, if ever, survive to maturity. Because we obtained this result in a wide variety of geographically scattered populations it is unlikely that δ is overestimated because of violations of the assumptions underlying the marker-based estimator, such as the lack of inbreeding equilibrium or disequilibrium between marker genes and deleterious alleles (Charlesworth 1991). High estimates of δ have also been obtained for other species of *Aquilegia* using both allozyme markers ($\delta > 0.9$ for two populations of *A. vulgaris* and two populations of *A. viscosa*, C. R. Herlihy, S. Lavergne & C. G. Eckert, unpubl. data 2004; $\delta = 0.72$ for one population of *A. caerulea*, Montalvo 1994) and microsatellite markers ($\delta \approx 1$ for *A. pubescens* and *A. formosa*, S. A. Hodges, pers. comm., 2004), as well as from experimental comparisons of selfed versus outcrossed progeny over part of their life cycle under field conditions ($\delta = 0.54$ for one population of *A. caerulea*, Montalvo 1994). Direct comparisons of the performance of selfed versus outcrossed progeny conducted under field conditions are certainly required to verify our genetic estimates of δ , but at present we have no reason to suspect that inbreeding depression is not strong in *A. canadensis*.

The conclusion that selfing is maladaptive in *A. canadensis* is based on comparing intact plants capable of substantial autonomous autogamy with emasculated phenotypes rendered incapable of autogamy. This comparison is only evolutionarily relevant if populations of *A. canadensis* could evolve mechanisms to eliminate or largely avoid autogamy. In many perfect-flowered hermaphroditic plant species, self-pollination is limited developmentally by stigmas becoming receptive either before or after anthers have shed pollen (dichogamy), or physically by bearing receptive stigmas and dehiscing anthers at different positions within flowers (herkogamy). Both these mechanisms are associated with reduced selfing in a wide range of species (e.g. Schoen 1982; Belousoff & Shore 1995; Karron *et al.* 1997; Brunet & Eckert 1998; Elle & Hare 2002) and they both appear to be evolutionarily feasible ways of reducing selfing in *A. canadensis*. The genus *Aquilegia* includes closely related species that appear to be strongly dichogamous. Furthermore, some species produce protogynous flowers that present receptive stigmas before anther dehiscence (Chase & Raven

1975; Miller 1978), whereas others have protandrous flowers in which pollen is shed before stigmas become receptive (Miller 1978, 1985; Miller & Willard 1983; Brunet & Eckert 1998). The variability of dichogamy in the genus along with evidence suggesting that these species differentiated rapidly and recently (Hodges & Arnold 1994) suggest that the degree of dichogamy can be readily altered by selection in this group. *Aquilegia canadensis* was first described as protogynous based on the observation that stigmas are exerted from flowers a few days before stamens reflex downwards to present dehiscing anthers (Schneck 1901). However, analyses of pollination and pollen tube growth revealed that stigmas only become receptive and start receiving pollen when anthers begin to shed pollen (Griffin *et al.* 2000). Moreover, flowers rendered protogynous experimentally by removing the first anthers to dehisce did not self-fertilize less than control flowers from which randomly chosen anthers were removed, suggesting that a protogynous mutant would not benefit from reduced selfing and/or seed discounting in natural populations of *A. canadensis* (Griffin *et al.* 2000).

We have also investigated the feasibility of herkogamy as a mechanism to reduce selfing (C. R. Herlihy & C. G. Eckert, unpubl. data, 2004). Natural populations of *A. canadensis* exhibit striking variation in herkogamy. Floral measurements from 19 widely distributed natural populations revealed that herkogamy varies much more than other floral traits (mean population-level CV = 58% for herkogamy, 10% for pistil length and 9% for spur length). Furthermore, a greater proportion of this phenotypic variation in herkogamy is distributed among individual plants as opposed to among flowers within plants (mean among-plant variance component = 72% for herkogamy, 45% for pistil length and 41% for spur length). This marked phenotypic variation in herkogamy also appears to have a substantial genetic component. Herkogamy varied significantly among naturally pollinated seed families grown in a common environment. Moreover, there was a strong positive correlation between maternal herkogamy measured in the field and progeny herkogamy measured in the greenhouse for most populations. Finally, we used a categorical analysis in which plants were classified as possessing flowers with high or low herkogamy to show that increased herkogamy was associated with reduced selfing in 13 of 19 populations examined (Fig. 9). These results strongly suggest that natural selection could act on phenotypic variation in herkogamy to significantly reduce self-fertilization in these populations of *A. canadensis*. Yet, natural populations exhibit extraordinary variation in herkogamy.

Our cost-benefit analysis of self-fertilization in *A. canadensis* focused primarily on the consequences for female fitness. However, the evolution of selfing will

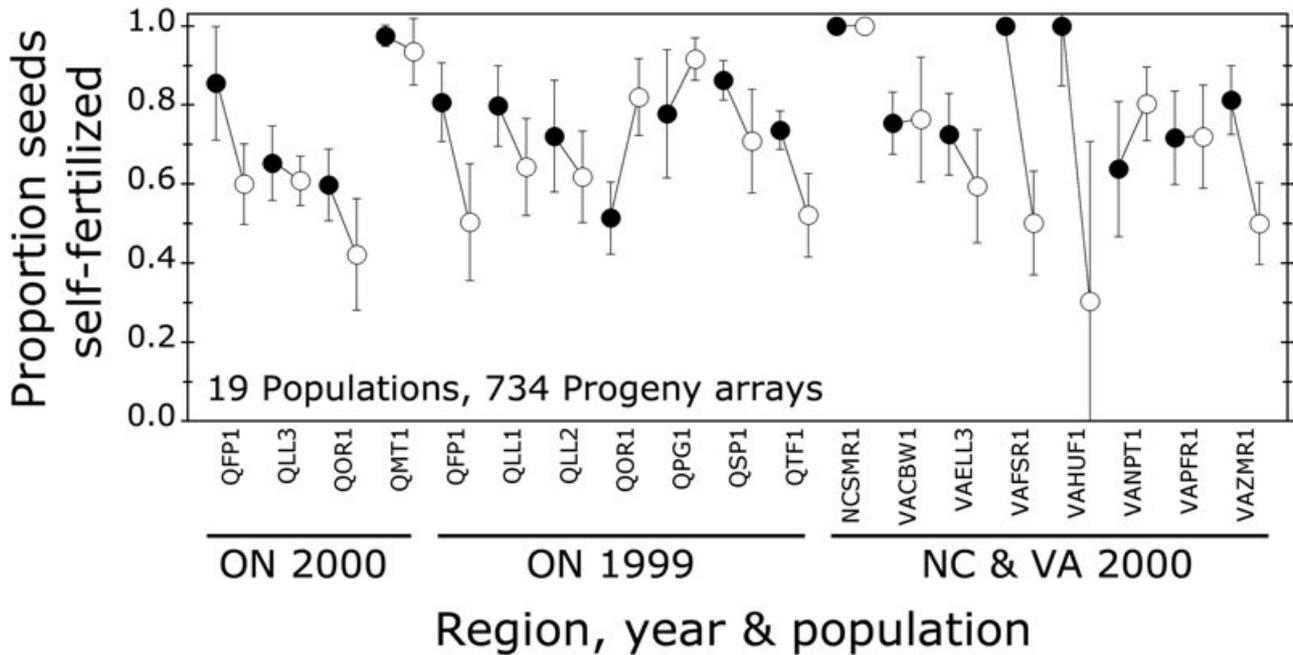


Fig. 9 Herkogamy, the spatial separation between dehiscing anthers and receptive stigmas within flowers, reduces self-fertilization in most natural populations of *Aquilegia canadensis*. Within each population maternal plants were divided into equally sized high (○) and low (●) herkogamy groups. Points are total selfing estimated using maximum likelihood for the high and low herkogamy plants jointly (joined by a line). Populations are grouped by region (ON = Ontario, Canada, in the northern portion of the geographic range, VA & NC = Virginia and North Carolina, USA, near the geographic center of the range). Error bars are ± 1 SE. Redrawn from C. R. Herlihy & C. G. Eckert (unpubl. data, 2004).

also be influenced by how it covaries with outcross siring success. In general, it is thought that, if anything, outcross siring should be compromised by selfing (i.e. pollen discounting Holsinger 1996). However, it is possible that the opposite may apply if a floral morphology that increases female selfing also increases outcross siring success (Harder & Wilson 1998; Johnston 1998). For example, Harder and Thomson (1989) showed by tracking pollen removal and deposition in *Erythronium grandiflorum* that pollen deposited on a bee's body so that it was most likely to become involved in self-pollination was also most likely to be deposited on the stigmas of subsequently visited plants. It is conceivable, therefore, that flowers of *A. canadensis* with anthers and stigmas in close proximity would sire more outcrossed progeny than those where anthers are positioned away from stigmas. However, herkogamy is determined primarily by stigma position rather than anther position in this species. Furthermore, pistil length, the main determinant of stigma position and herkogamy, correlates positively with both stamen number, pollen per stamen and total pollen per flower (C. R. Herlihy & C. G. Eckert, unpubl. data, 2004). It appears unlikely that selfing is maintained by a trade-off between male and female outcross success.

Adaptive explanations

Substantial phenotypic and genetic variation in herkogamy that significantly influences self-fertilization may suggest that predominant selfing represents an equilibrium condition in *A. canadensis*. As it has become apparent that a substantial proportion of flowering plants mix self-fertilization and outcrossing many theoretical explanations for the evolutionary stability of mixed mating systems have been advanced (reviewed by Jarne & Charlesworth 1993; Holsinger 1996; Charlesworth & Charlesworth 1998). However, a broad perusal of the theoretical literature does not yield any ready explanations for the evolutionary maintenance of high levels of selfing in the face of strong inbreeding depression, particularly if selfing causes severe seed discounting. For example, mixed mating might be evolutionarily stable if the consequences of selfing are frequency- or density-dependent (Chang & Rausher 1998; Cheptou & Dieckmann 2002), vary among lineages or populations (Uyenoyama *et al.* 1993; Cheptou & Mathias 2001), or differ for female versus male fertility (Rausher & Chang 1999). However, this usually only occurs when inbreeding depression is much weaker (i.e. $\delta < 0.5$) than it appears to be in *A. canadensis*. Such strong inbreeding depression in chronically selfing populations

can be explained under some conditions (Lande *et al.* 1994; Charlesworth & Charlesworth 1998; Morgan 2001). However, its occurrence, whatever the reason, clearly presents a challenge for any adaptive explanation of high selfing.

One possibility that we have not directly tested involves selection for self-fertilization at the metapopulation level. An obvious extension of the reproductive assurance hypothesis is that selfing might be favored during episodes of colonization when opportunities for outcrossing are low (Baker 1955). In this way, selfing might be favored at the metapopulation level even if it is disadvantageous within individual populations. It is tempting to view populations of *A. canadensis* as patches within a metapopulation because they are typically small and appear vulnerable to extinction during episodes of late spring drought that, based on bitter experience, seem fairly frequent in rock outcrop habitats with thin soils. Although we have never witnessed an extinction in more than 100 population years of monitoring we have observed abrupt year-to-year changes in population size and density (C. R. Herlihy & C. G. Eckert, unpubl. data, 2004, see also Mavragnis & Eckert 2001).

However, theoretical investigations of how metapopulation dynamics affect the evolution of selfing versus outcrossing reveal that, in addition to reproductive assurance mechanisms, selection also favors high seed production because only seeds engage in colonization of vacant habitat patches (Barrett & Pannell 1999). Hence in species with strong inbreeding depression, such as *A. canadensis*, outcrossing may be selected at the metapopulation level because it yields more viable seed than selfing, even though selfing provides reproductive assurance. It is also possible that outcrossed progeny may fare better in establishing after dispersal from a local habitat patch (Holsinger 1986). As a result, outcrossing should be favored in metapopulations where patch occupancy is relatively high, even if extinction is common, whereas selection for reproductive assurance via selfing will be strongest when a species is sparsely distributed across the landscape (Pannell & Barrett 1998). Populations of *A. canadensis* are very common in eastern Ontario, which is where we have studied the species in most detail. Furthermore, the proportion of seeds produced through outcrossing is highest in large, dense, open populations (Routley *et al.* 1999; Herlihy & Eckert in press a) and population size correlates positively with the per-capita production of viable seeds (Mavragnis & Eckert 2001). All this suggests that metapopulation dynamics if they actually occur in *A. canadensis* are likely to favor outcrossing rather than self-fertilization.

The goal of our research with *A. canadensis* was to test the simple adaptationist's expectation that in populations at equilibrium the costs of a particular mating strategy should be balanced by its benefits, and that no other rea-

sonable strategy should appear to have a more favorable cost-benefit ratio. Although our experimental approaches have shed new light on functional aspects of plant mating systems and provided empirical evidence for some major selective factors impinging on the evolution of selfing, the costs of selfing appear to greatly outweigh the benefits and our expectation of costs = benefits is not supported for *A. canadensis*. It may be the case that for many species the cost-benefit expectation is realized. For example, data from several species suggest that high levels of outcrossing are maintained by strong inbreeding depression or that predominant selfing is associated with very weak inbreeding depression (see Husband & Schemske 1996). There are also species like *Decodon verticillatus* where, although 30% of seeds are produced through selfing despite very strong inbreeding depression ($\delta \approx 0.9$), most selfing occurs through geitonogamy. Hence, selfing can be viewed as an incidental cost of other life history traits (i.e. large size, clonal propagation and mass-flowering, Eckert 2000). However, the adaptive significance of mixed mating systems where substantial selfing occurs through autogamy has not been fully explained for any species. Furthermore, the perplexing combination of high levels of selfing combined with strong inbreeding depression, as represented by *A. canadensis* and other species of *Aquilegia*, may not be uncommon (see fig. 2 in Husband & Schemske 1996). Determining the ecological and evolutionary processes responsible for these enigmatic mating systems remains a major challenge for plant evolutionary biologists.

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