Frequency-dependent variation in reproductive success in *Narcissus*: implications for the maintenance of stigma-height dimorphism

John D. Thompson[1*], Spencer C. H. Barrett[2] and Angela M. Baker[2]†

[1]*Centre d'Ecologie Fonctionnelle et Evolutive, CNRS, 1919 Route de Mende, 34293 Montpellier Cedex 5, France*
[2]*Department of Botany, University of Toronto, 25 Willcocks Street, Toronto, ON M5S 3B2, Canada*

Negative frequency-dependent selection is a major selective force maintaining sexual polymorphisms. However, empirical demonstrations of frequency-dependent reproductive success are rare, particularly in plants. We investigate this problem by manipulating the frequencies of style morphs in a natural population of *Narcissus assoanus*, a self-incompatible herb with style-length dimorphism and intra-morph compatibility. We predicted that the reproductive success of morphs would vary negatively with their frequency because of the effects of morph-specific differences in sex-organ position on patterns of pollen transfer. This prediction was generally supported. The fruit and seed set of the two morphs did not differ significantly in plots with 1 : 1 morph ratios. However, short-styled plants produced significantly fewer seeds than long-styled plants in monomorphic plots, and significantly more seeds than long-styled plants in plots with 'long-biased' morph ratios. These patterns indicate that in the absence of physiological barriers to intra-morph mating, negative frequency-dependent selection contributes to the maintenance of stylar polymorphism through inter-morph pollen transfer. Our experimental results also provide insights into the mechanisms governing the biased style-morph ratios in populations of *Narcissus* species.

Keywords: Amaryllidaceae; frequency-dependent selection; pollination; stigma-height dimorphism

1. INTRODUCTION

Frequency-dependent selection is of fundamental importance for the evolutionary maintenance of genetic polymorphisms (Clarke & O'Donald 1964; Endler 1986). In flowering plants, negative frequency-dependent selection maintains sexual polymorphisms because cross-compatible mating types benefit reproductively from the presence of other morphs. Thus, the fitness of a sexual morph will depend on the frequencies of alternate morphs in a population. This form of selection commonly leads to an equilibrium in which all morphs have equivalent fitness (Fisher 1930).

To understand the precise mechanisms underlying frequency-dependent mating, the relative frequencies of different morphs need to be manipulated in natural conditions. This is particularly important for the study of sexual polymorphisms in animal-pollinated plants whose reproductive success is closely tied to rates of pollen transfer. In plants, empirical evidence for negative frequency-dependent selection is limited to studies of either temporal variation in morph ratios (e.g. Curtis & Curtis 1985; Eckert *et al.* 1996) or reproductive success in artificial populations (Smithson & MacNair 1996; McCauley & Brock 1998). To our knowledge, no study has manipulated the ratios of sexual morphs in order to investigate how reproductive success may be negatively frequency dependent in natural conditions. This experimental approach could also reveal why several plant species with sexual polymor-phisms exhibit wide variation in morph ratios in natural populations (reviewed in Arroyo *et al.* 2002).

Species with stigma-height dimorphism provide useful systems in which to explore the role of negative frequency-dependent selection on the maintenance of sexual polymorphisms (Lloyd & Webb 1992*a,b*; Barrett *et al.* 1996). In such species, populations comprise two floral morphs that differ significantly in stigma height, but in which the anthers are located at similar positions. In long-styled plants (hereafter L-morph) the stigma is positioned above the anthers and in short-styled plants (hereafter S-morph) the stigma is positioned below the anthers (figure 1). Stigma-height dimorphism occurs in approximately a dozen species of insect-pollinated *Narcissus* and has evolved independently in three sections of the genus (Barrett *et al.* 1996). In *Narcissus*, plants are usually self-incompatible, but fully cross-compatible with all other individuals in a population, regardless of morph identity. In this regard they differ from typical heterostylous species, which usually exhibit both self- and intra-morph incompatibility. The potential for intra-morph mating in *Narcissus* populations has important implications for the stability of the polymorphism, because morph-specific differences in intra-morph mating and fertility may explain why morph ratios deviate significantly from 1 : 1 in several species (Barrett *et al.* 1996; Baker *et al.* 2000*b*; Arroyo *et al.* 2002). Although 1 : 1 morph ratios occur in populations of some species, populations commonly contain 2–3 times as many plants of the L-morph as the S-morph, or contain only the L-morph. Such unequal morph ratios contrast with the 1 : 1 ratios typical of distylous species, in which the negative frequency-dependent selection associated with intra-morph incompatibility causes any deviation in morph ratio to return to 1 : 1 after each cycle of

*Author for correspondence (john.thompson@cefe.cnrs-mop.fr).
† Present address: Department of Biology, University of Oulu, PO Box 3000, Fin 90014, Finland.

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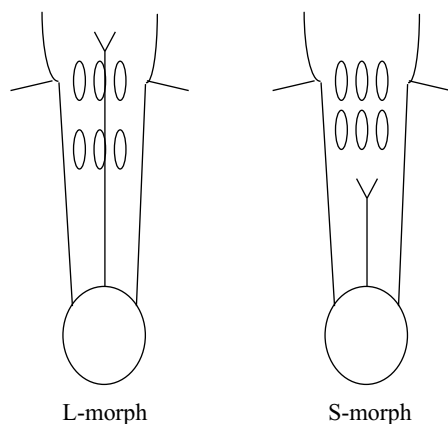DOI 10.1098/rspb.2002.2318

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Figure 1. Diagram of the relative position of stigmas and anthers in long-styled (L-morph) and short-styled (S-morph) flowers of *Narcissus assoanus*.

mating (Charlesworth & Charlesworth 1979). In terms of morphology, mating and morph ratios, stigma-height dimorphism is distinct from classical heterostyly.

The underlying ecological and genetic mechanisms that cause biased morph ratios in *Narcissus* remain to be fully explained. Barrett *et al.* (1996) proposed that effective pollen transfer among plants of the L-morph is likely to be more proficient because herkogamy is relatively weak in this morph and stigmas and anthers contact similar locations on the bodies of visiting insect pollinators. By contrast, the well-developed herkogamy of the S-morph may reduce the precision of pollen transfer among plants of this morph (Barrett *et al.* 1996; Baker *et al.* 2000b,c). Theoretical models indicate that greater intra-morph mating in the L-morph relative to the S-morph will result in populations in which the L-morph predominates (Barrett *et al.* 1996; Baker *et al.* 2000c).

We report the results of a field experiment that addresses these issues using *Narcissus assoanus*. By manipulating the morph composition of patches in a natural population we investigate the influence of morph structure on pollen transfer within and between morphs. We used variation in female fertility (fruit and seed set) to assess the effectiveness of pollen transfer in three contrasting treatments: monomorphic plots, L-biased plots and plots with 1 : 1 morph ratios. We tested four predictions: (i) plants in monomorphic plots have lower fertility than plants in dimorphic plots due to less effective pollen transfer; (ii) in monomorphic plots, the S-morph has lower fertility than the L-morph because greater herkogamy of the S-morph causes reduced cross-pollen transfer; (iii) the fertility of the S-morph exceeds that of the L-morph in L-biased plots because of negative frequency-dependent pollen transfer; (iv) the L- and S-morphs have equivalent fertility in plots with 1 : 1 morph ratios. Because *N. assoanus* is frequently pollen limited (Baker *et al.* 2000a), variation in female fertility may be strongly influenced by patterns of pollen transfer within and between morphs.

2. MATERIAL AND METHODS

(a) *The study system*

Narcissus assoanus is a diminutive perennial geophyte common in southern France and Spain in lowland garrigues vegetation

and upland grassland on limestone. Each year, flowering plants in this region usually produce a single yellow, insect-pollinated flower with a bowl-shaped corona and a long narrow floral tube (figure 1). Both style morphs produce small amounts of seed set on selfing, indicating moderate self-sterility, and within-morph and between-morph crosses produce equivalent amounts of seed (Baker *et al.* 2000b,c). Our study was undertaken from March to May 1999 in a natural population of *N. assoanus* at Ceyrac, *ca.* 40 km north of Montpellier in southern France. We chose this site for two reasons. (i) The population is very large covering an extensive area and containing many thousands of plants. High flowering density allowed us to manipulate the morph ratios of local patches. (ii) The natural style-morph ratio of the Ceyrac population is 1 : 1. This allowed us to establish monomorphic and dimorphic patches at densities typical for the species in the study region.

(b) *Experimental treatments*

On 19 March, as flowering began at the site, we randomly set out five replicate plots of each of three morph-ratio treatments in areas where many flower buds were emerging. The treatments were as follows. (i) Monomorphic plots in which only one morph was allowed to flower. (ii) L-biased plots with a ratio of flowers of the L- and S-morphs of 2 : 1 (i.e. the average morph ratio for lowland populations in this region). (iii) 1 : 1 plots with an equal number of flowers of the L- and S-morphs. We did not include an S-biased morph ratio, because populations with this morph ratio have not been observed in *N. assoanus* (Baker *et al.* 2000b). The area of each plot was slightly less than 1 m² and was surrounded by a 1 m border in which we removed all flower buds of *N. assoanus* during the course of the experiment. On average, plots of the four treatments produced equivalent number of flowers (ANOVA, $F_{2,17} = 2.4$, $p > 0.1$: PROC MIXED in SAS (2001)), with means (minimum–maximum) of 27 (17–58) in monomorphic plots, 41 (26–63) in L-biased plots and 30 (18–54) in 1 : 1 plots. As required by the experimental design, flower number differed significantly between morphs in the L-biased treatment ($F_{1,8} = 18.9$, $p < 0.01$), but not in monomorphic ($F_{1,8} = 0.2$, $p > 0.5$) or 1 : 1 ($F_{1,8} = 0.01$, $p > 0.5$) plots.

Observations of pollinator foraging (cleopatra butterflies (*Gonepteryx cleopatra*: Lepidoptera), hawkmoths (*Macroglossum stellatarum*: Lepidoptera) and, to a lesser extent, bees (*Anthophora* sp.: Hymenoptera)) indicated that pollinators frequently visited many flowers before leaving a plot. We thus expected that pollen delivery to stigmas from outside the plots would not be high enough to erase any reproductive signal arising from differential pollen transfer associated with our experimental treatments.

In the period 19 March to 6 April (except 25–27 March when it rained) we visited all plots every 2 days before 1000 to maintain the required flowering morph ratios by bud removal before pollinator activity. All experimental flowers were marked at the base of the stem with white tape to identify their morph. The absence of a tag allowed us to identify newly opened flowers during each visit. Stems with more than one flower (less than 5% of plants in this population) were removed. The experiment involved a total of 335 flowers of the L-morph and 291 flowers of the S-morph.

In mid-April we visited the site to record fruit set and damage by herbivory. On 14 May we checked all marked stems of plants in the experimental plots and harvested all fruits to assess fruit set, seed set and ovule number. During October 1999 seeds of

each fruit were planted in a 1 : 1 : 1 mix of sand, humus and garden soil in individual compartments in seed trays and randomly positioned in an experimental glasshouse at the CEFE-CNRS in Montpellier. Germination was recorded in April 2000 and April 2001.

(c) *Data analysis*

We analysed the proportional data for fruit set per flower, seed set per ovule and germination per seed by using generalized linear models with a binomial error distribution (McCullagh & Nelder 1989; PROC GENMOD in SAS (2001)). We did not perform an overall analysis on the whole dataset because both morphs were not present in all plots. We thus analysed fertility in two ways. First, we assessed treatment effects independently for each morph, using *a posteriori* contrasts to determine which treatments differed significantly. In these analyses the plot effect was nested within treatment. Second, to assess whether morphs differed in fertility we analysed the data separately for each treatment. For the analysis of the monomorphic treatment, plots were nested within morphs whereas in the analyses of dimorphic treatments, plots were treated as a fixed effect. Ovule number does not differ ($F_{1,437} = 0.54$, $p > 0.05$) between the L- and S-morphs in the study population.

3. RESULTS

Fruit set varied significantly among the morph-ratio treatments for the S-morph (d.f. = 2; $G = 12.4$, $p < 0.01$), but not the L-morph (d.f. = 2; $G = 3.08$, $p > 0.1$; figure 2*a*). Contrasts among treatments for the S-morph showed that fruit set was significantly higher in the L-biased treatment than in both the monomorphic (d.f. = 1; $G = 12.01$, $p < 0.001$), and 1 : 1 plots (d.f. = 1; $G = 4.01$, $p < 0.05$; figure 2*a*). There was no significant difference in S-morph fruit set between the monomorphic and 1 : 1 morph-ratio treatments (d.f. = 2; $G = 2.66$, $p > 0.1$). Correction of a threshold p-value of 0.05 to account for three contrasts (using the Dunn–Sidak method) gives a p-value of 0.017. At this level only the contrast between monomorphic and L-biased treatments remained significant. Fruit set did not vary significantly among plots within each treatment. Comparisons between style morphs within each treatment revealed that the S-morph set significantly fewer fruit than the L-morph in monomorphic plots (d.f. = 1; $G = 6.13$, $p < 0.05$) but the two morphs had equivalent fruit set in the L-biased and 1 : 1 plots (d.f. = 1; $G = 0.15$, $p < 0.5$ in both treatments; figure 2*a*).

Proportional seed set differed significantly between treatments in the L-morph (d.f. = 2; $G = 9.45$, $p < 0.05$) and the S-morph (d.f. = 2; $G = 17.18$, $p < 0.001$; figure 2*b*). Both morphs showed significant variation among plots within treatments ($p < 0.001$). The L-morph set significantly more seeds in 1 : 1 plots than in both the L-biased (d.f. = 1; $G = 9.28$, $p < 0.05$) and monomorphic (d.f. = 1; $G = 5.44$, $p < 0.05$) plots, which did not differ from each other (d.f. = 1; $G = 0.63$, $p > 0.1$). By contrast, the S-morph set significantly fewer seeds in monomorphic plots than in either the L-biased (d.f. = 1; $G = 17.09$, $p < 0.001$) or 1 : 1 plots (d.f. = 1; $G = 4.94$, $p < 0.05$), which did not differ from each other (d.f. = 1; $G = 2.93$, $p < 0.05$). After correction of p-values only the contrast between monomorphic and L-biased plots was significant.
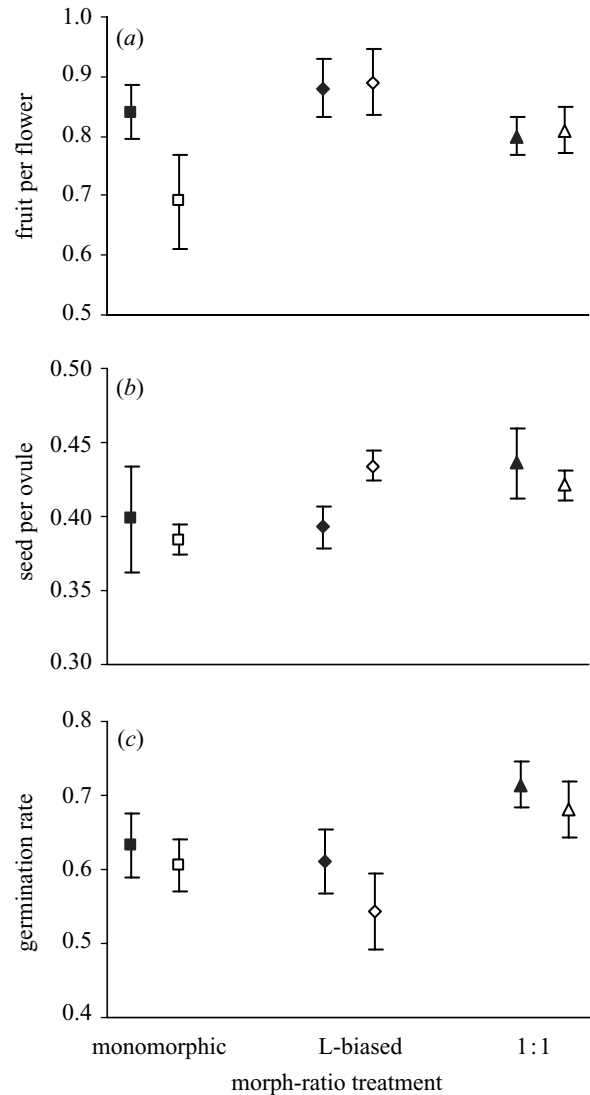

Figure 2. The influence of style-morph ratios on components of reproductive success (mean ± s.e.m. in the L- (filled symbols) and S-morphs (open symbols) of *Narcissus assoanus*. The three experimental morph-ratio treatments (monomorphic: squares—plots containing a single style morph; L-biased: diamonds—plots with a 2 : 1 ratio of the L- and S-morphs; 1 : 1: triangles—plots with an equal proportion of the L- and S-morphs) were established in a natural population at Ceyrac, southern France. (*a*) Proportion of flowers setting fruit; (*b*) proportion of ovules setting seed; (*c*) proportion of germinating seeds.

The S-morph set significantly more seed than the L-morph in the L-biased plots (d.f. = 1, $G = 19.34$, $p < 0.001$), but not in the monomorphic (d.f. = 1; $G = 2.39$, $p > 0.1$) or 1 : 1 (d.f. = 1; $G = 0.94$, $p > 0.1$) plots.

Seed germination also differed significantly among treatments for both the L-morph (d.f. = 2; $G = 38.05$, $p < 0.001$) and the S-morph (d.f. = 2; $G = 37.93$, $p < 0.001$; figure 2*c*). For both morphs, relatively more seeds germinated in 1 : 1 plots than in either monomorphic plots (L-morph, d.f. = 1, $G = 16.04$, $p < 0.001$; S-morph, d.f. = 1, $G = 18.38$, $p < 0.001$) and L-biased plots (L-morph, d.f. = 1, $G = 38.04$, $p < 0.001$; S-morph, d.f. = 1, $G = 36.48$, $p < 0.001$), which did not differ ($p > 0.05$: figure 2*c*). The L-morph showed significantly higher seed germination than the S-morph in all three treatments
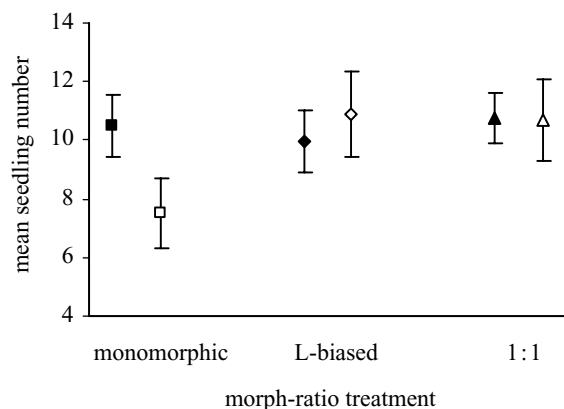
Figure 3. The number of germinating seeds produced per flower (mean ± s.e.m.) in the L- (filled symbols) and S-morphs (open symbols) of *Narcissus assoanus*. The three experimental morph-ratio treatments (monomorphic: squares—plots containing a single style morph; L-biased: diamonds—plots with a 2 : 1 ratio of the L- and S-morphs; 1 : 1: triangles—plots with an equal proportion of the L- and S-morphs) were established in a natural population at Ceyrac, southern France.

(monomorphic plots, d.f. = 1, $G = 4.64$, $p < 0.05$; L-biased plots, d.f. = 1, $G = 8.82$, $p < 0.01$; 1 : 1 plots, d.f. = 1, $G = 4.83$, $p < 0.05$; figure 2c). There was no significant correlation between seed number and germination rate in any of the morph by treatment combinations.

To compare the female fertility of the two style morphs in relation to morph-ratio variation we calculated the total number of germinating seeds produced by each marked flower. The distribution of these data involved two major peaks, one corresponding to flowers that produced no fruit and the second associated with a near-normal distribution of all non-zero values. Although this bimodal distribution prevents statistical analysis, the S-morph suffered 28% lower fertility than the L-morph in monomorphic plots, but realized 10% higher fertility in L-biased plots and equivalent fertility in 1 : 1 plots (figure 3).

4. DISCUSSION

The frequency-dependent variation in female fertility that we observed in *N. assoanus* supports our predictions about differences in cross-pollen transfer between morphs. The S-morph had lower fertility in monomorphic plots relative to L-biased and 1 : 1 plots. In the last two treatments, the S-morph had higher fertility in L-biased plots than in 1 : 1 plots. By contrast, the L-morph had lower fertility in both monomorphic and L-biased treatments than in 1 : 1 plots. Associated with these different responses to morph-ratio variation, the S-morph had lower fertility than the L-morph in monomorphic plots but greater fertility than the L-morph in L-biased plots. As predicted, in 1 : 1 plots the two morphs had equivalent fertility. In the following sections we discuss the implications of these results for the maintenance of stigma-height dimorphism and the biased style-morph ratios in populations of *N. assoanus* and other species in this genus.

(a) Sex-organ position and context-dependent pollen transfer

The position of sex organs in animal-pollinated flowers plays an important part in the dispersal of pollen to conspecific stigmas. In species with a polymorphism for sex-organ position, opportunities exist for negative frequency-dependent pollen transfer. For example, in heterostylous plants pollen transfer between plants in a population containing a single style morph should be less proficient than in a polymorphic population because of the absence of stigma–anther reciprocity (fig. 1 in Barrett 2002). Experimental studies (Kohn & Barrett 1992) and fertility variation in natural populations (Wyatt & Hellwig 1979) support this expectation.

We interpret the differences in fertility observed between monomorphic and dimorphic treatments as resulting from variation in the amounts of cross-pollen transfer. In monomorphic plots the fertility of the S-morph was 28% lower than that of the L-morph (figure 3), primarily because of reduced fruit set (figure 2a). The lower fertility of the S-morph probably occurred because well-developed herkogamy reduced the precision of cross-pollen transfer, resulting in insufficient pollen deposition to maximize fruit and seed set. By contrast, pollen transfer in the L-morph is not constrained by well-developed herkogamy and stigmas and anthers contact similar locations on the bodies of visiting pollinators. Monomorphic populations composed of the L-morph are common in several sexually dimorphic *Narcissus* species (e.g. *N. dubius*; Baker et al. (2000a) and *N. papyraceus*; Arroyo et al. (2002)), but have not been observed in *N. assoanus* despite surveys of 60 populations (Baker et al. 2000a; J. D. Thompson, unpublished data). Theory predicts that a rare-morph advantage of the type we found in L-biased plots accentuates with strongly biased morph ratios (Clarke 1975; Endler 1986). If this is the case in *N. assoanus*, it may help to explain why no populations of this species appear to have lost the S-morph (Baker et al. 2000a).

(b) Negative frequency-dependent selection and the maintenance of sexual polymorphisms in plants

By manipulating the local frequencies of the L- and S-morphs in a *N. assoanus* population and leaving plants to be pollinated naturally, we demonstrated significant negative frequency-dependent variation in the maternal fertility of the two style morphs. Moreover, the specific patterns of female fertility that we detected were largely congruent with predictions based on a previous model of pollen transfer in *Narcissus* inferred from morph-specific differences in floral morphology (Barrett et al. 1996). We obtained these results despite the potentially confounding influence of pollen transfer from plants outside our experimental plots. The variation we observed presumably arose because pollinator service was not sufficient to maximize seed set at the site. Populations of *N. assoanus* frequently receive very low pollinator service and earlier studies close to the Ceyrac site indicated that seed set was pollen limited in some years (Baker et al. 2000a). Although it is often assumed that heterostyly and stigma-height dimorphism largely function to increase fitness through male reproductive function by reducing pollen wastage (Lloyd & Webb 1992a,b; Barrett et al. 2000, but see Ganders 1975),

our results indicate that stylar polymorphism may also benefit maternal fitness in pollen-limited conditions.

Our results provide an experimental demonstration in a natural population of how negative frequency-dependent selection may act to stabilize plant sexual polymorphisms. In L-biased plots the S-morph had a rare-morph advantage, realizing significantly higher reproductive success than the more abundant L-morph. This advantage should increase the frequency of the rare morph. Indirect evidence for a frequency-dependent advantage to the rare morph has also been detected in tristylous *Lythrum salicaria*, in which the rare morph increased significantly in frequency over time, as predicted theoretically (Eckert *et al.* 1996). Temporal surveys of morph-ratio variation in populations of *N. assoanus* which had L-biased morph ratios in the mid-1990s (see Baker *et al.* 2000*b*) would be useful to determine whether the fertility differences we detected in our experiments translate into changes in morph ratio. Interestingly, our earlier investigations (Baker *et al.* 2000*c*) of variation in female fertility in several dimorphic populations of *N. assoanus* with L-biased ratios failed to detect morph-specific differences of the type we observed in our experiment. Therefore, the fitness of the morphs may already have equalized in these populations, with L-biased morph ratios representing a stable equilibrium.

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