



Title	When spring ephemerals fail to meet pollinators : mechanism of phenological mismatch and its impact on plant reproduction
Author(s)	Kudo, Gaku; Cooper, Elisabeth J.
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1 **When spring ephemerals fail to meet pollinators: mechanism of phenological**
2 **mismatch and its impact on plant reproduction**

3

4 Gaku Kudo^{1*} and Elisabeth J Cooper²

5

6 ¹ Faculty of Environmental Earth Science, Hokkaido University, Sapporo 060-0810,
7 Japan

8 ² Institute for Arctic and Marine Biology, UiT-The Arctic University of Norway, NO-9037
9 Tromsø, Norway

10

11 *Author for correspondence (gaku@ees.hokudai.ac.jp)

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13

14

15 **Abstract**

16

17 The flowering phenology of early-blooming plants is largely determined by snowmelt
18 timing in high-latitude and high-altitude ecosystems. When the synchrony of flowering
19 and pollinator emergence is disturbed by climate change, seed production may be
20 restricted due to insufficient pollination success. We revealed the mechanism of
21 phenological mismatch between a spring ephemeral (*Corydalis ambigua*) and its
22 pollinator (overwintered bumble bees), and its impact on plant reproduction, based on
23 19 years of monitoring and a snow removal experiment in a cool-temperate forest in
24 northern Japan. Early snowmelt increased the risk of phenological mismatch under
25 natural conditions. Seed production was limited by pollination success over the three
26 years of pollination experiment and decreased when flowering occurred prior to bee
27 emergence. Similar trends were detected on modification of flowering phenology
28 through snow removal. Following snowmelt, the length of the pre-flowering period
29 strongly depended on the ambient surface temperature, ranging from 4 days (at >7°C)
30 to 26 days (at 2.5°C). Flowering onset was explained with an accumulated surface
31 degree-day model. Bumble bees emerged when soil temperature reached 6°C, which
32 was predictable by an accumulated soil degree-day model, although foraging activity
33 after emergence might depend on air temperature. These results indicate that
34 phenological mismatch tends to occur when snow melts early but subsequent soil
35 warming progresses slowly. Thus, modification of the snowmelt regime could be a
36 major driver disturbing spring phenology in northern ecosystems.

37

38 **Keywords:** *Bombus*, global warming, phenological mismatch, pollinator, snowmelt,
39 spring ephemeral

40

41 1. Background

42

43 The phenology of diverse organisms has changed in response to ongoing global
44 warming [1–3]. If the environmental cues determining phenological events differ or the
45 sensitivity to environmental cues varies among species, phenological synchrony
46 between interacting species may be disturbed by climate change [4,5]. Plant-pollinator
47 interactions are a key mutualism in terrestrial ecosystems. Phenological mismatch
48 disrupts these mutualistic relationships when the temporal overlap of flowering and
49 pollinator activity is decreased by phenological modifications, and it may result in
50 population declines in plants and/or insects [4]. The possibility of plant-pollinator
51 phenological mismatch with changing climate is widely discussed. Significant
52 phenological mismatch was reported between specific plants and pollinators in some
53 studies [6,7], while less significant or unclear trends were found in other studies that
54 examined assemblages of interacting species [3,8,9]. This discrepancy suggests that
55 phenological mismatch can occur between particular interacting species but broader
56 assemblages are more robust. [9,10]. Although phenological shifts in response to
57 climatic change are well known, our knowledge about the mechanism and ecological
58 impacts of phenological mismatch is more limited [10–12].

59 In addition to the analyses of historical records and long-term monitoring of
60 phenologies of interacting species, experimental regulations of phenologies are
61 effective approaches to test the occurrence of phenological mismatch [10, 12, 13].
62 Several experimental studies investigated this using artificial regulation of flowering
63 phenology [14–16], while experimental studies controlling the timing of pollinator
64 emergence are limited [17]. Furthermore, the ecological significance of phenological
65 mismatch in terms of fitness of interacting species is rarely evaluated [7,14]. To better
66 understand the prevalence and impact of phenological mismatch given ongoing
67 environmental change, it is crucial to clarify the factors governing the phenological
68 responses of interacting species and evaluate the effect of mismatch on fitness.

69 Synchrony of interacting species is sensitive to climate fluctuations, especially
70 when development occurs rapidly during short growing seasons, and so even small
71 differences in phenological responses may cause significant mismatch. Flowering
72 phenology in arctic, alpine, and boreal ecosystems is strongly influenced by warming
73 [16, 18]. Furthermore, the vulnerability of phenological events varies temporally, and
74 spring phenologies are most susceptible to climate fluctuations [1,2,14,19]. Spring
75 ephemerals, that have a short growing period between snowmelt and canopy closure
76 of overstory vegetation, grow fast and have potentially high reproductive activity [20]

77 but their pollination success is a primary factor limiting seed production [20,21]. They
78 are therefore most at risk from such a phenological mismatch.

79 Bumble bees (*Bombus* spp., Apidae) are important pollinators for many plant
80 species in temperate, alpine, and subarctic ecosystems [22]. In early spring,
81 overwintered queens visit spring ephemerals for nectar before establishing the colony,
82 and the timing of queen bee emergence can strongly affect the pollination success of
83 early-blooming plants [6,7]. Subsequent colony development determines the amount of
84 floral resources (pollen and nectar) required, and the availability of floral resources
85 during the colony development influences the number of workers and production of
86 new queen and male bees [22]. This cascade effect forms the link between flowering
87 phenology, plant, and pollinator populations [21]. Any degradation of phenological
88 matching between spring ephemerals (as a nectar resource) and queen bees may
89 therefore have negative impacts, not only on the pollination success of spring
90 ephemerals, but also on colony development and its subsequent pollination service to
91 late-blooming, bumble bee-pollinated plants.

92 Our previous study [7] conducted in natural cool-temperate forests of Japan
93 reported that flowering onset of a spring ephemeral (*Corydalis ambigua*) and
94 emergence of queen bees were related in different ways to the timing of snowmelt. The
95 phenological mismatch between them increased with earlier snowmelt time when
96 flowering onset was accelerated more rapidly than queen bee emergence, resulting in
97 lower pollination success in early springs [7]. Since that study was based on the
98 observation of natural populations without any experimental treatment, the
99 determinants of flowering phenology and emergence timing of queen bees were not
100 clearly defined, and any generalization regarding the impacts of phenological mismatch
101 on pollination service to spring ephemerals was limited.

102 In the present study, in addition to long-term monitoring of natural conditions (19
103 years), we conducted a snow removal experiment to manipulate flowering phenology of
104 *C. ambigua* for three years in order to reveal the mechanism of phenological mismatch
105 and its ecological impacts on pollination success. The aims of this study were to: (1)
106 Record the spring phenology of *C. ambigua* and its queen bee pollinator and describe
107 the relationship between the snowmelt timing, degree of phenological mismatch, and
108 seed production, using (a) long-term monitoring data and (b) experimental
109 manipulation of snowmelt. (2) Clarify the environmental cues that determine flowering
110 onset and queen bee emergence and the mechanism of phenological mismatch. We
111 hypothesized that the flowering phenology of the spring ephemeral is determined by
112 the combination of snowmelt timing and subsequent ambient surface temperature,

113 while the emergence of bumble bees from hibernation may be determined by the soil
114 temperature that overwintering bees experience [23].

115

116 **2. Methods**

117

118 **(a) Study site and system**

119 This study was conducted in a natural deciduous forest in Nopporo (43°25'N,
120 143°32'E), Hokkaido, northern Japan. This forest is located on a flat area at 50–75 m
121 elevation (figure S1). Snow usually covers the ground from early December to early
122 April, and the soil does not freeze at this time due to the insulating layer of snow;
123 maximum winter snow depth is 80–100 cm. Annual mean air temperature is 7.1°C,
124 ranging from –6.3°C (January) to 20.6°C (August), and annual precipitation is 930 mm.
125 Leaf emergence of canopy trees usually occurs in mid-May, and the understory is
126 shaded by closed canopy until mid-October. From the snowmelt in April to canopy
127 closure in late May, flowering of spring bloomers progresses sequentially among
128 species, including *Adonis ramose*, *Petasites japonicus* var. *giganteus*, *Corydalis*
129 *ambigua*, *Trillium apetalon*, and *Anemone flaccida*, in that order.

130 *Corydalis ambigua* Chem. Et Schlecht (Papaveraceae) is a common spring
131 ephemeral species in northern Japan. Each plant produces one or two inflorescences
132 and each of the three to 20 zygomorphic flowers has a spur in which nectar collects.
133 There are some variations in flower color but it is commonly mauve or purple. This
134 species is self-incompatible and dominantly visited by bumble bees [23]. Shoots
135 emerge soon after snowmelt, flowering season is usually from mid-April to early May,
136 and aboveground parts die after seed dispersal in late May. Thus, it has a typical life-
137 history of spring ephemerals. It is a perennial, non-clonal species.

138 Queens of the bumble bee, *Bombus hypocrita sapporoensis* Cockerell, a major
139 pollinator of *C. ambigua*, usually emerge from hibernation coincident with flowering of
140 this plant [7,21]. Due to high nectar production and formation of dense populations, *C.*
141 *ambigua* is the most important nectar resource for queen bees soon after emergence
142 [21]. Queen bees usually suck nectar by perforating spurs of flowers and seldom visit
143 legitimately but they are an available pollinator owing to accidental pollen removal and
144 deposition during nectar robbing [24]. It has been shown that *B. hypocrita* carried out
145 about 90% of pollinator visits to *C. ambigua* flowers and the remaining 10% of visitors
146 were queens of *B. ardens sakagamii* and *B. diversus tersatus* [25].

147

148 **(b) Monitoring of plants and pollinators**

149 Monitoring of the flowering period of *C. ambigua* and date of first emergence of queen
150 bees was conducted during 1999–2017. Flowering phenology was observed within a
151 20 m x 20 m area in the central part of a large population (>1 ha). At the same time,
152 seed-set rates under natural pollination were recorded for 30–60 plants randomly
153 selected every year except for 2004. During the flowering period, the number of flowers
154 of tagged plants was recorded and all fruits (pods) were harvested before seed
155 dispersal. Sampled pods were carefully opened in the laboratory, and the number of
156 mature seeds and undeveloped ovules were counted. Seed-set rate at the
157 inflorescence level was calculated as a ratio of matured seed number to total ovule
158 number. Individual flowers have 9.1 ovules on average, ranging from 4 to 14. Ovule
159 production of aborted flowers was estimated from the mean number of ovules per pod
160 of the same inflorescence.

161 The emergence of queen bees was observed by walking along a 1.2 km trail in the
162 forest providing access to the study site (figure S1). Searching for bee emergence
163 started when snow melted at the trail, and normally we carried out a survey every other
164 day, but not when it was rainy, snowy or cool (< 5°C). Observation was conducted by
165 1–3 people (including G. Kudo), and observation periods were continued until the first
166 queen bee was observed along the trail. We used 1–3 hours each time to search for
167 flower visitation or flying queen bees and for foraging scars on *C. ambigua* flowers
168 along the trail. Since *C. ambigua* is the earliest major nectar resource for overwintered
169 bees, the first detection of nectar robbing scars reflects the time of emergence from
170 hibernation when flowering occurred ahead of bee emergence. There may be some
171 time lag between the time of emergence and the start of nectar robbing. However, we
172 assumed that the time-lag effect would be small because we commonly detected first
173 flying and robbing scars on the same day or robbing scars prior to flying, but seldom
174 flying prior to robbing scars when flowering of *C. ambigua* had started. This suggests
175 quick learning of nectar robbing soon after emergence. Before the flowering in the
176 study site, we carefully checked *C. ambigua* flowers blooming at the forest edges,
177 where, due to earlier snowmelt, flowering progresses earlier than in the central part of
178 the forest. Before the onset of flowering of *C. ambigua* even in the forest edges, only
179 *Petasites japonicus* var. *giganteus* (Compositae) is available as a floral resource for
180 queen bees, although visits of queen bees to this species are occasional. Thus, we
181 also carefully checked flowers of this species for bee presence before the flowering of
182 *C. ambigua*.

183 Air temperature (at 1.5 m) and soil temperature (at 5 cm depth) were recorded at
184 the automatic weather station (see figure S1) at one-hour intervals since 2010 using a

185 datalogger (Hobo, Onset Co., USA). The air temperature sensor was shielded from
186 direct solar radiation.

187

188 **(c) Snow removal experiment**

189 We conducted a three-year snow removal experiment from 2014 to 2016. In November
190 2013, we randomly selected three locations within a 50 m x 50 m site in a large *C.*
191 *ambigua* population, and marked a pair of fixed plots at each location (i.e. six plots in
192 total; figure S1). Within each pair these were randomly allocated to control (C1 to C3)
193 and snow removal treatments (R1 to R3). These treatments were conducted at exactly
194 the same plots throughout the experimental period. The plot size was 5 m x 5 m, which
195 is fairly large for manipulative experiments. Plot size was decided to be as large as
196 practically possible to avoid the strong edge-effect common with smaller plots [26],
197 such as limitations of the effect of snow removal (e.g. wind-enhanced refilling of snow
198 back onto the plot; or flooding from melting of surrounding snow [27], with potential
199 subsequent freezing thus creating an ice layer; or insufficient area to enable adequate
200 soil response to exposure to subsequent air temperature), insufficient number of
201 flowering plants to study, and limited pollinator attraction (small floral patch size may
202 not be attractive to bees [28]). The plots in each pair were c 3 m apart at their closest
203 edge, and the pairs were c 35 m from each other. Since overwintering buds of *C.*
204 *ambigua* are located around soil-surface at the time of snowmelt, development of
205 shoots after snowmelt may be influenced by surface temperatures. A data logger
206 (Tidbit V2, Onset Co., USA) was therefore fixed at the centre of each plot to record
207 hourly soil-surface temperature. The logger sensors were set under litter layer to shield
208 from solar radiation.

209 Snow was removed from the plots (figure S2) by manual shoveling with a spade,
210 leaving 10 cm remaining to protect plants under snow, and removing an area 50 cm
211 wider than the plot border, to avoid potential edge-effects. In 2014 snow was removed
212 in mid-February but subsequent snowfall refilled the plots and it was necessary to
213 remove snow again in mid-March. In 2015 and 2016, snow removal was therefore
214 carried out only once a year in mid-March, which was sufficient. Surface temperature
215 under snow was continuously kept around 0–1°C throughout the winter irrespective of
216 snow depth in this site. The snow removal treatment of this study therefore did not
217 influence the thermal conditions during the snow-covered period. Snowmelt timing was
218 determined for each plot as the date when the surface temperature suddenly rose
219 above 0–2°C and began to fluctuate (see figure S3).

220 To test whether the timing of flowering in the removal plots was purely dependent
221 on snowmelt timing, we did not apply the removal treatment in 2017, but conducted all

222 plant and bee observations as described below. Since there were no significant
223 differences in the control and removal plots for any of the measured variables in 2017
224 (see descriptions below), all six plots were thus treated statistically as intact controls in
225 that year.

226 After snowmelt, we counted the number of inflorescences during the flowering
227 period in each plot at 1-4 day intervals. We randomly selected 20 plants producing
228 inflorescences in each plot before flowering and marked them with numbered tags,
229 recorded the number of flowers opening at 1-4 day intervals, and harvested pods at
230 fruiting before seed dispersal. Seed-set rates were measured as mentioned above.

231 To clarify the potential seed-set ability of plants without pollen limitation, we
232 conducted a hand-pollination treatment in 2014–2016 for plants growing outside of the
233 experimental plots in order to minimize the artificial disturbance of the experimental
234 plots. We selected 20 plants arbitrarily at flowering within a fixed 5 m x 5 m area (HP
235 plot, figure S1), and hand-pollinated all flowers using pollen from multiple (3–5) plants >
236 5 m from the recipient plants (and not from the control or removal plots). Then, the
237 seed-set rates were measured as mentioned above.

238 In 2016, we observed the bumble bee visitation frequency during flowering for 1 to
239 3 hours on clear days, for 11 days in total, from 5 April until 9 May. We selected the
240 plot with the densest inflorescences in each observation day (R1 and R2 in the early
241 flowering season, and subsequently C1 and C2), and counted the bumble bee visits to
242 the plot per hour.

243

244 **(d) Analysis**

245 Linear regressions were used to analyze the relationship between date (as day of year,
246 DoY) of snowmelt and: flowering onset or bee emergence, or phenological mismatch in
247 the long-term dataset (1999–2017). Mismatch (in number of days) was calculated as
248 the date of flowering onset in the study area minus that of bee emergence in the forest
249 (negative value when flowering occurred prior to bee emergence). Variation in naturally
250 pollinated seed-set (seed/ovule ratio per inflorescence) in response to mismatch was
251 analyzed with a generalized linear model (GLM) with a binomial error distribution and
252 logit-link function in which mismatch was the explanatory variable.

253 Flowering progress within the experimental plots was fitted to a unimodal function
254 of DoY using a GLM with a Poisson error distribution and log-link function, in which the
255 number of open inflorescences was the response variable and DoY with a quadratic
256 term was the explanatory variable. Based on this function, we defined (1) the flowering
257 onset as the DoY on which the number of open inflorescences reached 10% of that
258 plot's maximum inflorescence number for that year, and (2) the flowering period as the

259 length of time (in days) that the inflorescence number was greater than 10% of the total
260 inflorescence number of a plot. The end of flowering was therefore defined as the DoY
261 when the number of flowers decreased to 10% of the maximum plot value. We used
262 these estimated values of flowering properties for the analyses instead of observed
263 values since our observation frequency was not consistent within and across flowering
264 seasons. The relationship between flowering onset and flowering period within plots
265 was analyzed by the comparison of determination coefficient (R^2) across plots and
266 years.

267 We analyzed the effects of snow removal on flowering onset, mismatch, and seed-
268 set using generalized linear mixed-effect models (GLMMs). We set two random
269 intercepts in the GLMMs; the first term is location of each pair of control and removal
270 plots (figure S1) and the second term is year (2014–2017) in which treatment (control,
271 snow removal) is nested. We incorporated the nested random effect because our
272 experimental design was not balanced throughout the years, i.e. snow removal
273 treatment was not performed in 2017 and all of the six plots were used as a control
274 treatment in that year, after checking that there were no differences between the
275 control and removal plots in any measured parameter for 2017. In the pre-analysis for
276 2017 data, we conducted a GLMM for each of flowering onset, mismatch, and seed-set
277 to test that there were no differences between the values for the control and removal
278 plots in 2017. However, there were potential limitations in our experimental design in
279 terms of the small number of levels for random effects and unbalanced allocation of
280 treatments to the experimental plots over years. These limitations might reduce the
281 statistical power, but results obtained in our analyses seemed to adequately reflect the
282 patterns that we detected in the experiment. Variation in the flowering onset of
283 individual plants and mismatch were explored using a GLMM with a Gamma error
284 distribution and log-link function in which treatment was the explanatory variable. Since
285 mismatch varied from –9 to 11 days among plants, observed values of mismatch were
286 transformed into positive values by adding 10 for fitting to a Gamma distribution model.
287 Variation in seed set was analyzed by GLMMs with a binomial error distribution in
288 which effects of treatment and mismatch were separately analyzed because these
289 variables are collinear. First, the effect of treatment on seed set was analyzed. Then,
290 the effect of mismatch on seed set was analyzed for each treatment.

291 The extent that seed-set was pollen limited tested by comparing seed-set of hand-
292 pollinated (n=16–19) and naturally pollinated plants (in control plots, n=20 per plot)
293 using a GLMM with a binomial error distribution in which treatment (hand pollination,
294 control) and year (2014–2016) were explanatory variables, and plot (HP, C1, C2, C3)
295 was incorporated as a random factor.

296 The temperature dependence of the pre-flowering period (i.e. the number of days
297 between snowmelt and flowering onset) in the experimental plots was determined with
298 a linear regression between the pre-flowering period and mean daily surface
299 temperature during the pre-flowering period. Furthermore, we calculated the
300 accumulated degree-days (DD) for flowering onset from snowmelt day to flowering
301 onset day in every plot, using a threshold value of 1°C, since the surface was
302 maintained around 0–1°C before snow melt (figure S3).

303 Similarly, we evaluated the relationship between the date at which the soil attained
304 a given temperature (within the range of 5–7°C), and the date of first bee observation
305 during 2010–2017. The temperature giving the smallest mean deviation from observed
306 emergence dates was selected as the determinant for bee emergence (i.e. threshold
307 mean temperature estimator). We also calculated accumulated DD for emergence from
308 soil data using a 2°C threshold temperature since soil was maintained below 2°C
309 before snow melt (figure S3). Using the mean accumulated DD over 8 years, we
310 calculated the expected bee emergence day with reference to the soil temperature
311 record in each year. Comparing the deviation between observed bee emergence day
312 and estimated emergence day by the threshold mean temperature or accumulated DD
313 estimator, we evaluated which estimator best fit the emergence date.

314 After hibernation, however, the foraging activity of bumble bees is likely to be
315 weather dependent, and that may also affect the timing of first observation. We
316 therefore tested the temperature dependence and seasonal progress of bee activity
317 using 2016 flower visitation data, with a GLM with a Poisson error distribution, where
318 number of bee visits per plot per hour was the response variable and air temperature
319 and DoY were explanatory variables.

320 All statistical analyses were performed using an open source system, R version
321 3.4.4 (R Development Core Team, 2018, <https://www.r-project.org>). We conducted
322 GLMs using the R function “glm”, and GLMMs using the R function “glmer” in the library
323 of “lme4” for the analyses. Wald test (binomial and Poisson distribution) or *t* test
324 (Gamma distribution) was performed to test for significance in the GLMs and GLMMs.

325

326 **3. Results**

327

328 **(a) Phenological mismatch under natural conditions**

329 The 19-yr monitoring dataset revealed that both flowering onset of *C. ambigua* and first
330 emergence day of bumble bees occurred earlier when snow melted earlier ($R^2 = 0.91$
331 and 0.72, $df = 17$, $p < 0.001$, respectively; figure 1a, figure S4). However, the slope of
332 the regression line was steeper for flowering onset. As a result, phenological mismatch

333 was larger in early snowmelt years ($R^2 = 0.39$, $df = 17$, $p = 0.002$; figure 1b) in which
334 flowering of *C. ambigua* started up to one week earlier than bee emergence. Seed-set
335 with natural pollination varied depending on the extent of mismatch ($df = 17$, $z = 9.22$, p
336 < 0.001 by GLM; figure 1c), and was about 60% when mismatch was small, but
337 decreased to around 30% with 7-days mismatch.

338

339 **(b) Responses of flowering phenology and reproduction to snow removal**

340 During the experimental period (2014–2017), snowmelt timing in control plots varied
341 from year to year; ranging from 30 March to 24 April (table S1). Following manual
342 removal, snowmelt was advanced by 12–28 days (table S1, figure S4).

343 In the pre-analysis for 2017 data in which the snow-removal treatment was not
344 performed, GLMMs of flowering onset, mismatch, and seed-set revealed that all of the
345 variables did not differ significantly between the control and removal plots although
346 mean seed-set rates tended to be larger in the control plots ($df = 114$, $t = 0.4$, $p = 0.66$
347 for flowering onset, $df = 114$, $t = 0.42$, $p = 0.67$ for mismatch days, and $df = 115$, $z = -$
348 1.87 , $p = 0.06$ for seed-set rate). Thus, data from all plots in 2017 were considered as
349 controls for subsequent analyses. Flowering onset in the control plots varied from 12
350 April to 27 April among years (table S1). Snow removal advanced flowering onset by
351 5.1 days on average, ranging from 3 to 8 days (figure 2a, figure S5). Flowering onset
352 varied significantly between the treatments ($p < 0.0001$; table S2a). The length of
353 flowering periods varied from 15 to 24 days across plots and years, and was extended
354 when flowering started early in the season (flowering period length in relation to
355 flowering onset, $R^2 = 0.72$, see figure S6 for details). In the controls, flowering started
356 after bee emergence in 2014, 2015, and 2017, but concurrently with emergence in
357 2016 (figure 2b; see also figure S5). In the removal treatment, however, flowering
358 started concurrently with bee emergence in 2014 and 2015, but before emergence in
359 2016, and so mismatch varied significantly between treatments ($p < 0.0001$; table S2b).

360 Seed-set in hand-pollinated plants was 83–88% (figure 2c), indicating a high
361 potential seed-set in *C. ambigua*. Seed-set with natural pollination was 65–74% and
362 therefore 16–23% lower than that of hand-pollinated plants ($p < 0.0001$; table S3); both
363 varied among years. The GLMM revealed that seed-set success with natural pollination
364 was significantly lower in the removal treatment than control ($p = 0.013$; table S2c). The
365 effect of mismatch on seed-set was apparent when flowering occurred prior to bee
366 emergence in both of the treatments as shown in figure 3 ($p < 0.0001$; table S2c).

367

368 **(c) Environmental cues for flowering phenology and bee emergence**

369 The length of pre-flowering period of *C. ambigua* was highly correlated with surface
370 temperature within the range from 2 to 7°C ($R^2 = 0.97$, $df = 21$, $p < 0.0001$; figure 4a).
371 Pre-flowering period was shorted by 4.9 days per 1 degree warming; it took 26 days at
372 2.5°C and 15 days at 5°C, while it took only 4–5 days at > 7°C. Therefore, flowering
373 onset was strongly determined by the timing of snowmelt and subsequent ambient
374 temperature. Accumulated surface DD for flowering onset calculated for every plot was
375 49.4 ± 7.7 degree (mean \pm sd; table S4). We also calculated accumulated soil DD for
376 control plots (because soil temperature was measured under snow-intact condition); it
377 was 31.2 ± 10.5 degree and more variable than that using surface temperature.

378 In the analysis of threshold mean temperature for bee emergence, the date when
379 soil temperature reached 6°C best described the first observation date of bumble bees
380 ($R^2 = 0.88$; figure 4b), while a deviation of 1.0 ± 2.4 (mean \pm sd) days between
381 expected and observed values (table S5). Accumulated DD for bee emergence
382 calculated from the soil temperature was 29.1 ± 10.8 degree. Correlation of the
383 accumulated soil DD and the observed bee emergence date ($R^2 = 0.89$) had an even
384 lower deviation, i.e. 0.0 ± 3.0 days (table S5), indicating that the bee emergence was
385 best predicted by accumulated soil temperature. We also estimated bee emergence
386 date using accumulated air temperature (with 2°C threshold value): the deviation from
387 the observed emergence date was -1.25 ± 3.3 days. This indicates that soil
388 temperature is an effective estimator of bee emergence more than air temperature.

389 During the observation of bumble bee foraging activity in 2016, the first bee was
390 sighted on 18 April in the forest, but on that date, no bees were observed visiting the
391 plots despite flowering onset in all plots (figure S7). Visitation frequency at the plots
392 peaked 4 days later on 22 April, and continuous visits were observed after that. Bee
393 visitation frequency significantly increased with ambient air temperature ($df = 8$, $z =$
394 5.19 , $p < 0.0001$) and seasonal progress ($df = 8$, $z = 2.05$, $p = 0.041$).

395

396 **4. Discussion**

397

398 Our long-term monitoring of the flowering onset of a spring ephemeral, emergence of
399 queen bees, and seed-set success clearly indicate that the phenological events were
400 strongly related to the time of snowmelt. These trends were confirmed by the snow
401 removal experiment. The flowering phenology of *C. ambigua* was determined by
402 snowmelt time and subsequent ambient temperature, while bee emergence seemed to
403 depend on belowground temperature although foraging activity was influenced by air
404 temperature. The phenological mismatch between the spring ephemerals and their

405 pollinators might occur when soil warming progresses slowly after snowmelt due to
406 cooler ambient temperatures.

407

408 **(a) Importance of snowmelt time as a trigger of phenological mismatch**

409 Timing of snowmelt is an important predictor of spring events in high-latitude and high-
410 altitude environments both for plants and insects [9]. This is because spring
411 phenologies are strongly determined by the thermal requirements of various organisms
412 and snow creates a specific thermal environment at the local scale. Due to the snow's
413 insulation, the soil and surface at our site was maintained constantly at 0–2°C
414 throughout the winter (figure S3). After spring snowmelt, the surface is abruptly
415 exposed to fluctuating air temperature and quickly warms, while the soil gradually
416 warms with smaller daily fluctuations. Thus, there is a time lag for soil warming after
417 snowmelt (figure S8), and this difference between the rate of warming of the surface
418 and soil appears to be the driving factor behind the phenological mismatch. During the
419 experimental period in this study, 30 accumulated DD were attained seven days later in
420 the soil than at the surface when snow melted early in April (2015–2017), while there
421 were only three days difference when snow melted after mid-April (2014). This
422 indicates that the time lag for soil warming would be larger in spring with early
423 snowmelt.

424 Phenological mismatch between interacting species may occur when the species
425 use different environmental cues as a determinant of phenological events or when
426 responsiveness to a specific cue is different between species [5,29]. Although the
427 spring emergence of pollinators may shift earlier in response to warmer spring
428 temperatures and earlier snowmelt in high-latitude and high-altitude ecosystems [8,9],
429 little is known regarding the environmental determinants of their emergence after
430 hibernation. Overwintered queen bees are known to emerge when soils reached 5–
431 9°C, depending on species [23]. In this study, the date when soil attained 6°C was
432 closely related to the bee emergence date in the forest, although accumulated soil
433 temperature was a more reliable predictor of bumble bee emergence rather than a
434 single soil temperature (see table S5), similar to what has been reported for trap-
435 nesting bee emergence [17].

436 Since soils gradually warm after snowmelt (figure S3), bee emergence timing may
437 be more synchronous than that of flowering. The threshold temperature and/or
438 effective degree-days may be species-specific; *B. hypocrita sapporoensis* is the
439 earliest bumble bee species in this region, and may have a lower thermal requirement
440 to break diapause than other bumble bee species. Even after emergence, however,
441 foraging activity of bumble bees is influenced by the weather, and cool conditions

442 decrease flower visitation frequency. In 2016, bee emergence occurred 4 days later
443 than expected from the accumulated soil DD estimator (figure S8). This might be
444 explained by cool air for several days ($< 6^{\circ}\text{C}$) before emergence and so activity of bees
445 that had ended their hibernation might have been lower than normal. Thus, both the
446 timing of diapause termination and the weather at that time (and shortly after) affect the
447 availability of pollinators in early spring. Furthermore, thermal conditions of the soil may
448 also vary with micro-topography, snowmelt time, and depth of soil in which bumble
449 bees are overwintering. This variation may also explain some of the discrepancies
450 between predicted and observed bee emergence dates. We need more information on
451 the overwintering ecology of bumble bees for a greater understanding of the
452 determinants of emergence time.

453 Pre-flowering period of *C. ambigua* is highly air temperature dependent, and
454 ranged from 4 days at $>7^{\circ}\text{C}$ to 26 days at 2.5°C . Similarly, earlier flowering onset than
455 pollinator emergence is reported in a subalpine meadows of the Rocky Mountains,
456 since higher threshold temperature for diapause termination of bees was required than
457 that for development of early-bloomers [17]. As air temperature generally increases as
458 the season progresses in spring, the pre-flowering period becomes shorter when
459 snowmelt is delayed, and this may buffer the yearly variation in flowering time caused
460 by the fluctuation of snowmelt date [16]. However, spring temperatures often vary daily
461 and only a few warm days can rapidly advance plant phenology. Therefore, both
462 snowmelt timing and the subsequent air temperature are important environmental cues
463 for flowering phenology of spring ephemerals.

464

465 **(b) Ecological significance of phenological mismatch between plant and** 466 **pollinators**

467 Despite many studies of phenological shifts with a warmer climate, there are only a few
468 studies examining the effects of mismatch on plant reproduction are limited [7,14,15].
469 As hand-pollinated plants in our study had continuously high seed-set, any variation in
470 seed-set with natural pollination reflected pollination failure. Our study clearly
471 demonstrated that phenological mismatch between flowering onset and bee
472 emergence strongly related to the seed-set success of *C. ambigua*, and indicates that
473 risk of mismatch is higher in earlier spring, i.e. years with earlier snowmelt. The strong
474 impact on fitness seen here may be more apparent in specialist relationships than
475 generalist relationships between interacting species [12]. Overwintering bumble bee
476 queens are specialist pollinators for *C. ambigua*, which is self-incompatible and relies
477 on visitation by queen bees for seed production. These specific biological situations

478 make the pollination relationship between *C. ambigua* and bumble bees sensitive to
479 phenological variation.

480 Our experiment, however, may have overestimated the negative effect of
481 mismatch if the early appearance of relatively small flowering patches (i.e. in the snow
482 removal plots) occurred earlier than flowering in the general area, making them
483 unapparent or less attractive for bumble bees. As shown in figure 3, plants of the snow
484 removal plots tended to show lower seed-set success than control plants even with the
485 same number of days of mismatch. This might reflect the negative effect of isolated
486 patches, i.e. Allee effect (a positive effect of density). This bias in the snow removal
487 experiment (i.e. small flowering patches available in the snow removal treatment) may
488 be more important in determining the plant seed set results rather than mismatch per
489 se. Such an intrinsic limitation in the experimental control of flowering phenology is
490 outlined by Forrest [10]. Even though our experiment involves some artificial bias,
491 however, its results clearly reflect the pattern observed in natural conditions (figure 1c).

492 The length of the flowering period depended on the date of flowering onset; the
493 flowering period was longer when flowering started earlier (figure S6a). This variation
494 might reflect a seasonal trend in pollination success because pollinated inflorescences
495 terminate their flowering quickly, while unpollinated inflorescences extend their
496 flowering period to increase pollination success [30]. Thus, the longer flowering period
497 in early-flowering plots might be caused by low pollination success due to phenological
498 mismatch. The flowering period of *C. ambigua* lasted 2–3 weeks, while the extent of
499 mismatch was usually less than 10 days. Nevertheless, only several days' mismatch
500 significantly decreased seed-set when flowering occurred prior to bee emergence; the
501 potential ability of seed production may decrease daily, due to rapid physiological aging
502 in spring ephemerals [31,32]. If so, the extension of the flowering period cannot fully
503 compensate for seed-set success when flowering occurs earlier than pollinator
504 emergence. Also in our experiment, seed-set tended to decrease with an increase in
505 flowering period at the plot level (figure S6b).

506

507 **(c) Implications of phenological mismatch in spring ephemerals**

508 Our study predicts that the risk of mismatch may increase if snowmelt starts occurring
509 earlier. Spring ephemerals are particularly vulnerable as their high potential
510 reproduction may be limited by insufficient pollination, thereby reducing seed
511 production [7,25]. Experimentally limited seed supply decreased a *C. ambigua*
512 population within several years (G. Kudo, unpublished data) and limits the distributions
513 of several understory herbs [33]. Thus, a continuous reduction in pollination may

514 decrease seed production, restrict seedling establishment, and change population
515 dynamics if the frequency of mismatch increases with earlier springs [34].

516 Since bumble bees are generalist pollinators, they can select any available plant
517 species suitable for resources [22]. However, spring ephemerals are important floral
518 resources for overwintered queens soon after hibernation [6,21], and early-season
519 floral resources affect the establishment and development of colonies [35,36].
520 *Corydalis ambigua* is a very important nectar resource in spring due to its dense
521 populations in the deciduous forest ecosystem as well as its large nectar production
522 [21]. Any degradation of *C. ambigua* populations in the foraging site would therefore be
523 detrimental for bumble bees.

524 At the same time, the possibility of adaptive evolution of flowering onset to climatic
525 change should be considered [37]. If seed-set success is related to flowering
526 phenology, selective forces should act on flowering onset to maintain phenological
527 matching with pollinator emergence. The possibility of genetic adaptation of flowering
528 phenology to climate change may depend on the life history of individual species, and it
529 is expected to be high in short-lived species with sufficient genetic variation.
530 Furthermore, phenotypic variation in phenological traits is large in species inhabiting a
531 range of climate conditions, such as along an elevational gradient [reviewed in 38].
532 *Corydalis ambigua* is a relatively short-lived perennial plant. It grows in a range of
533 snowmelt conditions and timing of flowering varies among local populations [7]. Thus
534 the sensitivity of mismatch to climate also varies among populations, and local
535 adaptation in flowering phenology may be possible. Evaluation of the selective forces
536 acting on phenological traits and the possibility of evolutionary responses to climate
537 change are therefore important issues in global change biology.
538

539 **Data accessibility.** <https://datadryad.org/review?doi=doi:10.5061/dryad.q4fm37m>

540

541 **Authors' contributions.** GK and EJC planned this study, GK led the field
542 manipulations and survey, data analyses and writing the manuscript; EJC contributed
543 in the field and with writing the manuscript.

544

545 **Competing interests.** We have no competing interests.

546

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549

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554

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669

670 Figure captions

671

672 Figure 1. (a) The relationship between date of snowmelt (day of year) and flowering
673 onset of *Corydalis ambigua* (solid line and circles) and bumble bee emergence
674 (dashed line and crosses), (b) the relationship between snowmelt and phenological
675 mismatch between flowering onset and bee emergence, and (c) the relationship
676 between phenological mismatch and seed-set rate for 19 years (1999–2017). Linear
677 regression lines (a and b) and a logistic regression curve obtained by GLM (c) are
678 shown. $R^2 = 0.91$, $p < 0.001$ for flowering onset and $R^2 = 0.72$, $p < 0.0001$ for bee
679 emergence in (a); $R^2 = 0.39$, $p = 0.002$ in (b); $z = 9.22$, $p < 0.001$ in (c).

680

681 Figure 2. (a) Flowering onset (day of year), (b) phenological mismatch (days) between
682 flowering onset and bee emergence, and (c) seed-set rate of the hand-pollinated plants
683 (HP), control plots (C), and snow removal plots (R) during the experimental period
684 (2014–2017). The snow removal treatment was conducted during 2014–2016, and all
685 plots were used as control in 2017. mean \pm se. See Table S2 for statistical results.

686

687 Figure 3. The relationship between phenological mismatch and seed-set rates of
688 individual plants in the control (open circles and dashed line) and snow removal
689 treatments (closed circles and solid line). mean \pm se. Data during the experimental
690 period (2014–2017) are pooled. A logistic regression curve is indicated separately for
691 each treatment.

692

693 Figure 4. (a) The relationship between daily mean surface temperature and pre-
694 flowering days after snowmelt in the experimental plots and (b) the relationship
695 between the date on which soil temperature attained 6°C and first observation date of
696 bumble bees during 2010–2017 in the study forest. In (a) a linear regression between
697 surface temperature and pre-flowering period was performed for the range of 2–7°C; y
698 $= -4.9x + 39.2$, $R^2 = 0.96$, $p < 0.0001$. In (b) the dashed relationship represents the 1:1
699 line.

700

Fig. 1 (Kudo & Cooper)

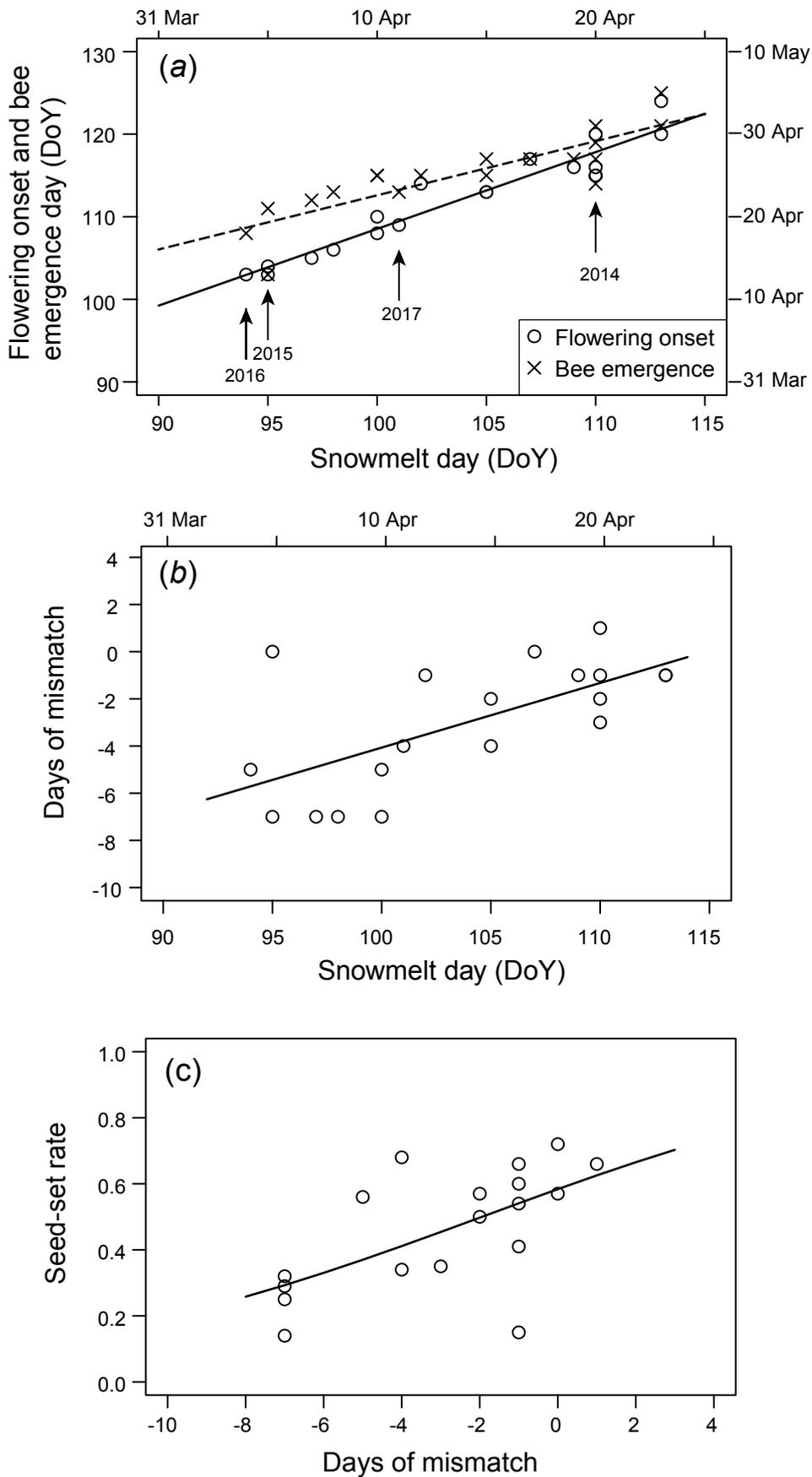


Fig.2 (Kudo & Cooper)

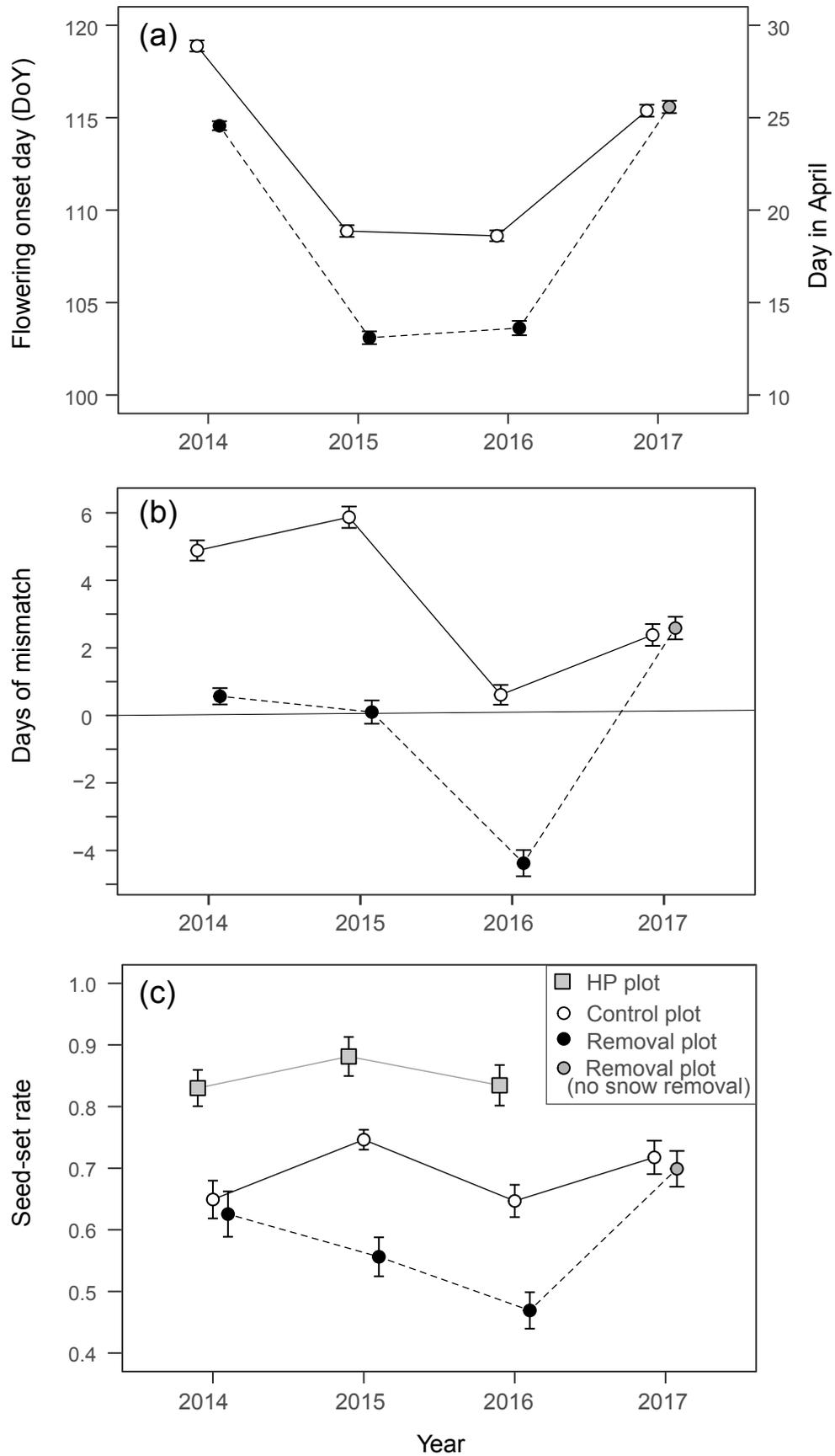


Fig. 3 (Kudo & Cooper)

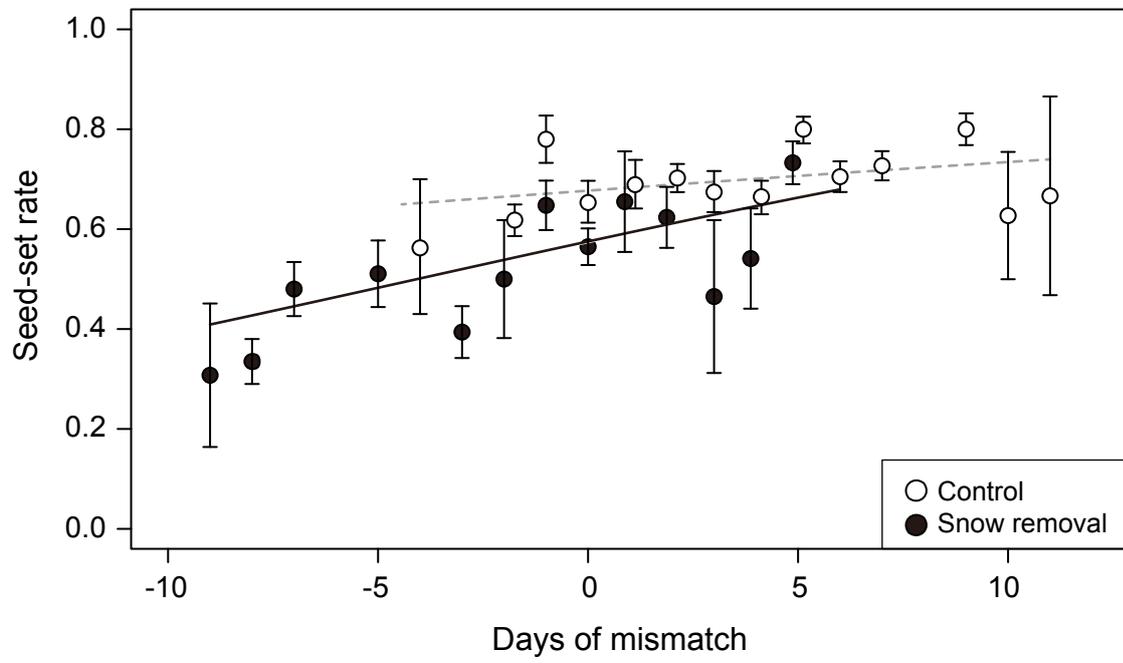


Fig. 4 (Kudo & Cooper)

