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ELEVATED ATMOSPHERIC CO₂ IMPAIRS APHID ESCAPE RESPONSES TO PREDATORS AND CONSPECIFIC ALARM SIGNALS

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1 **ABSTRACT** – Research into the impacts of atmospheric change on predator-prey interactions
2 has mainly focused on density dependent responses and trophic linkages. As yet, the chemical
3 ecology underpinning predator-prey interactions has received little attention in environmental
4 change research. Group living animals have evolved behavioural mechanisms to escape
5 predation, including chemical alarm signalling. Chemical alarm signalling between conspecific
6 prey could be susceptible to environmental change if the physiology and behaviour of these
7 organisms are affected by changes in dietary quality resulting from environmental change.
8 Using *Rubus idaeus* plants, we show that elevated concentrations of atmospheric CO₂ (eCO₂)
9 severely impaired escape responses of the aphid *Amphorophora idaei* to predation by ladybird
10 larvae (*Harmonia axyridis*). Escape responses to ladybirds was reduced by >50% after aphids
11 had been reared on plants grown under eCO₂. This behavioural response was rapidly induced,
12 occurring within 24h of being transferred to plants grown at eCO₂ and, once induced, persisted
13 even after aphids were transferred to plants grown at ambient CO₂. Escape responses were
14 impaired due to reduced sensitivity to aphid alarm pheromone, (*E*)-β-farnesene, via an
15 undefined plant-mediated mechanism. Aphid abundance often increases under eCO₂, however,
16 reduced efficacy of conspecific signalling may increase aphid vulnerability to predation,
17 highlighting the need to study the chemical ecology of predator-prey interactions under
18 environmental change.

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20 **Key Words** – Aphid, chemical signals, climate change, tri-trophic interactions, pheromones

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28 INTRODUCTION

29 Animals that live in groups have evolved numerous behavioural mechanisms for escaping
30 predation, ranging from aggregation for protection (e.g. the 'selfish herd' hypothesis (Hamilton,
31 1971)) to more 'altruistic' alarm signalling to conspecifics (Zuberbuehler, 2009). The latter has
32 evolved in many different invertebrate and vertebrate taxa, employing a variety of acoustic,
33 visual and chemical cues to warn conspecifics of a predation risk (Ruxton et al., 2004). Alarm
34 signalling between individuals via pheromones is particularly prevalent amongst insects (Blum,
35 1969).

36 Predicting how ecosystems will respond to climate change requires greater understanding of
37 the impacts on community processes, like herbivore prey-predator interactions (Jamieson et al.,
38 2012; Facey et al., 2014). Atmospheric carbon dioxide (CO₂) has reached 400ppm and is
39 predicted to increase to 421-936 ppm by 2100 (IPCC, 2013). This may have profound
40 consequences for predator-prey interactions (Robinson et al., 2012; Facey et al., 2014).

41 Experimental studies have hitherto focussed on density dependent population responses and
42 trophic interactions (e.g. Chen et al., 2005; Hentley et al., 2014). Elevated atmospheric CO₂
43 (eCO₂) could, however, also alter behavioural aspects of predator-prey interactions, such as
44 how prey respond to conspecific chemical signals (e.g. pheromones) to avoid predators. Such
45 communication could be indirectly altered by eCO₂ affecting the physiology of herbivorous prey
46 via changes to their plant resources (Zavala et al., 2013).

47 Aphids emit the alarm pheromone (*E*)-β-farnesene (*E*βF) to alert conspecifics of imminent
48 attack (Bowers et al., 1972). This pheromone facilitates a variety of evasive tactics used by
49 aphids, including cessation of feeding, walking from the signal source or dropping from the
50 plant (Pickett et al., 1992; Vandermoten et al., 2012). Reduced evasion responses of aphids
51 when physically disturbed by the experimenter have been shown under eCO₂ (e.g. squeezing
52 with forceps, Awmack et al., 1997 or prodding the thorax, Mondor et al., 2004). While these
53 studies did not explicitly link this to *E*βF, a subsequent study that subjected wheat aphids

54 (*Sitobium avenae*) to periodic release of $E\beta F$ showed population declines at ambient
55 atmospheric CO_2 (aCO_2), but no change at eCO_2 (Sun et al., 2010). They suggested that this was
56 due to aphids becoming insensitive to $E\beta F$ under eCO_2 (Sun et al., 2010). To date, however, the
57 behavioural response of aphids to both $E\beta F$ and predators when feeding on intact plants under
58 eCO_2 has not been investigated; previous studies have used excised leaves (Awmack et al., 1997;
59 Mondor et al., 2004) or focused solely on the response to $E\beta F$ (Sun et al., 2010).

60 We used the large raspberry aphid (*Amphorophora idaei*) feeding on red raspberry (*Rubus*
61 *idaeus*) as a model system. Densities of *A. idaei* increase in response to eCO_2 on some *R. idaeus*
62 genotypes (Martin and Johnson, 2011), but predation at eCO_2 can negate this increase (Hentley
63 et al., 2014) potentially due to increased susceptibility of aphid prey. We therefore hypothesised
64 that aphids have diminished escape responses to predator attack under eCO_2 , compared to
65 aCO_2 , which will be underpinned by reduced sensitivity to $E\beta F$.

66

67 **MATERIALS AND METHODS**

68 *Insects, plants and environmental chamber conditions*

69 The large raspberry aphid (*Amphorophora idaei*) population was initiated from field aphids and
70 then maintained in the laboratory for multiple generations. Cultures were maintained at $18 \pm$
71 $1^\circ C$ with a 16 h photoperiod. The predatory harlequin ladybird (*Harmonia axyridis*) cultures
72 were first collected from lime trees (*Tilia* spp.) in Oxfordshire, UK. The population was then
73 maintained in the same environment as the aphid cultures (full details of collection and
74 maintenance given in Hentley et al., 2014). Prior to the experiment, insect cultures were reared
75 for at least four generations at aCO_2 ($390 \pm 50 \mu mol/mol$) and eCO_2 ($650 \pm 50 \mu mol/mol$)
76 conditions in four environmentally controlled chambers (two per CO_2 regime, all at $20 \pm 4^\circ C$,
77 50-70% relative humidity and 16h photoperiod). These chambers (full details in Hentley et al.,
78 2014) were also used for growing plants. Forty *Rubus idaeus* (cv. Glen Clova) were grown from
79 rootstock; at approximately 6 weeks old (1 cm height) plants were transferred to 3L pots, and

80 randomly assigned to the four chambers. To minimize chamber effects, plants were moved
81 between corresponding treatment chambers once a week for five weeks prior to assays (*sensu*
82 Bezemer et al., 1998; Johnson and McNicol, 2010).

83 *Behavioural assays*

84 Behavioural assays used intact plants exposed to the different CO₂ treatments for five weeks.
85 Fully-crossed combinations (Fig. 1) of aphids and plants maintained under the two CO₂ regimes
86 were tested in response to stimuli from the presence of (i) ladybird (*Harmonia axyridis*)
87 predators, (ii) 200 ng of EβF in 5μl hexane solvent and (iii) control of 5μl hexane (both
88 analytical standard, Sigma-Aldrich, UK). For each assay, a leaf was randomly selected and 50
89 mixed-age, apterous aphids were confined to the underside of the leaf with a 20mm diameter
90 clip-cage for 24h. The cage was then removed and the number of aphids feeding counted, aphids
91 not feeding were removed prior to the onset of the assay. Assays proceeded as follows: (i) a
92 single fourth instar ladybird larva, starved for 24h, was introduced onto the leaf c. 2 cm from the
93 aphids and observed for 5 mins. The ladybird was replaced if it did not attack aphids after 5
94 mins. For the assays with (ii) EβF and (iii) the control hexane only, 5μl of the solution was
95 placed on the underside of the leaf surface, c. 2 cm from the aphid colony and, again, observed
96 for 5 mins for escape responses. For each assay, the proportion of aphids that stopped feeding
97 and showed predator avoidance behaviour (e.g. walking away, dropping) in response to stimuli
98 (i-iii) was quantified. Each assay was repeated 10 times.

99 *Statistical analysis*

100 The proportion of aphids responding to stimuli was modelled with generalised linear mixed
101 effect models fitting a binomial error distribution (GLIMMIX, SAS Institute). The random effect
102 was environmental chamber nested within date of bioassay. Mean temperature was fitted as a
103 fixed effect to account for spatial (between chambers) and temporal (between days) variation
104 during the experiment (Table 1-f). A minimum adequate model was obtained with forward
105 stepwise selection of fixed effects. F-ratio and p-values presented are adjusted for other

106 significant fitted terms (SAS type III), two-way interactions are only reported where $p < 0.05$.
107 Degrees of freedom were estimated with Satterthwaite's approximation.

108

109 **RESULTS**

110 Compared to aphids and plants under aCO₂ (Fig. 2-I), aphid escape responses to ladybirds were
111 significantly reduced when they had been reared under eCO₂ (Fig. 2-II & IV, Table 1-b & c), even
112 when transferred to plants grown under aCO₂ (Fig. 2-II). Moreover, aphids reared under aCO₂,
113 but subsequently transferred to plants grown under eCO₂ for <24h, showed the same reduction
114 in escape responses (Fig. 2-III, Table 1-d) as seen in aphids reared under eCO₂ (Fig. 2-II & IV).
115 Over twice as many aphids initiated escape responses when they fed under aCO₂ conditions
116 (Fig. 2a-I). Aphids exposed to their alarm pheromone *EβF*, exhibited a reduction in escape
117 behaviour, similar to when being attacked by the predator (Fig. 3a, Table 1-a & e). Aphids
118 showed no behavioural response to the control solvent hexane (Fig. 3b).

119

120 **DISCUSSION**

121 Aphid escape responses to predator presence or exposure to the aphid alarm pheromone *EβF*
122 were rapidly (within 24h) and significantly (>50%) impaired when feeding on a plant reared in
123 eCO₂, which persisted individual aphids reared in eCO₂ fed on plants grown at aCO₂.
124 Aphid behaviour often differs when feeding on experimentally excised leaves compared with
125 intact plants, because phloem hydraulics and chemistry are altered by excision (Van Emden and
126 Bashford, 1976; Douglas, 1993). This study, using whole plants, an insect predator, and a
127 controlled dose of alarm pheromone *EβF* - rather than a mechanical disturbance of aphids to
128 stimulate its release (as performed by Awmack et al., 1997; Mondor et al., 2004), - in a
129 reciprocal experimental design permitted us to conduct a more realistic test of eCO₂ impacts on
130 this tri-trophic interaction.

131 Impairment of aphid escape responses by eCO₂ is likely to have been mediated via changes in
132 the plant quality which in turn impacted aphids, this is because the impairment always occurred
133 when aphids had been, or were, feeding on plants grown at eCO₂. At least two possible
134 mechanisms may underpin reduced escape responses. Firstly, aphids may 'hold their ground'
135 rather than escape if feeding on a good quality host. In particular, *Amphorophora idaei*
136 performance is known to be enhanced by eCO₂ induced changes in plant suitability (Martin and
137 Johnson, 2011) and these species is known to continue feeding, even under threat, if the host
138 plant is of good quality (Mitchell et al., 2010). Alternatively, if eCO₂ reduces host plant quality,
139 then aphids can engage in more intense and sustained feeding activity (Sun and Ge, 2011; Guo et
140 al., 2013a; Guo et al., 2013b), akin to the compensatory feeding responses of chewing insect
141 herbivores (e.g. Docherty et al., 1996; Johnson et al., 2014a). Ingesting more phloem will
142 inevitably be energetically costly and may require deeper penetration of plant tissues, and
143 possibly the manipulation of the plants metabolism (Guo et al., 2013b). This investment may
144 result in aphids being either physiologically less able, or behaviourally less inclined, to abandon
145 a host plant. Either way, it is feasible that enhancement or deterioration in the nutritional
146 quality of plants grown under eCO₂ is enough to make aphids to continue feeding, even under
147 risk of predation.

148 Multi-trophic interactions must be accounted for to accurately predict the net effect of eCO₂ on
149 plants (Harrington et al., 1999; Robinson et al., 2012; Facey et al., 2014). Crops may become
150 more susceptible to insect pests, including aphids (Martin and Johnson, 2011; Johnson et al.,
151 2014b), in an eCO₂ environment. Top-down control of aphids by natural enemies is a major
152 factor in regulation of aphid populations (Dixon, 2000). Here we demonstrate that atmospheric
153 change modified the behavioural response of a herbivore when a conspecific is being attacked
154 by a natural enemy, which could increase the net impact of the predator. The lack of behavioural
155 response from conspecifics will increase prey availability for the natural enemy, but also limit
156 beneficial, non-consumptive effects, such as the herbivore dropping from the plant. Such

157 modified predator-prey interactions clearly have implications for crop security in a changing
158 world.

159 Together with previous evidence using other aphid species (Awmack et al., 1997; Mondor et al.,
160 2004; Sun et al., 2010), it seems that the interference effect of eCO₂ on aphid escape responses
161 is a general, and possibly widespread, reaction. However, further work is needed to establish
162 the mechanistic basis of how atmospheric change mediates the chemical ecology of predator-
163 prey interactions. Moreover, whether aphid populations will adapt to such atmospheric changes
164 over time to re-establish responsiveness to predator-related conspecific alarm signals remains
165 an unanswered question.

166

167

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171

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269 **Figure Legends**

270 Figure 1 Schematic of behavioural assays using reciprocal treatments of aphids and plants
271 maintained under aCO₂ and eCO₂

272

273 Figure 2 Mean (\pm S.E.) percentage of aphids reared at aCO₂ (grey bars) or eCO₂ (white bars)
274 showing escape responses to ladybird larva (*Harmonia axyridis*). Roman numerals refer to
275 treatment combinations (see Fig. 1)

276

277 Figure 3 Mean (\pm S.E.) percentage of aphids reared at aCO₂ (grey bars) or eCO₂ (white bars)
278 showing escape responses to a) E β F mixed with hexane solvent, and b) hexane alone. Roman
279 numerals refer to treatment combinations (see Fig. 1).

280 Table 1. Final GLMM results summary for aphid escape response to predator/ EβF stimulus and
 281 CO₂ regimes.

Response variable	Explanatory variables	Estimate	F _(ndf,ddf)	p
% aphids per plant showing escape behaviours	a) Stimulus <i>Hexane/Ladybird/ EβF</i>	<i>2.17 / 3.54 / 2.68</i>	6.46 _(2,6)	0.0031
	b) Plant growing environment <i>aCO₂/eCO₂</i>	<i>2.74/2.68</i>	15.28 _(1,6)	0.0089
	c) Aphid rearing environment <i>aCO₂/eCO₂</i>	<i>3.29/2.68</i>	21.43 _(1,145)	<0.0001
<i>Random effect estimate = 0.07 ± 0.07</i>	d) Plant growing * aphid rearing environment <i>aCO₂ plant * aCO₂ aphid / eCO₂ plant * aCO₂ aphid</i> <i>aCO₂ plant * eCO₂ aphid / eCO₂ plant * eCO₂ aphid</i>	<i>4.04 / 2.68</i> <i>2.68 / 2.68</i>	38.40 _(1,38)	<0.0001
	e) Aphid rearing environment * stimulus <i>aCO₂ * Hexane / aCO₂ * Ladybird / aCO₂ * EβF</i> <i>eCO₂ * Hexane / eCO₂ * Ladybird / eCO₂ * EβF</i>	<i>1.30 / 2.16 / 2.68</i> <i>2.68 / 2.68 / 2.68</i>	6.02 _(2,145)	0.0031
	f) Mean temperature	-0.275	5.41 _(1,5)	0.0544

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284

CO₂ conditions

Ambient CO₂
390 μmol/mol



Combination

aCO₂ aphid
- aCO₂ plant

eCO₂ aphid
- aCO₂ plant

aCO₂ aphid
- eCO₂ plant

eCO₂ aphid
- eCO₂ plant

Figure Label

I

II

III

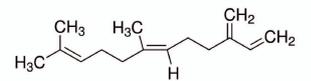
IV

Behavioural assays

(i) Ladybird larva



(ii) *E*-β-Farnesene
(dissolved in hexane)



(iii) Control (hexane)

