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1 **Can we predict the effects of multiple stressors on insects in a changing climate?**

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3

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11

**12 Abstract**

13 The responses of insects to climate change will depend on their responses to abiotic and  
14 biotic stressors in combination. We surveyed the literature, and although synergistic stressor  
15 interactions appear common among insects, the thin taxonomic spread of existing data means  
16 that more multi-stressor studies and new approaches are needed. We need to move beyond  
17 descriptions of the effects of multiple stressors to a mechanistic, predictive understanding.  
18 Further, we must identify which stressor interactions, and species' responses to them, are  
19 sufficiently generalizable (i.e. most or all species respond similarly to the same stressor  
20 combination), and thus predictable (for new combinations of stressors, or stressors acting via  
21 known mechanisms). We discuss experimental approaches that could facilitate this shift  
22 towards predictive understanding.

23

## 24 **Introduction**

25 Insects and other organisms must simultaneously respond to various, natural abiotic and  
26 biotic stressors, as well as to an increasing array of novel anthropogenic environmental  
27 stressors. It is often difficult to distinguish the origin of stress because natural abiotic or biotic  
28 stressors can be exacerbated by anthropogenic influences. Some of the most important  
29 human-induced or -accelerated environmental stressors include climate change, habitat  
30 fragmentation, chemical pollution and introduction of invasive species [1,2]. Different  
31 stressors will interact in various possible ways under climate change, and the responses and  
32 resistance of organisms to climate change will depend on their responses to combinations of  
33 stressors, which may or may not reflect responses to single stressors in isolation.

34

35 Multiple stressors have been studied systematically in aquatic ecosystems for non-insect  
36 organisms, especially in ecotoxicology [3–6] [7<sup>\*</sup>], where it appears that synergistic  
37 interactions (resulting in a greater-than-expected impact) among stressors may be the norm.  
38 Here we show that multiple stressor studies on insects are still relatively rare, and discuss best  
39 practices in experimental design for multiple stressor studies that aim to characterise  
40 responses and identify underlying mechanisms, with an overall goal of predicting *a priori* the  
41 outcomes of interacting stressors.

42

### 43 **What do we study when we study multiple stressors?**

44 We define a ‘stressor’ as any environmental or biotic factor with the potential to disrupt  
45 homeostasis, performance or fitness [8]. The consequences of exposure to multiple stressors  
46 are usually separated by ecologists and ecotoxicologists into additive and non-additive  
47 (synergistic or antagonistic) effects [3,9–12]. In this review, we use the terms “synergism”  
48 and “antagonism” as a convenient way to indicate stressor interactions that result in greater or

49 lesser effects than the additive sum of effects produced by the stressors acting in isolation,  
50 respectively [see 5]. A range of statistical models have been used to identify non-additive  
51 effects, and, as a consequence, the strength and basis of conclusions may depend on the  
52 experimental context [9,12,13]. Some authors advocate using ‘synergism’ and ‘antagonism’  
53 only to refer to effects detected by means of additive ANOVA models in a full-factorial  
54 design [3,9,10,13,14]. By contrast, in some disciplines, for example toxicology, the mode of  
55 actions of multiple toxicants (stressors) are always assumed to be different, leading to  
56 multiplicative (rather than additive) effects, which therefore requires appropriate statistical  
57 analysis and terminology [9,15,16].

58

59 We informally surveyed the Anglophone insect literature (see supplementary material for  
60 details of the studies we identified). To narrow our criteria we focused on studies that  
61 manipulated different classes of stressors (e.g. temperature and pathogens) in full-factorial  
62 designs that allow estimation of non-additive results. A full-factorial two-stressor study  
63 would thus include four treatments; (1) neither treatment (control), (2) stressor A alone, (3)  
64 stressor B alone, and (4) both stressors, A and B, in combination. Despite the prevalence of  
65 multiple, interacting, stressors in nature, most insect studies explore effects of one stressor in  
66 isolation. At least 210 studies applying two stressors were not full-factorial in study design  
67 (e.g. lacking a stressor-free control or an individual stressor treatment). Some of those studies  
68 (e.g. those exploring interactions between temperature and pathogens and temperature and  
69 atmospheric gases) use an additional stressor to enhance the impact of a stressor of primary  
70 interest [17<sup>\*</sup>], and do not use a full-factorial experimental design. Such studies cannot predict  
71 the range of non-additive responses and do not provide material for future meta-analyses on  
72 stressor interactions. We found 133 full-factorial studies (listed in Supplementary Word file),  
73 covering 24 stressor pairs (Table S1; Table S2). Fewer than ten studies included three-

74 stressor combinations, and we found none including more than three stressors. The two-  
75 stressor studies were spread across 12 insect orders, 51 families and 100 species (Fig. S1).  
76 This coverage of insects is still low for making far-reaching generalisations, especially when  
77 the stressor outcomes are split among many stressor pairs.

78

79 The choice of endpoints in multiple stressor studies can substantially influence the  
80 conclusions [18<sup>\*</sup>,19]. Many studies used mortality as an endpoint, for example those that  
81 explored the combined lethality of chemical and pathogen/temperature exposures. Although  
82 these studies can reveal non-additive stressor interactions, in our opinion they cannot reveal  
83 their underlying mechanisms. Many two-stressor studies used sub-lethal, fitness-related,  
84 endpoints, such as growth or fecundity (Fig. S1), which likely reflect diversion of energy to  
85 repair stress damage and re-establish homeostasis [2,20,21]. We suggest that fitness-related  
86 endpoints can shed light on underlying mechanisms of ecologically-relevant interactions  
87 among stressors.

88

89 Although the studies we included were full-factorial in design, many were unable to directly  
90 identify synergy or antagonism because of the selected statistical approach (e.g. comparing  
91 combined effects only to the univariate effects of one stressor, but not both). Thus, the results  
92 can provide only the presumption of non-additive interactions between stressors (Fig. S1;  
93 Table S2). However, studies incorporating chemical stressors (especially chemical-  
94 temperature and chemical-pathogen interactions) more routinely used full-factorial statistical  
95 models. These studies reveal that chemicals, such as pesticides, often interact synergistically  
96 with temperature or pathogen stress. For example, in the blue-tailed damselfly *Ischnura*  
97 *elegans*, acetylcholinesterase inhibition by the pesticide chlorpyrifos was synergistically  
98 magnified when combined with heat stress [18<sup>\*</sup>]. This possible predominance of synergistic

99 interactions is consistent with observations for other animals [3,5]. We argue that the thin  
100 taxonomic spread of existing data makes the insect literature currently unsuitable for formal  
101 meta-analyses, but the accumulating number of primary full-factorial studies will eventually  
102 be useful to identify large-scale patterns in non-additive effects among insect species and  
103 stressors.

104

### 105 **Accounting for the multiple scales of multiple stressors**

106 The timing, intensity, duration, frequency and spatial distribution of stressor exposure will  
107 vary among stressors and among stressor events, complicating their inclusion when  
108 predicting organismal responses to multiple stressors and global change [2,22], particularly  
109 when complex systems can give rise to emergent phenomena [23]. Including all possible  
110 stressor combinations (and durations, sequences and intensities of stressor exposures) will  
111 rapidly become intractable, but the spatio-temporal complexity of multiple stressors can be  
112 reduced for experimental purposes by selecting combinations on the basis of their (co-)  
113 occurrence dynamics in nature, for example, using the fractional factorial approach espoused  
114 by Porter et al. (1984) [24\*\*].

115

116 The intensity (loosely, 'dose') of stressors can determine the outcome of interactions. This is  
117 best exemplified in the concept of hormesis, wherein small doses of stress can be beneficial,  
118 but large doses damaging [25]. This effect of intensity is prevalent in responses to many  
119 kinds of physical, chemical or biological stressors, for example, the heat shock response is  
120 induced at a threshold temperature [26], which can then substantially modify responses to  
121 other stressors; however, exposure below that threshold will have minimal effect, and  
122 exposure to higher temperatures may be lethal. Thus, identifying the minimum 'dose'  
123 required to elicit a stress response (or an interaction – which may be a higher or lower dose)

124 must be included in the design of experiments and choice of stressors. One approach to this  
125 is to standardise the impact of stressors (the ‘toxic unit approach’ of ecotoxicology) to allow  
126 interactions to be explored under conditions where each stressor has similar impact [cf. 27].  
127 However, this standardisation may only be ecologically-relevant if the stressors in nature are  
128 expected to co-occur at similar intensities.

129

130 The mechanisms underlying the physiological responses to stress can be tightly linked to the  
131 spatio-temporal dynamics of co-occurring stressors. For example, the physiological responses  
132 to thermal, drought or starvation stress, which often co-occur, are similar in insects [28–30].  
133 By contrast, insects may experience other stressors together for the first time under global  
134 change; for example, neonicotinoid insecticides and invasive pathogens [31]. Further,  
135 changing climates could change the timing and severity of overlapping interacting stressors  
136 [28\*]. If a change in the sequence of two stressors does not affect the outcome and the result  
137 is repeatable in one or several species, or when additional stressors are present, then the  
138 responses may have underlying shared mechanisms, and thus mutually-predictable responses,  
139 even if they have not previously co-occurred in nature [32,33].

140

#### 141 **Mechanisms underlying insect responses to multiple stressors**

142 Identifying the mechanisms underlying stressor interactions could help us to predict *a priori*  
143 the effects of novel combinations of stressors, or to generalise the effects of multiple stressors  
144 among taxa [2]. This is especially challenging when our understanding of the mechanistic  
145 basis for insects’ responses to even simple single stressors is incomplete. Predictable and  
146 generalizable responses to multiple stressors could arise through cross-tolerance – shared  
147 mechanisms of stress response that impart protection against multiple stressors once activated  
148 – or through cross-talk, whereby signaling responses to the first stress also activate resistance



149 to other stressors [28<sup>\*</sup>]. These underlying processes are generally conceived as adaptive,  
150 leading to antagonistic effects of combined stressors, to the benefit of the insect. For  
151 example, prior exposure to dehydration improves subsequent tolerance to (i.e. reduces the  
152 impact of) cold or heat in the Antarctic midge *Belgica antarctica*, the cross-tolerance  
153 facilitated by accumulation of trehalose [29]. In addition, cross-tolerance between low  
154 temperature and hypoxia (or other controlled atmospheres), and their underlying  
155 physiological and molecular mechanisms have been studied to some extent in insects  
156 [17<sup>\*</sup>,34–36]. For example, in the false codling moth *Thaumatotibia leucotreta* mild pre-  
157 treatments with chilling and hypoxia increased resistance to low temperatures and these  
158 responses were correlated with increased membrane fluidity and/or alterations in heat shock  
159 protein (HSP70) [36]. The antagonistic effects of cross-tolerance or cross talk are  
160 conceptually (and likely mechanistically) related to hormesis [25].

161

162 However, this adaptive framework based on shared responses to stressors predicts  
163 antagonistic responses to multiple stressors and thus appears to be at odds with the  
164 preponderance of synergistic effects of multiple stressors that we observed in our literature  
165 survey (Fig. S1). Synergistic stressor interactions in insects have been most commonly  
166 reported for chemical-temperature and chemical-pathogen pairs and the effects of other  
167 stressor pairs have been little-studied. Thus, we lack both either the breadth of descriptive  
168 data or (for many stressors) the mechanistic understanding of their mode of action necessary  
169 to make predictions within this framework. However, mechanism can predict synergistic  
170 responses to combined stressors, as in a scarabaeid beetle in which application of an  
171 insecticide weakens the immune system, leading to a synergistic interaction when the  
172 insecticide is applied in concert with a fungal pathogen [37]. These mechanism-based non-  
173 additive interactions can easily yield both synergistic and antagonistic results. For example,

174 cell membrane fluidity can determine cold tolerance in the collembolan *Folsomia candida*, so  
175 lipophilic contaminants can either increase or reduce cold tolerance, depending on each  
176 contaminants' impact on the phospholipid membrane – a property that can be predicted in  
177 advance [38<sup>\*</sup>]. Thus, predicting the impacts of multiple stressors based on mechanism may be  
178 primarily hampered by a lack of understanding of the mechanisms underlying the impact of  
179 each stressor in isolation.

180

### 181 **Can we generalize multiple stressor effects to yield predictions?**

182 Currently, the insect literature is dominated by descriptive studies that characterise the  
183 responses of a specific taxon to a specific combination of (usually two) stressors. When  
184 designed well, these studies can identify non-additive interactions, and hint at underlying  
185 mechanisms or pathways shared among stressors. However, the millions of insect species  
186 and thousands of stressors mean there are trillions of potential stressor-taxon combinations,  
187 so such descriptive studies fall short if we wish to account generally for multiple interacting  
188 stressors in our understanding of climate change. To make *a priori* predictions about the  
189 consequences of multiple interacting stressors, we first need to determine if the responses to  
190 multiple stressors are predictable from an understanding of univariate or bivariate responses,  
191 and second, determine whether such predictions are generalizable among taxa. If responses to  
192 stressors are predictable, then we can draw larger conclusions about responses to novel  
193 combinations of stressors (Fig. 1). Such predictability will likely arise when there are shared  
194 mechanisms (or perhaps signaling pathways) underlying responses to those stressors. Thus,  
195 univariate studies of single stressors, and the physiological and molecular mechanisms  
196 underlying insect responses to them, are essential.

197

198 We term responses to multiple stressors as ‘generalizable’ when most or all species exhibit  
199 broadly similar responses to the same stressor combination, but ‘idiosyncratic’ if different  
200 species respond differently or in a context-dependent manner (Fig. 2). The shared  
201 mechanisms that underlie many stress responses [29,36,39,40], and the associations of at least  
202 some stressors over evolutionary time [29] lead us to expect that at least some interactions  
203 among stressors will have impacts generalizable to higher taxonomic levels. There are well-  
204 established conceptual and analytical tools to assess physiological responses in a  
205 phylogenetic context [41,42]. Although these have largely been applied to individual  
206 stressors in insects [43–47], we expect that a phylogenetically-cogent approach, for example  
207 with *Drosophila* [47<sup>\*</sup>] will yield information on the prevalence of idiosyncratic vs.  
208 generalizable responses to multiple stressors.

209

210 An alternative approach is to understand the structure of multiple stressor responses. When  
211 exposed to increasing numbers of combinations of stressors, it is possible for the cumulative  
212 effects to saturate or accelerate (Fig. 3). A saturation of responses would arise if there are a  
213 limited number of possible interaction mechanisms among stressors, such that additional  
214 stressors have limited additional impact after some threshold. By contrast, if synergistic  
215 interactions combine and become increasingly synergistic with additional stress (or there are  
216 emergent properties), the effect of additional stressors may continue to increase. Increasing  
217 number of stressors in the green alga *Chlamydomonas reinhardtii* have limited impact on  
218 population growth after c. four stressors, suggesting a saturation structure to multiple  
219 interacting stressors in this species [48<sup>\*\*</sup>], and saturation also appears to apply to toxicants  
220 (the “funnel hypothesis”) [49<sup>\*\*</sup>]. However, such experiments have not, to our knowledge,

221 been conducted in insects, and even the few three-stressor studies we identified do not have  
222 sufficient stressors to tease these two possible responses apart.

223

## 224 **Conclusions**

225 Although we know a lot about how insects respond to single stresses, few studies have  
226 characterized responses to two stressors in combination, and studies that include three or  
227 more stressors are rare. Similarly, although the mechanisms underlying responses to  
228 univariate stressors have been explored, we cannot yet connect those mechanisms to the  
229 responses to stressors in combination. We suggest that using these data in phylogenetic or  
230 multiple stressor frameworks may allow determination of the predictability and  
231 generalizability of responses to multiple stressors, and that determining this will improve our  
232 ability to incorporate multiple stressors in more general models of global change.

233

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240

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- 243 • of special interest
- 244 •• of outstanding interest

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395

396

397

## 398 FIGURE LEGENDS

399

400 **Figure 1**

401 Shared response mechanisms can hypothetically be used to predict responses to novel stressor  
402 combinations. Stressor pairs P+Q and P+R share same mechanism (X) resulting in a shared  
403 response or outcome (O1). In this example, the new stressor pair T+S also share response  
404 mechanism X, so we predict the O1 -response.

405

406 **Figure 2**

407 Responses to interacting stressors can be generalizable or idiosyncratic. This may be evident  
408 when responses are compared across a phylogeny (A), where a strong phylogenetic constraint  
409 can imply a generalizable response. We speculate that generalizable responses arise when  
410 mechanisms are shared (Figure 1), but idiosyncratic, if the same mechanisms yield different  
411 responses (B), or if the mechanisms themselves are context-dependent (C).

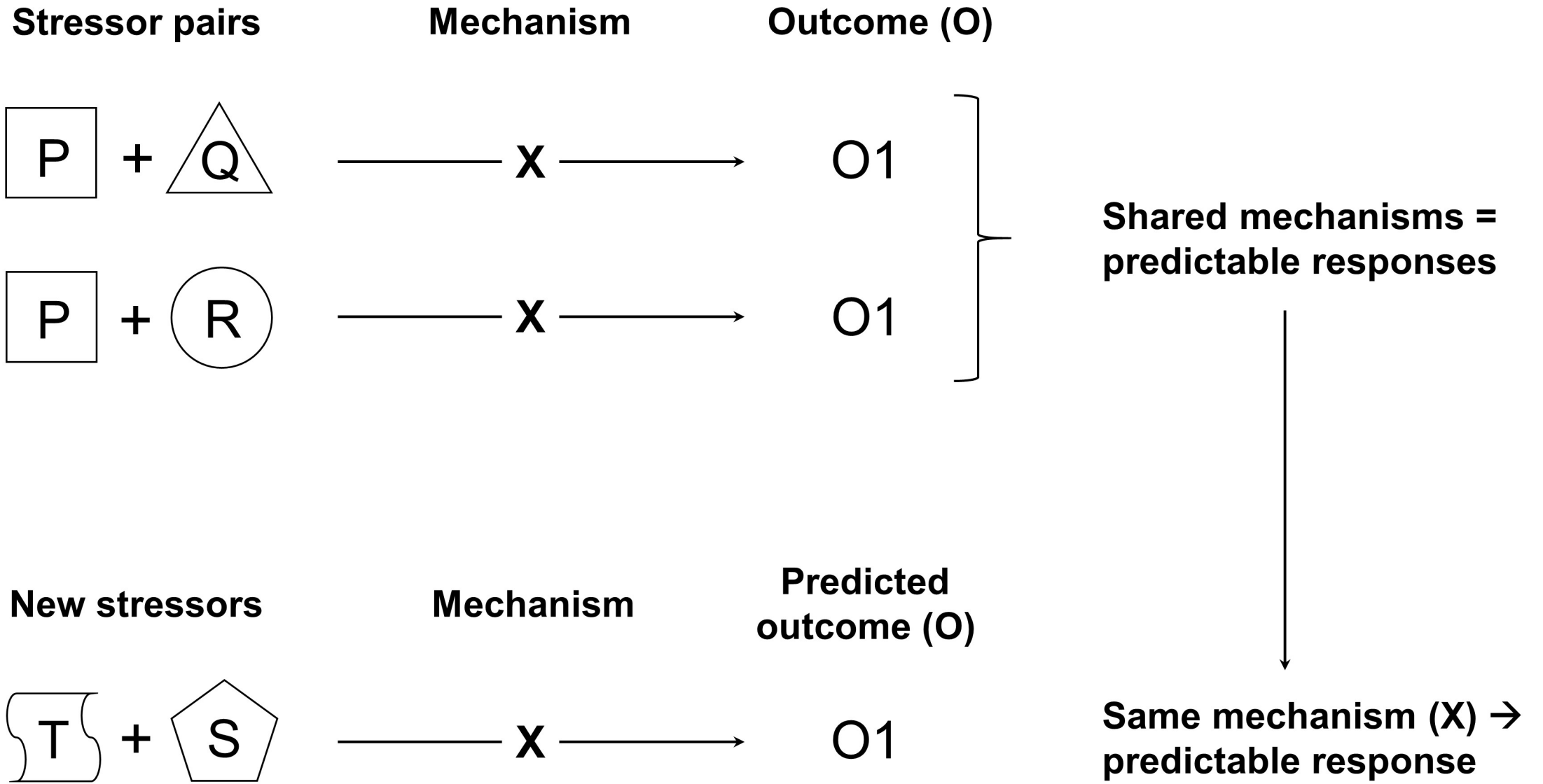
412

413 **Figure 3**

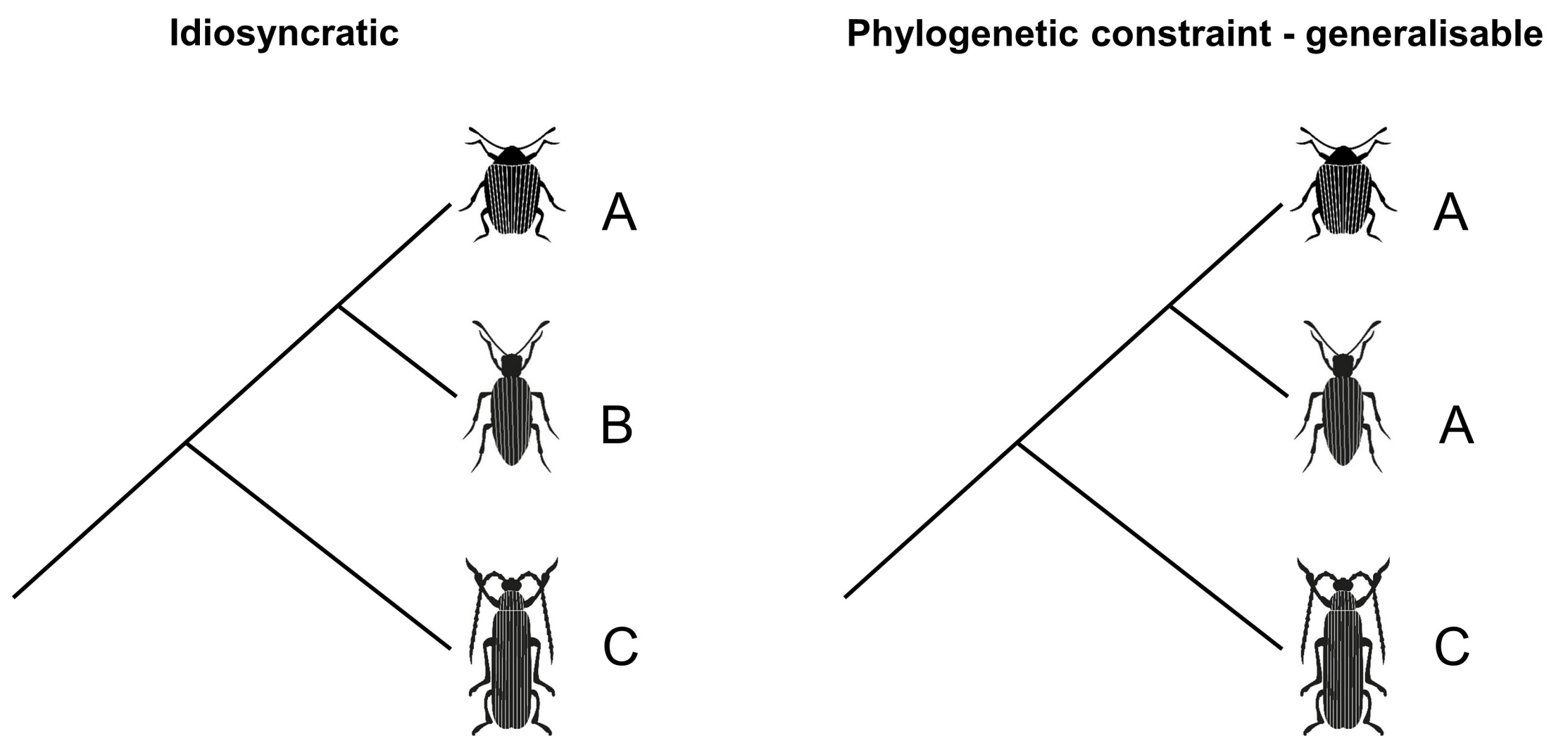
414 When exposed to increasing numbers of combinations of stressors, it is possible for the  
415 cumulative effects to saturate (A) because many stressors use same mechanism, or accelerate  
416 if synergistic interactions combine and become increasingly synergistic with additional  
417 stressors (B). In the case of saturation the number of tractable multi-stressor experiments  
418 could be reduced. In the negative scenario, increasing number of stressors result in  
419 acceleration of stress responses with high diversity of mechanisms and accumulation of  
420 synergism making the number of required experiments intractable.

421

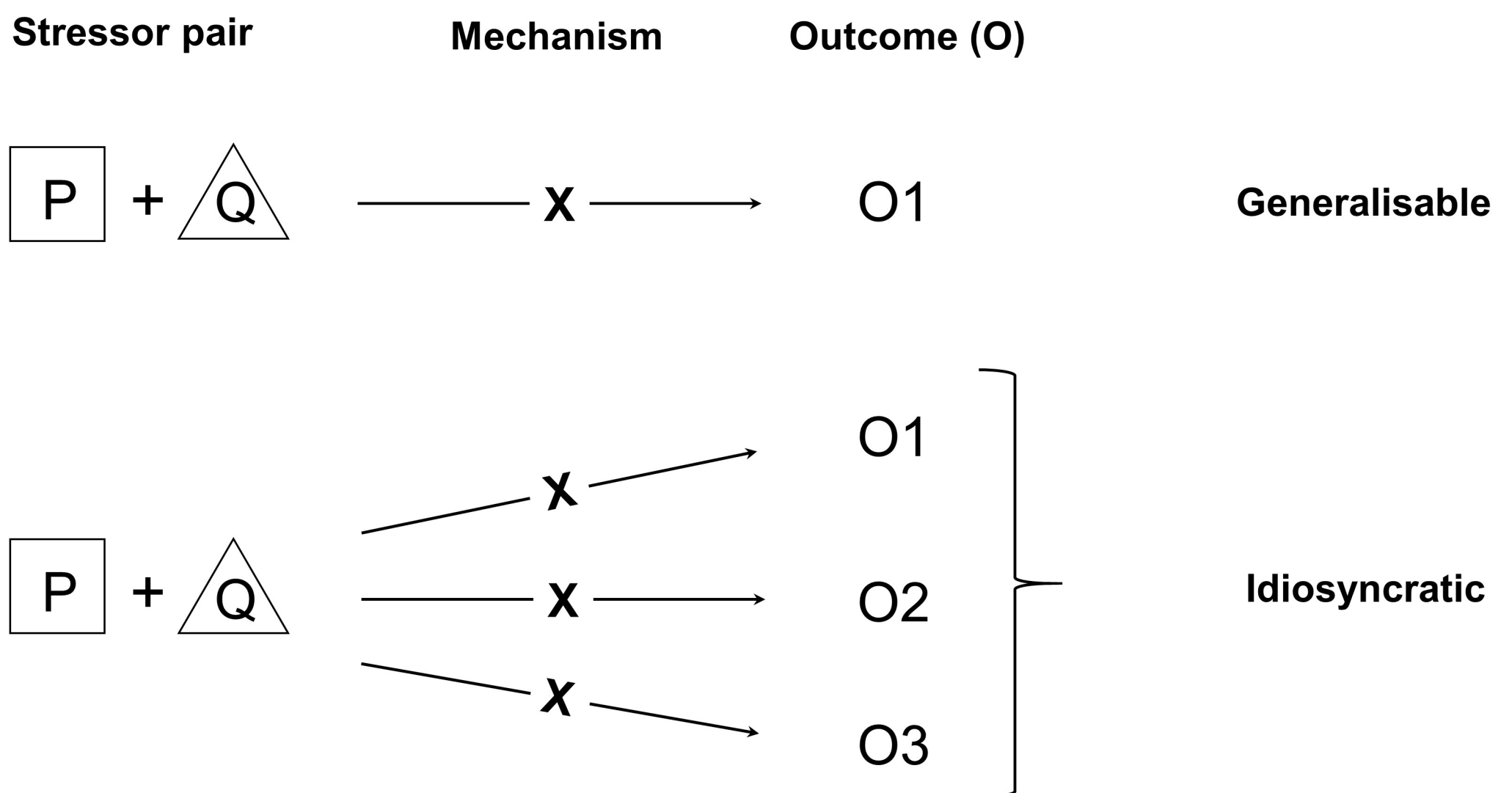
Figure 1



A)



B)



C)

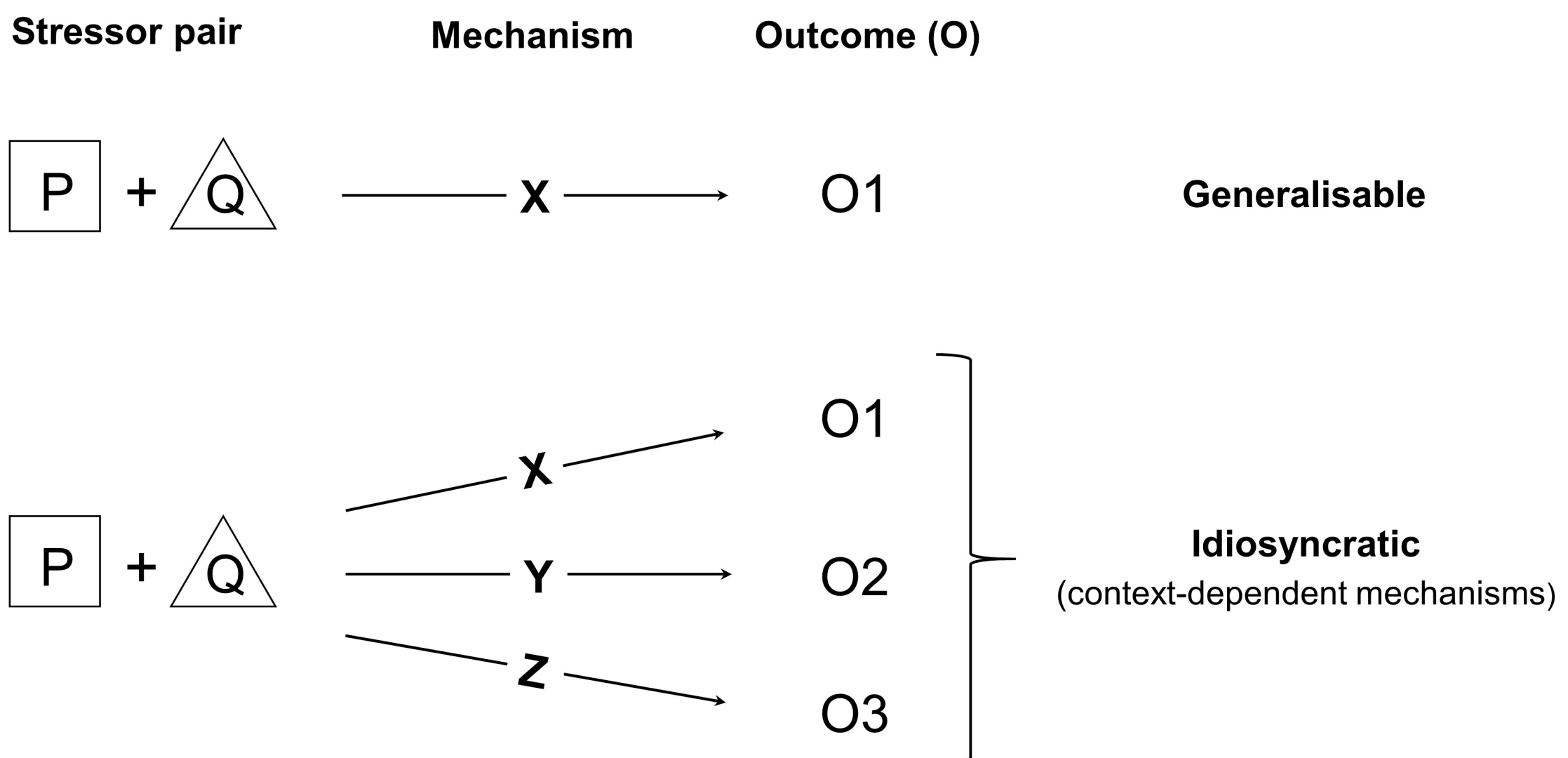
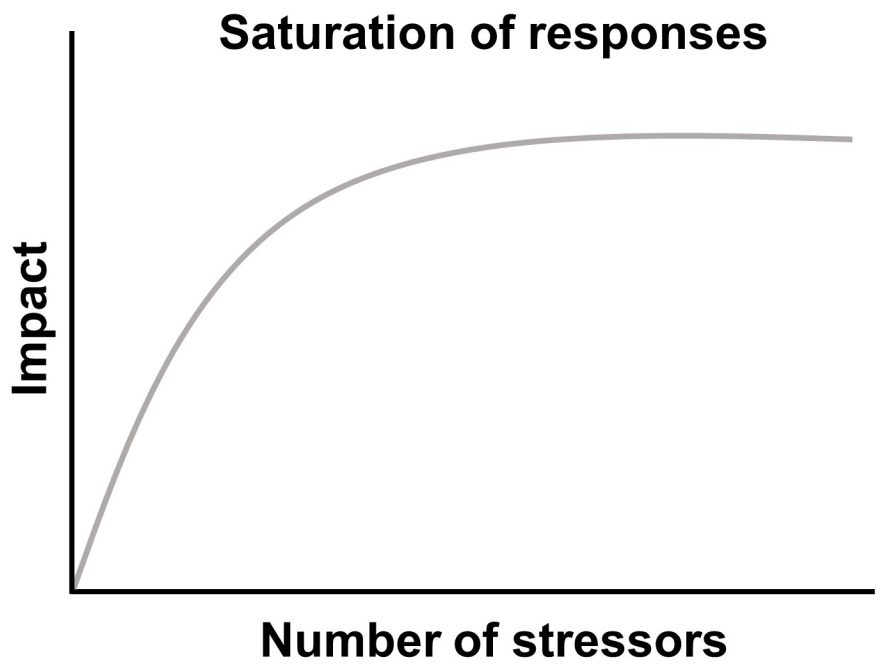


Figure 3

A )



B )

