

Rapid evolution generates synergism between multiple stressors: Linking theory and an evolution experiment

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Abstract

Global change encompasses many co-occurring anthropogenic stressors. Understanding the interactions between these multiple stressors, whether they be additive, antagonistic or synergistic, is critical for ecosystem managers when prioritizing which stressors to mitigate in the face of global change. While such interactions between stressors appear prevalent, it remains unclear if and how these interactions change over time, as the majority of multiple-stressor studies rarely span multiple generations of study organisms. Although meta-analyses have reported some intriguing temporal trends in stressor interactions, for example that synergism may take time to emerge, the mechanistic basis for such observations is unknown. In this study, by analysing data from an evolution experiment with the rotifer *Brachionus calyciflorus* (~35 generations and 31,320 observations), we show that adaptation to multiple stressors shifts stressor interactions towards synergism. We show that trade-offs, where populations cannot optimally perform multiple tasks (i.e. adapting to multiple stressors), generate this bias towards synergism. We also show that removal of stressors from evolved populations does not necessarily increase fitness and that there is variation in the evolutionary trajectories of populations that experienced the same stressor regimes. Our results highlight outstanding questions at the interface between evolution and global change biology, and illustrate the importance of considering rapid adaptation when managing or restoring ecosystems subjected to multiple stressors under global change.

KEYWORDS

adaptation, antagonism, *Brachionus calyciflorus*, contemporary evolution, global change factors, multiple drivers, stressor removal, synergism, trade-offs

1 | INTRODUCTION

Ecosystems experiencing global change are threatened by many different anthropogenic stressors. Predicting the combined effects of these multiple stressors is a deceptively challenging goal. A vast and diverse body of research has shown that the combined effect of stressors, or global change factors, often differs from what can

be expected based on individual effects. This discrepancy is what defines interactions between stressors. Antagonistic interactions cause combined effects to be less than expected, whereas synergistic interactions cause combined effects to be more than expected (Crain et al., 2008; Dieleman et al., 2012; Holmstrup et al., 2010; Jackson et al., 2016). Knowledge regarding the nature of stressor interactions can be used by ecosystem managers to prioritize which

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stressors to mitigate (Brown et al., 2013; Côté et al., 2016). Indeed, removing a stressor that interacts synergistically with other stressors will have a greater impact than removing a stressor that interacts antagonistically with other stressors (Brown et al., 2013; Falkenberg et al., 2013). There is, however, an overwhelming amount of context dependence in multiple-stressor research with seemingly few, if any, general patterns in the occurrence of antagonism or synergism (Côté et al., 2016; Kroeker et al., 2017; Orr et al., 2020). Moreover, interactions between stressors can change over time (Darling & Côté, 2008; Debecker et al., 2017; Lange et al., 2018), which further complicates efforts to understand and predict global change impacts.

Meta-analyses that investigated temporal patterns in stressor interactions have yielded conflicting results. In an analysis of 29 multiple-stressor studies on freshwater fish, the frequency of synergistic interactions increased with experimental duration (Lange et al., 2018). Conversely, a meta-analysis of 112 experiments that tested multiple-stressor effects on animal mortality found the opposite temporal trend that antagonism became more common as experimental duration increased (Darling & Côté, 2008). To reconcile these apparently opposing results, it is critical to understand the mechanisms that change stressor interactions over time. Indeed, recent progress has identified that stressor interactions can vary across different life stages (Fitzgerald et al., 2017; Przeslawski et al., 2015; Sniegula et al., 2017) and between parents and offspring due to transgenerational effects (Tran et al., 2018, 2019). Furthermore, adaptive evolution to one stressor can change its interactions with another stressor (Cambronero et al., 2018; Debecker et al., 2017; Zhang et al., 2018). Despite these advances, general expectations of how stressor interactions change over time remain elusive.

Here, we focus on rapid evolution, seeking a mechanistic understanding of its impacts on stressor interactions. Evolution can take place over ecological timescales and is therefore relevant to global change biology, restoration ecology and multiple-stressor research (Stockwell et al., 2003; Thompson, 1998; Zhang et al., 2019). Rapid evolution, or contemporary evolution, can occur within a month for organisms with fast lifecycles (Baym et al., 2016; Luijckx et al., 2017) and there is evidence of adaptation within seven generations for multiple species, including guppies (*Poecilia reticulata*), water fleas (*Daphnia pulex*) and flour beetles (*Tribolium castaneum*; Gorokhova et al., 2002; Reznick et al., 1997; Szűcs et al., 2017). Adaptation to global change can result in the 'evolutionary rescue' of populations and communities that would have otherwise gone extinct (Bell, 2017; Fugère et al., 2020; Hendry et al., 2017; Strauss et al., 2008). However, given that global change is comprised of many interacting stressors, it is crucial to understand whether, and how, rapid evolution to multiple stressors influences how those stressors interact.

Evolutionary theory can provide valuable insights into how adaptation to global change may alter stressor interactions. Performance trade-offs, where organisms cannot optimally perform multiple tasks, affect populations when adapting to multiple stressors (Agrawal et al., 2010; Hiltunen et al., 2018; Sheftel et al., 2018; Shoval et al., 2012; Tikhonov et al., 2020). Several evolutionary mechanisms can produce a trade-off, making it difficult for organisms to adapt to

multiple stressors at once. Antagonistic pleiotropy, where one gene controls multiple and opposing phenotypes (Williams, 1957), may cause the adaptation to one stressor to decrease an organism's tolerance to another (Anderson et al., 2013). For example, amphibian populations adapted to pesticides can have lower tolerance to certain parasites (Hua et al., 2017). Linkage disequilibrium (the non-random association of alleles) and epistasis (interactions between genes) are further potential causes of evolutionary trade-offs in multiple-stressor environments (Østman et al., 2012; Roff & Fairbairn, 2007). Genetic correlations between traits are also relevant when populations are adapting to multiple stressors (Agrawal et al., 2010; Agrawal & Stinchcombe, 2009). Finally, genetic bottlenecks associated with a reduction in population size due to intense stress may reduce genetic variation and impede adaptation to further stressors (Pedrosa et al., 2017; Ribeiro & Lopes, 2013). These trade-offs can be studied by plotting populations in performance space where each axis is defined by the adaptation of populations to a stressor. If evolved populations exhibit pareto optimality by falling on a convex hull of optimum strategies, known as the pareto front (Shoval et al., 2012; Tikhonov et al., 2020), adaptation to multiple stressors may be less than the sum of the adaptation to individual stressors, resulting in stressor interactions changing over time.

Here, we study the role of rapid evolution to multiple stressors in creating temporal variation in stressor interactions by analysing a dataset from an evolutionary experiment with the rotifer *Brachionus calyciflorus*, conducted by Luijckx et al. (2017). Rotifers are ecologically important organisms in freshwater ecosystems worldwide (Arndt, 1993) and are commonly used as model organisms to address ecological and evolutionary questions (Becks & Agrawal, 2010; Declerck & Papakostas, 2017). In the experiment, rotifers were grown in a full factorial design of three stressors (eight environments in total) for ~35 generations (70 days), creating eight evolved lines. Growth assays were conducted at the end of the experiment for each of the eight evolved lines of rotifers in each of the eight environments (64 combinations). We apply these data to a theoretical framework integrating the concepts of stressor interactions and performance trade-offs to explore general mechanisms of temporal trends in stressor interactions. We asked three questions at the interface of evolutionary biology and global change research. First, do interactions between stressors change over time due to adaptive evolution? Second, do trade-offs generate temporal variation in stressor interactions? Third, does removal of one or more stressors from populations evolved to those stressors increase or decrease their fitness?

2 | METHODS

2.1 | Evolution experiment and temporal variation in stressor interactions

We analysed data from an evolution experiment where the rotifer *B. calyciflorus* was allowed to adapt for 70 days (~35 generations) to individual and multiple stressors in a full factorial design.

For a detailed description of the experimental methods, we refer to Luijckx et al. (2017). In short, a population of rotifers (i.e. multiple genotypes) was hatched from sediment collected from Lake Onondaga, New York, and grown under standard laboratory conditions (constant light at 22°C) for 6 months (~100 generations) until the start of the experiment. In all, 32 rotifer populations were initiated (~6000 rotifers per population) from stock populations and exposed to eight different environments (four replicates per environment) where levels of salt (NaCl from 0 to 0.4 g/L), copper (0–1.25 μg of CuSO_4 per day) and temperature (reduced from 22 to 17.5°C) were altered in all possible combinations (Figure 1a). To study evolutionary effects, these levels of environmental change were chosen as proxies for environmental and chemical stressors because they were known to have negative individual effects, but non-lethal combined effects. Indeed, adaptation occurred in six of the seven stressor environments (Luijckx et al., 2017). Populations were maintained by providing 100 million algae per day and replacing 10% (50 ml) of the culture media every second day. Every 4 days, the size of all populations was standardized (to the third lowest population) to limit differences in evolutionary potential (i.e. the genetic variation available) between populations exposed to relatively benign environments and populations that experienced a large reduction in size due to high levels of stress (i.e. a genetic bottleneck). Adaptation during the experiment was monitored by

measuring both density (five 1 ml subsamples every 4 days) and lifetime reproductive success of individual rotifers (eight to 10 individuals per population for six timepoints). Growth assays were carried out at the end of the experiment where growth rates of 24 individuals from the 29 surviving populations (three populations from different treatments went extinct and were therefore not included in the analyses) were followed for 5 days in each of the eight environments (64 combinations of environment and evolved line with a total of 31,320 observations, Figure 1a). Data from growth assays were log transformed ($\ln(\text{data} + 1)$) and a linear regression was fitted against time in order to obtain population growth rates. Mean growth rates were then calculated for each combination of evolved line and environment (sample size of four, except for the evolved lines of copper, salt+copper and salt+temperature, where sample size was three). It should be noted that, while we have relatively few populations per treatment, high replication within each population ($n = 24$) allows us to detect differences within and between treatments.

The study of Luijckx et al. (2017) focused on the evolution of sexual reproduction. Here, we revisited the population growth rate data from that study by taking a multiple-stressor perspective. Our goal was to understand the role of rapid evolution in creating temporal variation of stressor interactions. The effects of stressors on non-evolved populations were determined using the growth

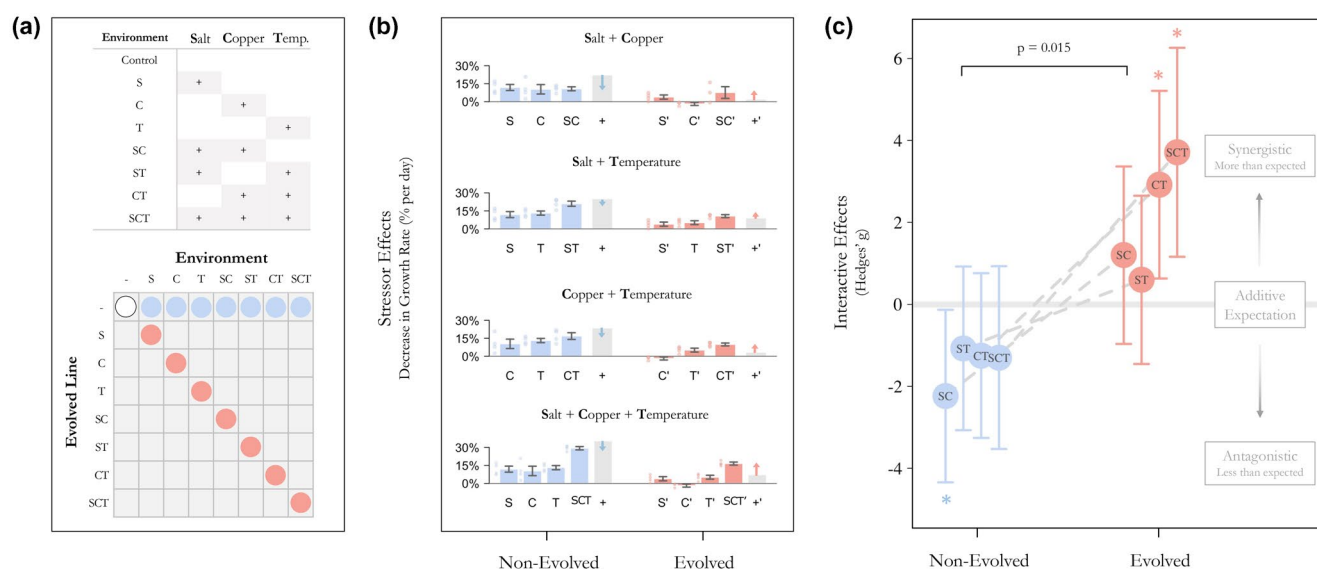


FIGURE 1 Bias towards synergism due to evolution. (a) A full factorial design of three stressors (salt, copper and temperature) created eight environments in which populations evolved. After evolution had occurred, growth rates of all populations in all environments were calculated. The white circle indicates how the baseline growth rate was determined. The blue and red circles represent non-evolved populations and evolved populations, respectively. (b) Individual and combined effects of stressors on the growth rates of non-evolved (blue) and evolved (red) populations for all combinations of stressors. The additive expectation for each combined effect is plotted in grey. Dots represent population replicates and error bars represent standard error. Arrows superimposed on the additive expectations represent interactive effects. Downward arrows indicate that the combined effect is less than expected (antagonism). Upward arrows indicate that the combined effect is more than expected (synergism). (c) Interactive effects (Hedges' g) with 95% confidence intervals for the four combinations of multiple stressors for non-evolved (blue) and evolved (red) populations. Interactive effects below and above the grey line (at $y = 0$) are antagonistic and synergistic, respectively. These effects are statistically significant (*) when 95% confidence intervals do not cross the grey line. Dashed lines connect evolved and non-evolved pairs of interactive effects. There is a clear shift from antagonism towards synergism due to evolution (paired t -test, $p = 0.015$)

rates of the control populations in the stressor environments (blue points in Figure 1a). The effects of stressors on evolved populations were determined using the growth rates of these populations in the stressor environment that they evolved in (red points in Figure 1a). Stressor interactions were identified based on an additive null model using standardized effect sizes (Hedges' g) with 95% confidence intervals for all four combinations of multiple stressors in non-evolved and evolved populations (Gurevitch et al., 2000). When the 95% confidence intervals crossed zero, stressor interactions were classified as additive. Negative and positive interactive effects whose confidence intervals did not cross zero were classified as antagonistic and synergistic, respectively (Hale et al., 2017; Jackson et al., 2016). To identify temporal changes in stressor interactions, interactive effects were compared between non-evolved and evolved populations using a two-sided paired samples t -test. This comparison (blue vs red points in Figure 1a) represents a scenario where a factorial study design is used to determine stressor interactions before and after evolution. One could also consider an alternative scenario where the null model used to predict the combined effect of stressors remains fixed at a point prior to the occurrence of evolution. Under such a scenario, one may expect combined effects to diminish due to evolution leading to a shift towards antagonism over time. Here, we focus on the first scenario which represents full factorial designs, the bedrock of multiple-stressor research, but we refer to Figure S1 and Section 4 for inferences and implications of the second scenario.

2.2 | Theoretical framework: Performance trade-offs should generate a bias towards synergism

To explore the role of performance trade-offs in creating temporal variation of stressor interactions, we developed a theoretical framework integrating the concepts of multiple-stressor interactions and performance trade-offs. First, by comparing the sum of adaptation to individual stressors and the adaptation to multiple stressors, we define and quantify a shift in stressor interactions (Figure 2a). We then considered performance space, where each axis is defined by the adaptation of populations to a single-stressor environment (Figure 2b). If a performance trade-off exists, populations cannot optimally adapt to multiple environments: for a population to improve its fitness in an environment, it will lose fitness in another. Evolved states would then, at best, lie on a convex surface of optimum strategies, known as the pareto front (Shoval et al., 2012; Tikhonov et al., 2020). We then compared the actual adaptation to multiple stressors (i.e. increase in fitness, ΔAB) and the taxi-cab distance (Δ^*AB) in performance space between non-evolved populations and populations evolved to multiple stressors (Figure 2). If adaptation to multiple stressors can be decomposed into adaptation to one stressor and adaptation to the other stressor (additive adaptation, see Note S1), then these two values (ΔAB and Δ^*AB) will be similar. We propose that if these two values are similar, and if there is evidence of performance trade-offs, then evolution will shift stressor interactions

towards synergism. Antagonistic interactions will become less antagonistic or even synergistic, while already synergistic interactions will become even more synergistic.

We then applied our theoretical framework to the population growth rate data. The growth rates of all populations in all environments are known (Figure 1a), allowing us to plot populations in performance space. To avoid artificially overestimating the strength of trade-offs, we had to account for the effect that the removal of a stressor can have on population fitness. For example, when populations evolved to salt+copper are grown in the salt environment, they will experience the removal of the copper stressor, which they are adapted to. When positioning these populations in performance space (the adaptation to salt alone on one axis and the adaptation to copper alone on the other), we must account for the change in fitness caused by the absence of one of the stressors. Otherwise, the trade-off observed could simply be entirely driven by the stressor removal (see Note S1 where we formalize this correction and clarify the underlying assumption of additive adaptation that it relies on). To test for the existence of performance trade-offs and the occurrence of a pareto front, we thus plotted populations in performance space, controlling for removal effects, for all combinations of stressors. We then compared the actual adaptation to multiple stressors (ΔAB) and the distance in performance space between non-evolved populations and populations evolved to multiple stressors (Δ^*AB) to determine whether performance trade-offs played a role in changing stressor interactions over time.

2.3 | Removal of stressors

To understand whether rapid evolution to stressors influences restoration efforts, we tested how removal of one or more stressors, after prolonged exposure, impacted population fitness. Specifically, we tested whether growth rates of evolved populations in their own environment differed from their growth rates in the control environment (i.e. when stressors were removed) using a two-sided paired t -test, which controlled for differences between populations. Finally, to determine whether the identity of the removed stressor was important, we compared the growth rates of the populations adapted to all three stressors when grown in the double stressor environments (e.g. lacking either temperature, salt or copper stressors) using a one-way ANOVA with Dunnett's 'many-to-one' post-hoc comparison (Dunnett, 1955). The assumptions of statistical tests (e.g. normality, homogeneity of variances, independence of data) were met and the data and code used for all statistical analyses and figures are available on GitHub: (<https://github.com/jamesaorr/evolution-multiple-stressors>).

3 | RESULTS

Adaptive evolution to all individual and multiple-stressor environments occurred within 1 month (~20 generations, except potentially

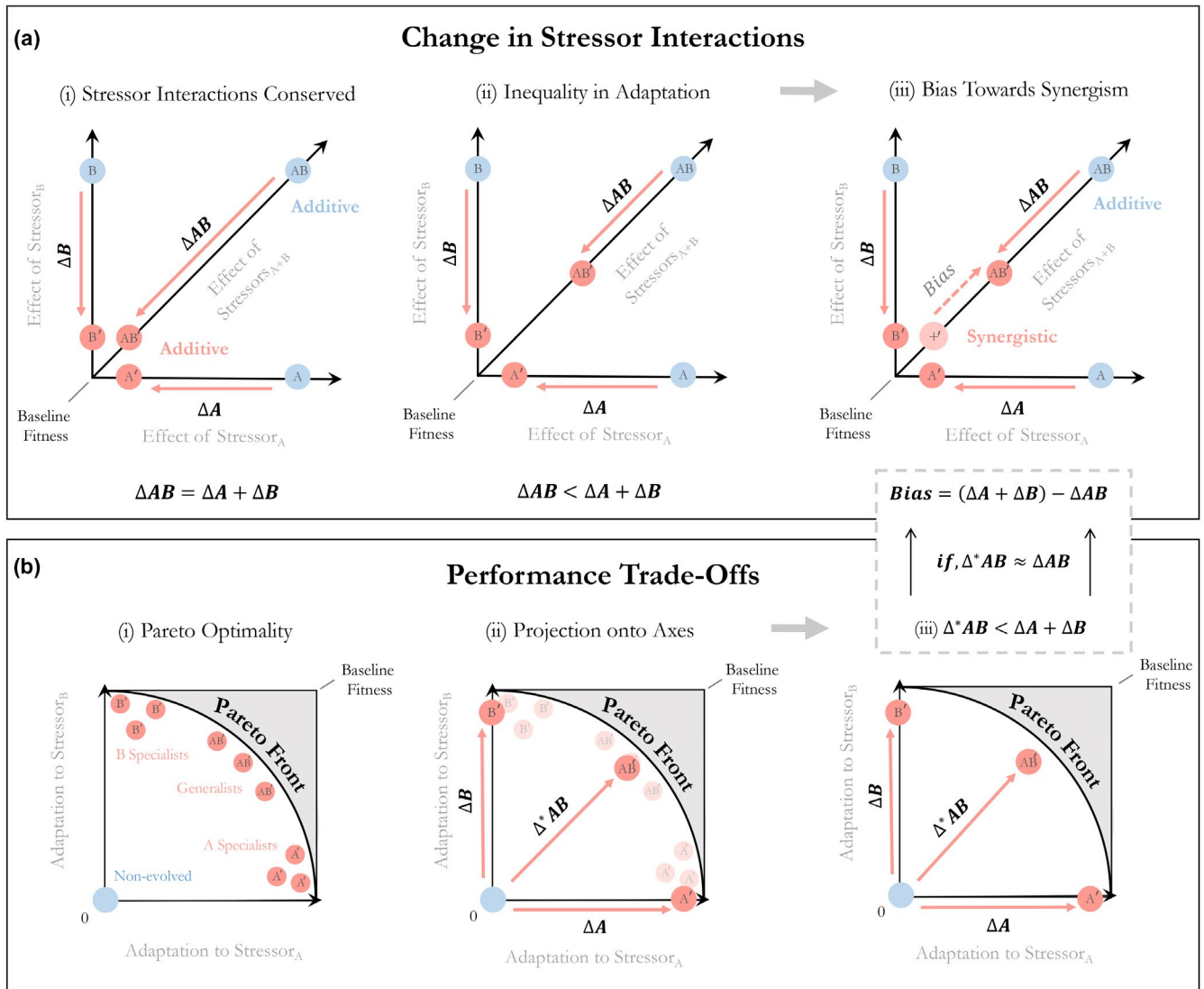


FIGURE 2 The link between performance trade-offs and changes in stressor interactions. (a) The effects of two stressors, A and B, and their combined effect, AB, define three separate axes to visualize additivity between stressors. The effect of stressors on non-evolved (blue) and evolved (red) populations can only fall on these axes. Adaptation (i.e. reduction in effect of stressors) is represented as red arrows and is quantified by the difference (e.g. ΔA) between non-evolved (e.g. A) and evolved (e.g. A') populations. (i) If adaptation to multiple stressors is equal to the sum of the adaptation to individual stressors ($\Delta AB = \Delta A + \Delta B$), then interactions between stressors (additive in this example) are conserved across evolution. (ii) However, if adaptation to multiple stressors is less than the sum of adaptation to individual stressors ($\Delta AB < \Delta A + \Delta B$), then (iii) interactions will shift towards synergism. The magnitude of this bias towards synergism is equal to the difference between the adaptation to multiple stressors and the sum of the adaptation to individual stressors ($\text{Bias} = (\Delta A + \Delta B) - \Delta AB$). (b) Non-evolved populations and populations evolved to A, B or AB environments are plotted in performance space, where each axis is defined by the adaptation to an individual stressor. Populations can fall anywhere in this space. (i) If there is a performance trade-off, if populations cannot optimally adapt to multiple environments, evolved populations will, at best, be restricted to the Pareto front, the convex hull of optimum strategies. (ii) Projecting the populations evolved to A or B onto their respective axes will recover the magnitude of adaptation to individual stressors (ΔA , ΔB). The taxi-cab distance in performance space between non-evolved populations and populations evolved to multiple stressors ($\Delta^* AB$) is different from the actual adaptation to multiple stressors (ΔAB). (iii) A performance trade-off results in this distance being less than the sum of the adaptation to individual stressors ($\Delta^* AB < \Delta A + \Delta B$). If the distance in performance space between evolved and non-evolved states is roughly equal to the adaptation to multiple stressors ($\Delta^* AB \approx \Delta AB$), then a performance trade-off will generate a bias towards synergism

the multiple-stressor combination of salt+copper). For non-evolved populations, the combined effects of stressors were less than expected due to antagonistic interactions (blue points in Figure 1c). For evolved populations, however, the combined effects of stressors

were more than expected due to synergistic interactions (red points in Figure 1c). Although not all stressor interactions were statistically detectable (1/4 non-evolved and 2/4 evolved), there was a clear shift towards synergism in the interactive effect sizes between

non-evolved ($M = -1.46$, $SD = 0.52$) and evolved ($M = 2.11$, $SD = 1.45$) populations ($t_3 = -5.03$, $p = 0.015$).

Adaptation to multiple stressors (diagonal red arrows in Figure 3a) was always less than the sum of the adaptation to individual stressors (vertical and horizontal red arrows in Figure 3a). For example, populations adapted to copper or salt stressors regained all (C to C') or most (S to S') of the fitness lost due to the stressors, but fitness of population adapting simultaneously to both stressors (SC to SC') improved little (Figure 3a(i)). This inequality, between the magnitude of the diagonal red arrows and the sum of the magnitudes of the horizontal and vertical arrows in Figure 3a

(e.g. $\Delta SC < \Delta S + \Delta C$), explains the shift towards synergism. In fact, as this inequality increases, the shift towards synergism also increases. The three-stressor combination had the greatest inequality between the adaptation to multiple stressors and the sum of the adaptation to individual stressors (Video S1) and therefore experienced the greatest shift towards synergism (steepest slope in Figure 1c). Plotting populations in performance space showed no clear evidence of pareto optimality (Figure S2; Video S2a—we return to this point in the Section 4). For all combinations of stressors, however, mean values of populations from the same evolved line revealed performance trade-offs in the adaptation to multiple

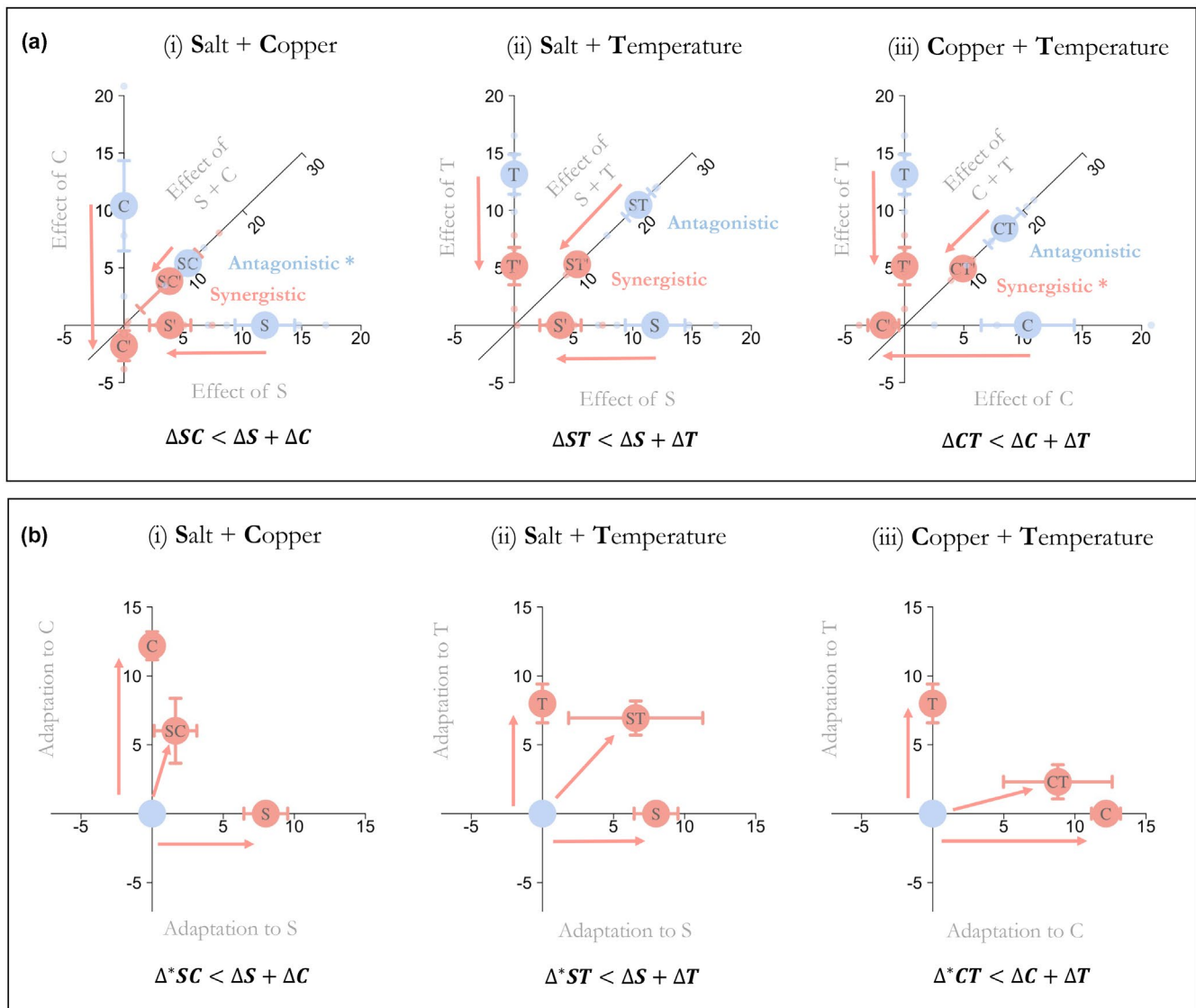


FIGURE 3 Empirical test of the link between performance trade-offs and changes in stressor interactions. (a) Population growth rate data for all pairs of stressors applied to the framework presented in Figure 2a. Effects of stressors are quantified by decreases in growth rates. Large points represent mean effects of stressors, error bars represent standard error and small points represent the effect of stressors on individual populations. For all pairs of stressors, there is a bias towards synergism due to evolution (as in Figure 1c). (b) As in Figure 2b, mean population growth rates with standard error are plotted and the populations evolved to individual stressors are projected onto their respective axis. For all pairs of stressors, the taxi-cab distance in performance space between evolved and non-evolved states is less than the sum of the adaptation to individual stressors (e.g. $\Delta^* SC < \Delta S + \Delta C$), revealing performance trade-offs

stressors (Figure 3b; Video S2b). Furthermore, the actual adaptation to multiple stressors was similar to the distance between non-evolved and evolved populations in performance space (Figure 4),

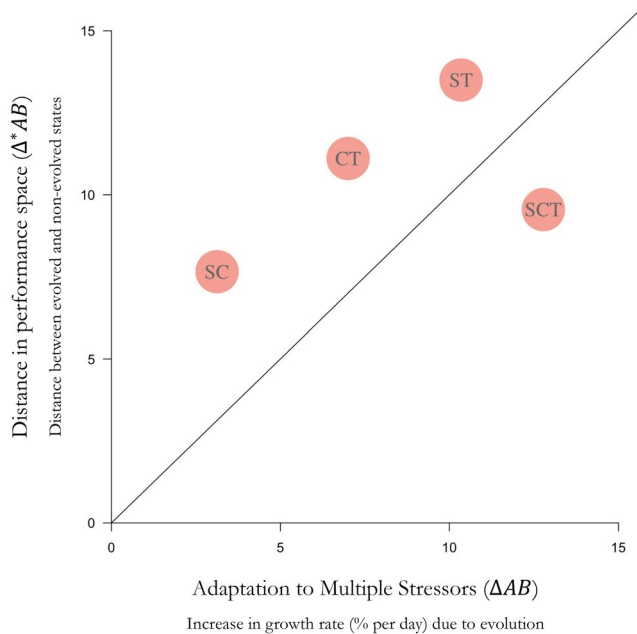


FIGURE 4 Comparison of magnitude of adaptation to multiple stressors and the taxi-cab distance between non-evolved and evolved populations in performance space. For each combination of multiple stressor (SC, ST, CT and SCT), the magnitude of adaptation (increase in growth rates) and the taxi-cab distance in performance space are plotted. The black diagonal line is the 1:1 line

thus making performance trade-offs a relevant mechanism to explain stressor interactions.

Removal of stressors from populations exposed to those stressors did not necessarily increase fitness (Figure 5). Growth rates of populations grown in the absence of stressors were not significantly different from those where stressors were present ($t_{24} = 0.39$, $p = 0.699$). Moreover, when only considering populations that were exposed to one or two stressors, growth rates (% increase per day) of evolved rotifers decreased by 2.2% (95 CI -0.2 , $+4.5$) when stressors were removed, although this result was not statistically significant ($t_{20} = 1.88$, $p = 0.074$, note the contrasting pattern in Figure 5a for the triple stressor evolved line, which was excluded from this second analysis). Growth rates of the populations evolved to three stressors ($M = 5.4$, $SD = 1.9$) were not significantly impacted when the salt ($M = 7.2$, $SD = 3.3$, $p = 0.83$) or copper ($M = 2.7$, $SD = 2.9$, $p = 0.59$) stressors were removed, but their growth rates increased by 6.9 (± 2.5 SE) when the temperature stressor was removed ($M = 12.3$, $SD = 5.3$, $p = 0.046$; Figure 5b).

4 | DISCUSSION

In an evolution experiment with the rotifer *B. calyciflorus*, we found that rapid evolution to multiple stressors created an emergent bias towards synergistic stressor interactions. Our results support the notion that rapid, contemporary evolution should be accounted for to accurately predict the impacts of multiple stressors. The observed bias towards synergism was independent of how the stressors themselves were interacting (e.g. via physicochemical effects;

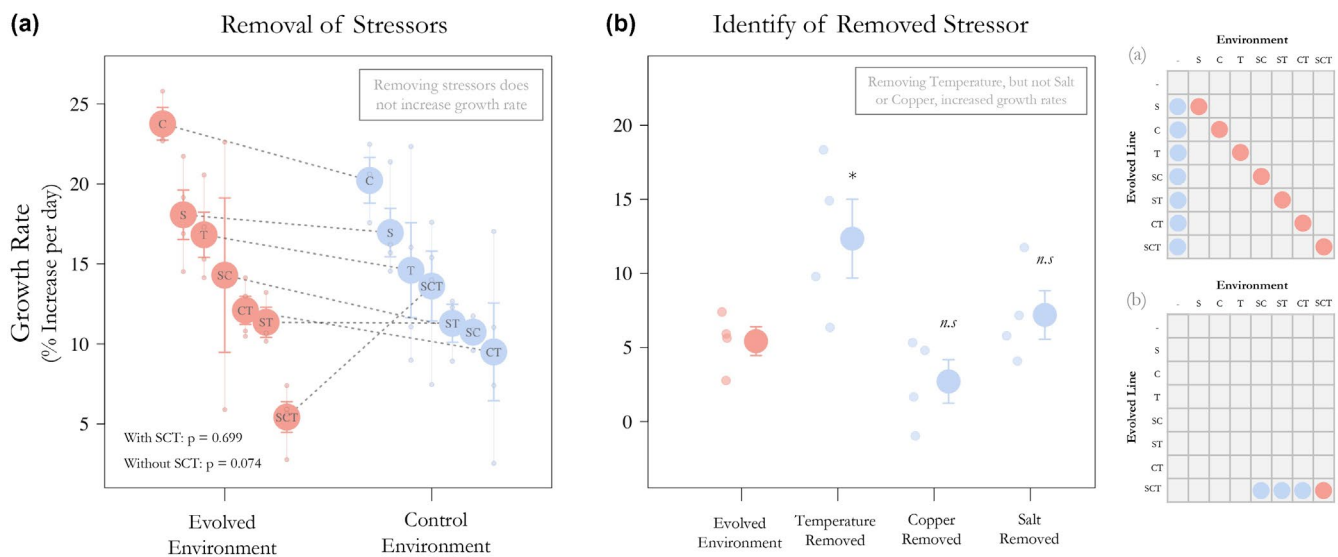


FIGURE 5 Removing stressors from evolved populations does not necessarily increase fitness. (a) Mean growth rates of populations in their own environment (red) and in the control environment (blue) (i.e. when stressors have been removed). Small dots represent population replicates, error bars represent standard error and dashed lines connect each pair that is being compared. Removal of stressors does not increase growth rate. (b) Mean growth rates of populations evolved to all three stressors in their own environment and in all double stressor environments. Small dots represent population replicates and error bars represent standard error. Removal of temperature, but not copper or salt, increased growth rates (*significant difference, n.s. = not significant). The comparisons being made in (a) and (b) are illustrated using the growth assay schema from Figure 1a

sensu Boyd & Brown, 2015), but was instead, a consequence of an inequality between the adaptation to multiple stressors and the sum of the adaptation to individual stressors. This effect co-occurred with a reduction of the combined effect of stressors due to adaptation. The synergism associated with trade-offs is therefore very different to the classic notion of synergism in ecology (Brook et al., 2008; Sih et al., 2004). Crucially, this highlights the importance of having a mechanistic understanding of stressor interactions when making management decisions. This is particularly relevant when the management of local stressors is influenced by the identity of their interactions (antagonistic or synergistic) with global stressors such as climate change (Brown et al., 2013, 2014). Removal of a stressor should not be blindly prioritized based on the emergent synergism due to evolutionary trade-offs, but should rather be based on the magnitude of effects and on an understanding of the mechanisms of stressor interactions (Orr et al., 2020; Schäfer & Piggott, 2018). Furthermore, knowledge of how well adapted organisms are to existing stressors could inform decisions regarding the removal of stressors, as we found that removal of stressors from populations evolved to those stressors does not necessarily increase fitness. Evolution can change the optimum environmental conditions for populations, meaning that removal of a stressor could potentially be a stressor itself.

4.1 | Temporal variation in stressor interactions

Our main finding that adaptation to multiple stressors generates synergism (question 1) is relevant to a scenario where factorial designs are employed, the standard approach in multiple-stressor research, where evolution to both multiple and individual stressors can be considered. In this context, our results suggest that as study duration increases, the probability of synergism also increases. This corroborates the findings of a recent meta-analysis of the effects of multiple stressors on fish (Lange et al., 2018). Given enough time, however, we cannot rule out the possibility of evolution overcoming performance trade-offs (i.e. by compensatory mutations) and potentially removing all stressor effects and interactions. We also considered a second scenario, where evolution to multiple stressors, but not to individual stressors, is considered so the null models used to detect stressor interactions remain fixed over time (Figure S1). Although this is not how interactions between stressors are usually studied, it may be of general interest as it represents a situation where time-series data are collected in natural systems experiencing global change and may become relevant when comparing field and experimental studies of multiple stressors (e.g. Birk et al., 2020). Here, as the null model remains fixed (as the sum of the initial effects of stressors) but the combined effect of stressors is reduced over time due to adaptation, stressor interactions will be shifted in the opposite direction, towards antagonism. This may be relevant to the results of a meta-analysis of terrestrial global change experiments, where effect sizes decreased with experimental duration (Leuzinger et al., 2011). In multiple-stressor research, factorial designs are the

rule, so the first scenario, where we generally predict a bias towards synergism over time, is more relevant. Nonetheless, we cannot rule out the possibility of adaptation to multiple stressors being greater than the sum of adaptation to individual stressors, which would bias stressor interactions towards antagonism (Figure S3). We reasonably expect, however, that such instances will be the exception rather than the rule (Agrawal et al., 2010; Barrett et al., 2005). It should be noted that these ideas are not specific to adaptation through evolution, but equally apply at smaller temporal scales when considering adaptation via phenotypic plasticity or epigenetic modifications (Fox et al., 2019; Rey et al., 2016; Scoville & Pfrender, 2010). Some meta-analyses have suggested that synergistic interactions take time to develop (Darling & Côté, 2008; Lange et al., 2018). Despite all of the context dependence of multiple-stressor research, we have identified a general mechanism, adaptation to multiple stressors being less than the sum of the adaptation to individual stressors, that may explain this temporal trend.

4.2 | Performance trade-offs and synergism

Our results support the theory that performance trade-offs generate a bias towards synergistic stressor interactions (question 2). First, populations that adapted to multiple stressors showed clear performance trade-offs, demonstrated by a reduction of adaptation to single stressors (Figure 3b). Second, the similarity between the magnitude of adaptation to multiple stressors and the distance between evolved and non-evolved states in performance space (Figure 4) indicated that adaptation to multiple stressors can be approximately decomposed into the adaptation to one stressor and adaptation to the other stressor (additive adaptation). Combined, these two points reveal that performance trade-offs introduce a systematic bias towards synergism. Occurrence of performance trade-offs seems inevitable, yet the reality is more subtle. Indeed, adaptation to both stressors (no trade-off) is sometimes possible, at least for salt and copper. Although some populations that only evolved to a single stressor (e.g. salt) performed poorly in the presence of another 'unknown' stressor (e.g. copper), other populations performed surprisingly well when exposed to an 'unknown' stressor (Figure S2). Given the high replication within each population, this variation is probably not related to sample size, but may instead be caused by populations moving to different peaks within a (genetic or phenotypic) fitness landscape (Simpson, 1944; Wright, 1932). This suggests that, in our study, performance trade-offs are not necessarily related to an 'intrinsic' pareto optimality (i.e. an inherent constraint on performance in multiple-stressor environments), but may reflect evolutionary history. It is possible that evolution had not yet converged and that, if given enough time, populations would approach a convex hull in performance space. However, fitness remained constant for a number of generations after the initial adaptation (Luijckx et al., 2017). Finally, it should be noted that performance trade-offs, whatever their causes, will not be relevant if the multiple-stressor environment is very different

from a simple combination of the individual stressor environments, due to physicochemical stressor interactions (Boyd & Brown, 2015). For example, consider two chemical stressors whose combination is effectively a third, novel chemical stressor (e.g. chemical interactions between multiple pesticides; Hernández et al., 2017). This would typically result in adaptation to multiple stressors being greater than the distance between evolved and non-evolved states in performance space. If performance trade-offs do play a role in creating emergent synergism, it is interesting to consider the consequences of increasing the number of stressors, and therefore increasing the dimensions of performance space and of pareto fronts themselves (Tikhonov et al., 2020). This is an idea that deserves further study, especially considering the recent interest in using the number of stressors, rather than their identity, to predict ecological and evolutionary responses to global change (Brennan et al., 2017; Komatsu et al., 2019; Rillig et al., 2019).

4.3 | Removal of stressors

Another key insight, potentially complicating ecosystem management, is that removal of stressors from evolved populations did not necessarily increase fitness (question 3). Evolution to stressors shifted populations' optimum environmental conditions leaving them maladapted to the original environment (Luijckx et al., 2017). Indeed, environmental variability is one of the key mechanisms of maladaptation (i.e. 'moving target' in Brady et al., 2019). Similarly, how adaptation to a novel environment influences fitness in the original environment has long been of interest to evolutionary biologists (Bennett & Lenski, 2007; Hereford, 2009; Levins, 1968). A textbook example of adaptation to global change that mirrors our results is the rapid evolution of the peppered moth (*Biston betularia*) in response to the rise and fall of industrial pollution. The frequency of melanistic peppered moths, which use their colouration as camouflage to avoid predation, rose with increasing levels of soot during the industrial revolution. As this stressor was removed during the 20th century, the melanistic phenotype became rare as it was maladapted to the original environment (Cook et al., 2012; Kettlewell, 1958). Many studies have since explored the importance of considering adaptation to global change in the conservation and restoration of biodiversity (e.g. evolutionary rescue; Bell, 2017, adaptation to anthropogenic stressors; Rice & Emery, 2003; Stockwell et al., 2003 and role of eco-evolutionary dynamics; Hendry, 2020). Our study contributes to this interdisciplinary field by exploring the consequences of adaptation to co-occurring multiple stressors. Although multiple-stressor research primarily focuses on the effects of adding stressors, there have been some investigations into the effect of removing stressors (Brown et al., 2013; Falkenberg et al., 2013; Fong et al., 2018). However, to our knowledge, none of these studies have considered the consequences of adaptation to stressors. In our study, we found that when populations had evolved to all three stressors, the removal of different stressors had varying consequences.

Fitness increased if the temperature stressor was removed (thus increasing temperature), which is unsurprising considering the relationship between temperature and metabolism (Brown et al., 2004). However, removal of the salt or copper stressors did not increase fitness (Figure 5b) as populations had successfully adapted to these new conditions. In this case, if ecosystem managers only had enough resources to remove one of the three stressors, having a mechanistic understanding of these stressors would be critical when prioritizing which stressor to remove. In addition, when removing stressors (particularly temperature), there was variation in fitness between populations that had evolved in the same environments (Figure 5b). This variation in the evolutionary trajectories of populations under the same conditions has been previously observed (Bennett & Lenski, 2007). The different paths evolution can take to reach peaks in fitness landscapes may add uncertainty to predictions of the long-term impacts of global change. Future multiple-stressor removal experiments, and indeed ecosystem management, may be enhanced by considering adaptation to single and multiple stressors.

4.4 | Implications and limitations for global change research

Insights from evolutionary biology may be useful to global change ecologists, particularly considering the growing interest in the temporal dynamics of multiple stressors (Gunderson et al., 2016; Jackson et al., 2021; Orr et al., 2020; Ryo et al., 2019). From an evolutionary perspective, the ability of populations to adapt to stressors may be impacted by the timing of stressors. For instance, a recent mesocosm experiment showed that prior exposure to a stressor strongly predicted the evolutionary rescue of communities from that stressor at levels lethal to naïve communities (Fugère et al., 2020). In our study, stressors were applied simultaneously as constant press perturbations and the levels of environmental change were chosen to explore the role of evolution, rather than being based on specific global change scenarios. However, the rate of environmental change can have strong evolutionary and ecological consequences (Bell, 2017; Pinek et al., 2020). Indeed, it has been demonstrated theoretically and empirically that the probability of evolutionary rescue is higher when environmental change is gradual rather than abrupt as this allows time for advantageous mutations to arise (Bell, 2017; Lindsey et al., 2013). With mechanistic insights from evolutionary biology, future work could build on our proof of concept by investigating the extent of adaptation to more realistic stressor magnitudes and temporal regimes. Furthermore, our experimental design only allowed us to study the immediate effect of removing stressors. If given time to adapt, would these populations be able to evolve back to their original baseline fitness after the removal of stressors? This would depend in part on the available genetic variation. While stressors with large effects may impose greater selective pressures on populations and thus speed up evolution, large stressor impacts may also reduce population size and genetic variation (a genetic bottleneck)

which may impede further adaptation. Moreover, available genetic variation would be higher if adaptation to previous stressors occurred from standing genetic variation (soft selective sweep) than from a newly arisen mutation (hard selective sweep; Burke, 2012; Payne & Wagner, 2019; Pedrosa et al., 2017). Similarly, genetic variation may be maintained at higher levels for local stressors or those that only occur periodically as genetic variation is maintained at a higher level in spatially or temporally varying environments (Huang et al., 2016). Theories from evolutionary biology are clearly relevant to multiple-stressor research and may improve understanding and prediction of how populations will respond to future global change scenarios.

Our theoretical and empirical results, although based on a single-species evolution experiment, potentially have ramifications for the conservation of entire ecosystems facing global change. Rapid evolution will occur for species with short generations and fast turnover, such as bacteria, algae and the rotifers that we studied (Baym et al., 2016; Luijckx et al., 2017). These organisms are the foundations of food webs and their adaptation to stressors will have knock-on consequences for the larger, more charismatic species (Hiltunen et al., 2017; Jones & Ellner, 2007) that conservation and restoration projects are often focused on (Colléony et al., 2017). It is, however, unclear whether adaptive evolution to stressors would be as strong when organisms are entangled in a web of biotic interactions. Indeed, adaptation of bacteria to environmental change is restricted in complex communities (Scheuerl et al., 2020). Would the emergent synergism caused by adaptation to multiple stressors still exist when considering communities of species? Nonetheless, understanding the population-level consequences of rapid evolution to multiple stressors is required for accurate, mechanistic predictions of community-level responses to environmental change. This paper has raised a number of outstanding questions, that sit at the interface between ecology, evolution and global change biology, which we summarize in Box 1. Our results have revealed the importance of considering adaptation to global change when understanding and predicting the ecological impacts of multiple stressors.

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AUTHOR CONTRIBUTION

James A. Orr carried out the data analysis, created the figures and wrote the first draft of the manuscript. Pepijn Luijckx conducted the evolution experiment and provided the data. Andrew L. Jackson and

BOX 1 Outstanding questions

- Does the emergent bias towards synergism created by evolutionary trade-offs become stronger as the number of stressors increases?
- Is removal of stressors based on their interaction types (antagonism/synergism) an effective strategy when adaptation to stressors has occurred?
- If the stressors that populations have adapted to are removed, how long will it take for populations to adapt back to their original baseline fitness?
- How much variation is there in the evolutionary trajectories of populations that have experienced the same stressor regimes?
- Is adaptation to multiple stressors important when considering communities of species? How does adaptation to stressors scale up when considering ecosystem-level properties (diversity, function)?
- How does environmental complexity influence evolvability? Is environmental complexity more closely approximated by the number of stressors or by some form of stressor diversity?

Pepijn Luijckx supervised the data analysis. James A. Orr and Jean-François Arnoldi developed the framework integrating multiple-stressor interactions and evolutionary trade-offs. Jeremy J. Piggott and Pepijn Luijckx initiated and supervised the overall project. All authors contributed to the final manuscript.

DATA AVAILABILITY STATEMENT

The code and data used for all statistical analyses and figures are available in R notebooks on GitHub (<https://github.com/jamesaorr/evolution-multiple-stressors>).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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