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Multiple stressors within and across populations: how predictable and repeatable is population response, evolution, and adaptation?

A dissertation submitted in partial satisfaction
of the requirements for the degree
Doctor of Philosophy in Biology

by

Eleanor Shelly Diamant

ABSTRACT OF THE DISSERTATION

Multiple stressors within and across populations: how predictable and repeatable is population response, evolution, and adaptation?

by

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Doctor of Philosophy in Biology
University of California, Los Angeles, 2023
Professor Pamela J. Yeh, Chair

Anthropogenic change alters environments and introduces novel stressors to multiple species and communities. Understanding how populations shift and cope in response to these stressors is essential to test evolution in real-time and predict our impacts on natural populations. In urban landscapes, populations that survive undergo rapid evolutionary and behavioral changes in response to strong selective pressures (Chapter 1). I am interested in how these populations respond to multiple stressors and in the patterns and processes underlying adaptive response to strong anthropogenic change. I led a reanalysis of population response data to multiple stressors across species and habitats from the last 25 years of studies by applying a new and generalizable framework—Rescaled Bliss Independence (RBI)—to evaluate stressor interactions (Chapter 2). We found that antagonism and additivity, rather than synergy, are the most frequent interaction types and that our novel method is likely to re-classify previously synergistic interactions as non-synergistic. I applied the RBI to three stressor combinations and found that antagonism was the dominant net interaction type, while synergy was the dominant emergent interaction type (Chapter 3). Next, I delved into one species to assess how populations respond to strong anthropogenic change in the wild. I tested four independent urban colonist populations of Dark-eyed Juncos (*Junco hyemalis*) to test if the birds have adapted morphological traits similarly in comparison to three non-urban populations or if similar environmental differences can result in different adaptations (Chapter 4). I found that there are complex patterns where some traits converge in some cities, while others are associated with urbanization generally. I quantified behavioral plasticity in response to the change in human activity due to COVID-19 restrictions ("the anthropause") in one of the urban populations (Chapters 5 and 6). I found that fear response surprisingly decreased following campus re-opening, but didn't change during the anthropause, posing new hypotheses to the development of a trait essential for urban living. I also found that novel urban nesting did not change during the anthropause, suggesting that novel nesting behavior is driven by landscape or urban predator effects, not human disturbance. This dissertation in sum investigates the patterns and processes underlying population response to novel stressors, with an emphasis on determining the predictability of how organisms respond to the growth of urbanization, a present threat to biodiversity.

The dissertation of Eleanor Shelly Diamant is approved.

Ian MacGregor-Fors

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I dedicate this dissertation to the women in science who have come before me and paved the way for more science to grow and for my perspective to be held and celebrated. To Penny Fogel for your open heart and open door. I dedicate this dissertation to my 5th grade teacher, Ms. Leanne Statland Ellis who watered my love of nature and guided my environmentalism, to my high school teacher Ms. Martha Wheelock who inspired me to embrace my inner self, its connection to ecology and advocacy for all beings, and to build confidence in my abilities while making space to integrate perspectives from others. I dedicate this dissertation to my family members before me, who have overcome tremendous generational hurdles to make it possible for me to pursue my passions.

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Diamant, ES, M Walters, PJ Yeh. Urban juncos during the anthropause. Invited talk: UChicago and the Field Museum, April 2023, Chicago, IL.

Diamant, ES, I MacGregor-Fors, PJ Yeh. Before, during, and after the "anthropause": the impact of human activity on individual fear response in an urban bird. Invited talk: Santa Monica Bay Audubon Society, February 2023, virtual.

Diamant, ES, I MacGregor-Fors, PJ Yeh. Before, during, and after the "anthropause": the impact of human activity on individual fear response in an urban bird. Invited talk: International Ornithological Congress, August 2022, virtual.

Diamant, ES. Only one way to succeed in the city? Dark-eyed Junco adaptation across urban "replicates". Graduate student workshop talk: Urban Animals Past and Present, October 2018, UCLA, Los Angeles, CA

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Diamant, ES. The Sea Around Us: DDT, the Pacific, and our ecological webs. Guest Lecture: DESMA 160 Ecological Arts & Justice Fieldwork Practice. Spring 2023, UCLA, Los Angeles, CA.

Diamant, ES. Explorations in avian animal behavior; Or, how I learned to see things from the bird's-eye view. Guest Lecture: EEB 100L Introduction to Ecology and Behavior. March 2023, UCLA, Los Angeles, CA.

Diamant, ES, U Heise. Befriending LA birds. Guest Lecture: *Eyes in the Sky, Birds in the Heart and Mind* workshop series. Counterforce Lab, Design Media Arts. February 2023, UCLA, Los Angeles, CA.

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Chapter One: Background and Significance

Ecological communities and the populations that live within them are composed and affected by multiple interacting environmental drivers and stressors. Understanding how the interactions of multiple stressors – rather than individual drivers - affect natural populations' fitness, survival, and evolution is integral in understanding the dynamics of a given system and the implications of stressors on the fate of populations and species (Crain et al. 2008; Darling and Cote 2008). How stressors interact and how predictable those interactions may be is particularly relevant as anthropogenic impacts across the globe alter environmental stressors and introduce multiple disturbances across habitats and systems (Sala et al. 2000; Didham et al. 2007). After understanding how populations across taxa respond to multiple stressors, we must also ask if populations can respond differently to similar disturbances.

Urbanization provides a natural experiment where multiple similar interacting novel disturbances affect populations across the globe. Urbanization drastically changes a species' natural habitat, creating novel threats and disturbances when compared with a given species' evolutionary history and environmental tolerance (Bonier et al. 2007; Sol et al. 2014). Using populations of the same species across cities in a similar climate can help us determine if response within populations is predictable and repeatable. Untangling the effects of urbanization on biotic responses not only allows us to better predict and mitigate our effects on other species (Sih et al. 2011; McDonnell and Hahs 2015), but also provides an arena to study adaptation-in-action (Diamond 1986; Yeh and Price 2004) and speciation in action (Thompson et al. 2018). COVID-19 lockdowns have provided a once-in-a-century opportunity to test the effects of human activity on animal behavior in cities and provide a natural experiment to determine how birds are adjusting their behaviors throughout their lifetime ("plasticity") (Rutz et al. 2020). By understanding the pattern (e.g., predictability) and process (e.g., evolutionary and non-evolutionary, plasticity-mediated mechanisms) of response, we can determine the strength of urban selection pressures not only in changing organisms through plasticity and genetic evolution but also on how and if we can predict a population's ability to succeed in drastically modified environments. Together, we can test how and if urban environments have the potential to foster diversity within species despite their threat to biodiversity as a whole.

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REVIEW AND SYNTHESES

Using a newly introduced framework to measure ecological stressor interactions

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Abstract

Understanding how stressors combine to affect population abundances and trajectories is a fundamental ecological problem with increasingly important implications worldwide. Generalisations about interactions among stressors are challenging due to different categorisation methods and how stressors vary across species and systems. Here, we propose using a newly introduced framework to analyse data from the last 25 years on ecological stressor interactions, for example combined effects of temperature, salinity and nutrients on population survival and growth. We contrast our results with the most commonly used existing method – analysis of variance (ANOVA) – and show that ANOVA assumptions are often violated and have inherent limitations for detecting interactions. Moreover, we argue that rescaling – examining relative rather than absolute responses – is critical for ensuring that any interaction measure is independent of the strength of single-stressor effects. In contrast, non-rescaled measures – like ANOVA – find fewer interactions when single-stressor effects are weak. After re-examining 840 two-stressor combinations, we conclude that antagonism and additivity are the most frequent interaction types, in strong contrast to previous reports that synergy dominates yet supportive of more recent studies that find more antagonism. Consequently, measuring and re-assessing the frequency of stressor interaction types is imperative for a better understanding of how stressors affect populations.

Keywords

Antagonism, antibiotics, food webs, multiple stressors, synergy.

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INTRODUCTION

Ecological systems are comprised of many interacting species and functional groups that span trophic levels and that are impacted by a diverse – yet often correlated – set of environmental drivers such as temperature, precipitation and nutrients (Chapin *et al.* 2000; Halpern *et al.* 2008). When a system responds to multiple stressors or environmental drivers, not only can individual stressors affect the dynamics and outcome of a system, but the interactions among stressors can also strongly affect outcomes and may ultimately determine the fate of many populations and species (Bliss 1939; Loewe 1953; Crain *et al.* 2008; Darling & Côté 2008). Interactions are especially relevant for studies on conservation of populations that are often under threat from multiple stressors via global change such as temperature change, habitat loss, overexploitation, harvesting, invasive species and other disturbances (Sala *et al.* 2000; Didham *et al.* 2007).

Synergistic interactions – when the combination of stressors produces a response greater than expected if there are no interactions – have received more attention than antagonistic interactions for which the combination of stressors produces a response less than expected if there are no interactions. This is in part due to concerns and predictions that synergy among stressors will lead to faster and greater biodiversity loss (Sala & Knowlton 2006) by exacerbating the effects of anthropogenic disturbances (Chapin *et al.* 2000; Brook *et al.* 2008; Halpern *et al.* 2008), including those resulting from climate change (Harley *et al.* 2006). However, recent reviews (Côté *et al.* 2016; Jackson *et al.* 2016) find that there are more antagonisms than have been accounted for and that synergistic interactions are over-emphasised in the ecological literature. This may be partially due to improper use of null models and misconceptions about the directional effects of individual stressors. Resolving these differences suggests a need for a comprehensive approach to studying stressor interactions (Côté *et al.* 2016; Schäfer & Piggott 2018).

Interactions are still poorly understood, even though there have been many empirical studies that examine the nature of ecological interactions in the lab (Folt *et al.* 1999; Mora *et al.* 2007; Cramp *et al.* 2014) and in the wild (Ross *et al.* 2004; Christensen *et al.* 2006). Both synergistic (Relyea 2003; Gooding *et al.* 2009; Shears & Ross 2010; Camarero *et al.* 2011; Griffith *et al.* 2012; Metz *et al.* 2013; Carnell & Keough 2014; Dávalos *et al.* 2014; Chown *et al.* 2015; Pringle *et al.* 2015) and antagonistic interactions (Darling *et al.* 2010; Bansal

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et al. 2013; Annala *et al.* 2014; Lakeman-Fraser & Ewers 2014; O'Regan *et al.* 2014; Treasure & Chown 2014; Griffiths *et al.* 2015) have been predicted in a wide range of organisms and environments. Yet basic questions remain as follows: (1) Are most interactions synergistic, antagonistic or additive (i.e. no interaction between the stressors)? (2) How does the interaction classification method affect the findings? (3) Do some systems or some stressor types lend themselves to certain types of interactions?

Understanding interactions among multiple stressors and combining that information to make inferences on outcomes are often very challenging for several reasons. First, two stressors may individually cause opposite organismic responses, which complicates finding the null model (Côté *et al.* 2016). Second, multi-stressor interaction studies have used many interaction classification methods, but differences in these techniques are not well-known or explicit (Côté *et al.* 2016; Schäfer & Piggott 2018; Thompson *et al.* 2018a). Typically, interactions are defined as the deviation from the null-expectation that stressors do not interact (termed additivity). The direction of the deviation defines the interaction type: synergy if negative, antagonism if positive and additive if indistinguishable from 0. That is when the growth rate in the presence of combined stressors is less than the expectation under no interactions, this corresponds to synergy, and when the growth rate in the presence of combined stressors is more than the expectation under no interactions, this corresponds to antagonism.

Diverse examples of interaction measures built upon this standard definition include (1) statistical measures of ANalysis Of VAriance (ANOVA) and its extensions (such as multivariate analysis of variance, MANOVA and analysis of covariance, ANCOVA) for inferring the differences of means and variances among groups (Sokol-Hessner & Schmitz 2002; Van Son & Thiel 2006; Crain *et al.* 2008), and also include (2) additive interaction models for detecting the direction of interaction (Soluk & Collins 1988; Sih *et al.* 1998), such as Bliss Independence (BI, or epistasis independence) that is commonly used to infer the interactions between drugs (Bliss 1939) or genes (Segre *et al.* 2005).

Although ANOVA is one of the most commonly used methods in studies of multi-stressor combinations, we and others have identified limitations and potential problems of ANOVA – hidden replication, parametric assumptions and so on – in finding the interaction type and classifying the magnitude of interaction (See Box 1 and Fig. 1).

Indeed, ANOVA and most other approaches have rarely quantified or established a method for cleanly determining the magnitude of interaction, which often requires normalisation (rescaling) of the interaction measure (Sanjuán *et al.* 2004; Segre *et al.* 2005; Tekin *et al.* 2016). The rescaling concept is similar to the use of relative response rather than absolute response, where the absolute response is informative only when there is some baseline or reference case to consider. In relative response the baseline is typically the control or maximum response of a given individual or genotype (Segre *et al.* 2005). In interactions, the interaction measure is rescaled with extreme synergy (lethality) or antagonism (buffering) as a baseline (Segre *et al.* 2005; Yeh *et al.* 2006; Tekin *et al.* 2016). These are chosen as the baselines because lethality is the absolute lower bound, and buffering is a commonly observed case where one stressor completely buffers (i.e. masks) the effect of the other. Buffering does not represent a hard upper bound but most antagonistic interactions cluster around this case and it makes it straightforward to interpret cases of suppression where two drugs have less effect than a single drug.

The goal of rescaling is to have numerical values for interactions be fairly compared, independent of whether the single effects are strong or weak. That is whether or not there is an interaction between two stressors should not depend on the strengths of the single effects. A natural outcome of the rescaling is that the distribution changes, often from unimodal in the unrescaled space to multimodal in the rescaled space, enabling easier identification of cut-offs for interaction types (Segre *et al.* 2005; Tekin *et al.* 2016).

Rescaling is critical because many interactions can be missed when using only raw interaction measures. Specifically, even for clearly different interaction types, the values of interaction measures without rescaling are often the exact same. Hence, the actual interaction type cannot be reliably categorised using the raw interaction measure, leading to incorrect interpretations (Tekin *et al.* 2016).

For example if two stressors A and B change the growth response relative to the control (no stressor) to be 0.54 and 0.91, respectively, and the combined effect of these two stressors equals the stronger effect of the single stressors (i.e. 0.54), then one unrescaled interaction measure is (effect of two stressors combined) – (effect of stressor A)*(effect of stressor B) = 0.54 – (0.54) * (0.91) = 0.05. This value is very close to 0 – suggesting no interaction between stressors A and B. However, this scenario is clearly antagonistic because one stressor is fully masking the effect of the other stressor. Hence, the unrescaled value of the interaction measure of 0.05 would seem to indicate that this combination is additive, even though it is actually antagonistic. Conversely, it is possible to get the same raw interaction values from very different circumstances. For instance if two single stressors each yield a response of 0.70, and combined they yield a response of 0.54, this again produces an interaction value of 0.05 as in the previous scenario. Thus, unrescaled values correctly capture the direction (sign) of interactions but not their magnitudes. However, the *type* of interaction depends on both direction and magnitude.

Via rescaling, the first scenario above would correctly conclude that there is an antagonistic interaction, whereas the second scenario would show an additive interaction. The advantage of rescaling is that it reveals true interaction types in such cases by calculating the interaction effects relative to natural interaction baselines, such as lethality (extreme synergy) or buffering (antagonism). Consequently, rescaling gives accurate information about both the direction of interaction (synergy or antagonism) as well as the strength (see Methods for more details of calculations for rescaling).

These ecological disturbances and stressor interactions are analogous to drug–drug interactions, where pathogens encounter drugs that reduce their proliferation and survival capacity. In some cases, clinicians use drug combinations that

Box 1. Limitations of ANOVA (Analysis of Variance) for Interaction Classification

ANOVA is widely used to study interactions across fields, but it has several inherent limitations in terms of detecting interactions.

First**,** for any specific treatment – predator density, gene mutation, drug concentration – there are usually only a handful of replicates (most of the studies analyzed here ranged from 1-6 replicates, see Table S4). This number of measurements often gives a poor estimate of the variance in the response – such as growth rate or mortality rate – because more data are needed to obtain an accurate estimate for successively higher-order moments (mean to variance to skewness to kurtosis). Even if a handful of measurements are sufficient to obtain an accurate estimate of the mean (whose accuracy increases linearly with the number of data points), it may be insufficient for estimating the variance (whose accuracy increases as the square root of the number of data points). Because ANOVA relies on estimates of the variance, poor estimates of the variance will often lead to inaccurate or misleading results.

Frequently, hidden replication is the remedy used to justify use of ANOVA for studies with a limited number of replicates per treatment. Hidden replication assumes that the variance is the same across the different treatments, and therefore, that the number of data points used to estimate the variance is not just the replicates for each specific treatment, but the total number of measurements across the entire study. This typically corresponds to tens or hundreds of measurements and provides a much more accurate estimate of the assumed “constant variance.”

It is this assumption and the reliance on hidden replication that we challenge when applying and interpreting ANOVA. Indeed, this leads to our second point: Hidden replication only applies when no interaction exists (Welham *et al.* 2014). When an interaction is present, the variance across different treatments should be expected to change (see example where we look at two stressors as they vary in amount in Fig. 1). This variance invalidates the underlying assumption used to justify hidden replication and the application of ANOVA itself. That is, *ANOVA is only reliably applied either when no interaction exists at all, or when an interaction does exist but there are many replicates for each specific treatment*.

Because most ecological data are challenging to obtain, they typically have only one or a few replicates, so this hidden replication assumption is commonly – though implicitly – needed. When interactions are found by ANOVA, it is likely because the assumptions are so badly violated that any conclusion about the interaction type is difficult to trust (Pomerantz 1981; Billick & Case 1994; Gotelli *et al.* 1999).

Third, it is often necessary to apply a normalization (or rescaling) method relative to natural baselines of interactions to identify interaction types. Otherwise, it is possible to get identical interaction values for distinct interaction types (see example in Introduction). However, to the best of our knowledge, one cannot rescale when using ANOVA methods. Therefore, using ANOVA to identify interaction types is much more challenging (Segre *et al.* 2005; Tekin *et al.* 2018).

Fourth, ANOVA tests for significance by comparing variances that assume Gaussian or parametric distributions (Winer *et al.* 1991). To estimate the variance under non-normal distributions would require substantial amounts of data for each response measurement in the system – corresponding to responses of single stressors and combinations of stressors – and also a reformulation of ANOVA in terms of non-parametric statistics and alternative distributions for null models.

Finally, when stressors have large effects, the underlying additive model of ANOVA may not give plausible independence expectations to infer the interaction. For example, when isolated single predator species kill 60% and 70% of the prey population, the additive expectation corresponds to killing 130% of the prey population – the interaction model cannot create a feasible test (Sih *et al.* 1998). Such situations are often overcome by logarithmically transforming the data, which essentially alters the underlying additive model to a multiplicative model (Soluk & Collins 1988; Sih *et al.* 1998). Furthermore, in addition to population responses, species interactions can also cause non-additive responses to stressors (Thompson *et al.* 2018b).

In summary, the use of ANOVA when studying multi-stressor interactions raises some concerns, such as the need for a large amount of data to have confidence in the estimates of variance, the choice of data-analysis space (linear or log), and the inability to rescale. Ted Case and colleagues noted some of these limitations of ANOVA a few decades ago (Billick & Case 1994), but these concerns appear to have been subsequently ignored or become lost knowledge.

inhibit different pathways, effectively kill harmful pathogens and combat numerous cases of drug-resistant pathogens (Tumbarello *et al.* 2012). Inhibition of different pathways is conceptually analogous to natural systems in which larger and more complex organisms experience changes in different critical resources, such as nutrients, light, nesting sites, hiding sites or water. These analogies suggest that we could adapt the interaction measures used in one specific system of interactions to study another system. Indeed, using toxicology models for interactions has been suggested previously (National Academies of Sciences 2017; Schäfer & Piggott 2018).

Here, we propose the use of a newly introduced framework to study multiple stressor interactions that solves the problems listed above for ANOVA and other methods. This method has foundations in the study of drug and gene interactions, and it has a strong correspondence to the model of interactions in the study of multiple predator effects (MPE) (Sih *et al.* 1998). In particular, we use the Bliss Independence model (Bliss 1939) where the interactions are quantified relative to an additive expectation when the presence of one stressor does not affect the other stressor’s percent effect on the response of the organism. We accompany the Bliss

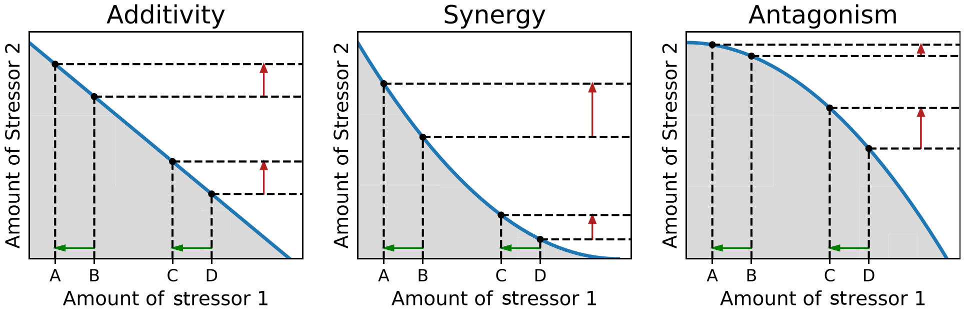

Figure 1 How Variance Changes with Stressor Amount Based on Loewe Interaction Type. Solid blue lines show stressor amounts needed to elicit a specific value of the response (e.g. kill the population) for each interaction type (left panel: additivity, middle panel: synergy, right panel: antagonism). We use these schematics to explain why variance is not constant across different treatments – thus violating the hidden replication assumptions – when interactions are present. Experimental errors in manipulating stressor amounts, measurement errors or slight inherent variation along one axis (e.g. x-axis, denoted change by green arrows) must result in shifts up or down the other axis (e.g. y-axis, denoted change by red arrows) in order to stay on the line and maintain the same response (e.g. kill rate or growth rate). The size of the shift up or down the y-axis depends not just on the shift along the x-axis, *but also the interaction type and the absolute amount of the stressor (low or high)*. If stressor X changes by a constant amount in the left panel (additivity) – from A to B at low stressor amounts and from C to D at high stressor amounts – the difference in stressor Y that is needed to compensate is the same, resulting on average in constant variance in the response across the line (homoskedascity). However, for synergy (middle panel), when the change from A to B is at low stressor amounts, the difference needed in stressor Y to compensate is much larger than the difference needed to compensate for changes from C to D at high stressor amounts, corresponding to the notable size difference in the red arrows. This yields a variance in the response that on average would change along the line (heteroskedascity). For antagonism (right panel), variance increases in the opposite direction. Changes from A to B at low stressor amounts require less change in stressor Y than that needed to compensate for changes from C to D at high concentration. Again, this is portrayed by the difference in size of the red arrows. Therefore, the variance in the response is not expected to stay the same under different treatment conditions (i.e. low versus high stressor amounts), violating the hidden-replication assumption for ANOVA (see Box 1 for details). Note these schematics correspond to definitions of interactions via Loewe additivity (Loewe 1953), which allows for measurements of interactions across a range of stressor amounts to illustrate how the shape of the response can lead to heteroskedasticity. This is a different interaction framework than the RBI framework we use elsewhere in this paper, which is defined for fixed stressor amounts.

Independence model with the rescaling already discussed and first introduced by Segre and colleagues (Segre *et al.* 2005). This framework provides a well-established notion of estimating significance when only small numbers of replicates are present (e.g. Segre *et al.* 2005; Lehár *et al.* 2007; Schmidt *et al.* 2013). Moreover, the Bliss framework is easy to understand and implement, providing straightforward generalisations when studying higher order (three or more) stressor interactions (See Supporting Information, and Tekin *et al.* 2018).

Using this framework, which we term the Rescaled Bliss Independence Model (RBI), we re-examine ecological data that we collected from literature published in the last 25 years (Supporting Information Fig. S1 and Table S1). The population-level responses – such as growth, survival and mortality – in the presence of stressors are similar to those in drug interaction studies. Using these metadata and this interaction classification scheme, we systematically investigate two-way interactions and compare the results of our framework with the results obtained in these previous studies. In particular, we ask the following questions: (1) Are our results different from the originally reported interaction types, and if so, how? and (2) Can we identify any patterns in these interactions? In doing so, we highlight examples where interactions switch direction – such as reported synergies being antagonistic or reported antagonisms being synergistic. Finally, we explore whether interaction findings vary substantially with the species' habitats – artificial laboratory-based, marine, freshwater, estuary or terrestrial – or with the taxa, such as unicellular versus multicellular.

MATERIALS AND METHODS

Study selection and criteria

Studies were first selected from those cited in three reviews of stressors and stressor interactions (Crain *et al.* 2008; Darling & Côté 2008; Ban *et al.* 2014). These reviews were chosen because they are highly cited, have already provided their own standardised methods of analysis, and contain some of the largest collections of studies on multiple stressors.

We then selected additional studies from literature searches using the *Web of Science* database [following (Darling & Côté 2008)]. Searches were conducted for studies published from January 1994 to August 2019 with the topics (inclusion in the paper keywords, title, and/or abstract): 'multiple stressors', 'multiple antagonism', 'multiple synergy' and 'multiple disturbance' (Fig. S1). The search results under each topic were then further narrowed down by using the topic categories that would more likely yield ecological studies: agriculture dairy animal science, biodiversity conservation, biology, biotechnology, applied microbiology, ecology, entomology, environmental sciences, evolutionary biology, genetics heredity, marine freshwater biology, mathematical computational biology, microbiology and zoology. Duplicate studies were removed. Only studies that investigated growth, mortality and survival at the population-level for a given species were included.

We then screened the remaining search results by systematically reading through the abstract and results sections of each study to identify the presence of our selection criteria. Studies selected were those that experimentally manipulated stressors in a factorial design and contained the presence of two stressors, had quantitative response variables, and had explicitly stated measurements for control treatments. Once studies that passed our initial screening were identified, we reevaluated each for eligibility in our meta-analysis. Studies found to be missing responses for control variables or individual stressors were excluded because they would not be viable for our data analysis.

From the remaining articles, we then extracted data from relevant figures and tables found in each study. The extracted data were tabulated into spreadsheets (see Table S2) and included stressor type, stressor units, responses for individual stressors, responses for combinations of stressors, responses for control variables, sample size, species of organism tested, species natural habitat and interaction type between stressors as concluded by the original authors (i.e. additive, synergistic or antagonistic). To compare interaction findings across distinct habitat classes and distinct types of organism studied, we classified species' natural habitats into five categories: artificial laboratory-based, marine, estuary, freshwater and terrestrial. Number of metadata across these habitat classes are as follows: artificial laboratory-based: 2, marine: 236, estuary: 29, freshwater: 382, terrestrial: 191 interactions. We also classified organism types into two different categories: unicellular and multicellular. There are 5 interactions tested in unicellular organisms and 835 in multicellular organisms in our metadata. In summary, multicellular organisms with terrestrial, estuary, marine or freshwater habitat classes cover the majority (99.4%) of metadata included in our study (data in Table S1).

Studies where no interactions were reported were further categorised by whether the authors looked for an interaction or not. Quantitative responses that we recorded were mean values that were averaged from raw values by the original authors or estimated from their figures. For responses that were presented in a time series format, we recorded the response at the latest time point.

To assess the variability in data measurements, which mainly rely on the mean response values due to limited data reports, we used a nonparametric bootstrapping technique across different studies in our meta-analysis. In doing so, we sampled studies with replacement and calculated the frequency of different interaction types under these samples (see sections below for details).

We focused on the response variables of survival, mortality and growth measurements, and we classified interactions using the methods described in the next section. Finally, calculating relative response values from the filtered data as described in the section 'Conversion of System Responses to Relative Response Values', we further confined the data set to include relative response measurements in the range of [0, 1.5]. The majority of the stressors we examined decreased responses, which would have given a relative response maximum of 1. But we also used studies with relative responses of up to 1.5 to be more inclusive. Those that resulted in relative responses larger than 1 meant that the population did better with the 'stressor' present than in no-stressor environments (see Fig. S2 for the distribution of relative response values). This final restriction on the response range led to an exclusion of only six papers from our meta-analysis. All studies included in our meta-analysis are given in Table S1 and summarised in Table 1 by reported and re-classified interaction type. We only analysed 2-stressor interactions because data for 3 + stressor interactions are limited (Table S3).

Conversion of system responses to relative response values

We calculated the relative responses (w_X) for survival and growth response types by dividing the absolute stressor response measurements (W_X) by the response of the control measurement (W_0), such that $w_X = W_X/W_0$. For response variables as mortality percentages, we first converted the data to survival percentages as (100 – mortality percentage) and calculated the relative response as described for survival response type. The last response variable included in our meta-analysis is the mortality rates over time. Because control represents the case with the lowest mortality rate over time, we calculated the relative response as the control response measurement over the response measurement of the stressor treatment. Note that we excluded the data when at least one of the measurements across the control, single stressors or the pairwise stressor was equal to 0 as it would yield infinite relative response values.

Classification of interactions

In studying the effects of single or multiple stressors, system responses – such as growth, survival or mortality rates – to stressors are frequently referred to as 'response variables'. The effect of a single stressor (X) is typically characterised by how much it alters the response measurement relative to the response in the absence of any stressor, that is the control treatment. We denoted this relative response measurement in the presence of the stressor X by w_X. Similarly, when there are multiple stressors (say X, Y) in the environment, the relative response in the presence of the combined stressors is denoted by w_{XY}, where the ordering of the indices is symmetric with respect to each other, that is $w_{XY} = w_{YX}$. When choosing a response variable that decreases with a stressor, the relative response of a control as represented by w_0 is equal to 1 by definition, and an extreme lethal stressor produces a response of 0. Therefore, for a typical stressor experiment, the relative response (w) would be in a range between 0 and 1. Therefore, interactions among stressors are characterised by a systematic investigation of how much response measurements in the presence of both stressors (w_{XY}) differ from a quantitative expectation based on responses in the presence of single stressors (w_X and w_Y) and no interaction between stressors. When the effect on the response of the combined stressors is greater or weaker than the null-hypothesis of no-interaction (i.e. additive) effect, the interaction is classified as synergistic or antagonistic respectively. Here, we followed the RBI model to characterise interactions, in which the no-interaction expectation – sometimes termed additive (with respect to selection coefficients) and sometimes termed multiplicative (with respect to measured responses) – is that the product of single-stressor relative responses is equal to the combined stressor effect, that

Table 1 Comparison counts for interaction type according to previously published results and our re-analysis using Rescaled Bliss Independence (RBI). 'Inconclusive' cases under RBI correspond to cases for which the outcome is not obvious due to response values being indistinguishable, whereas 'Inconclusive' cases under previously published results correspond to cases for which no explicit interaction type is reported. The table is summarised in Figure 4.

		Rescaled Bliss Independence (RBI)			
		Synergy	Additive	Antagonism	Inconclusive
Previously Published Results	Synergy	72	90	41	45
	Additive	10	23	11	9
	Antagonism	2	10	26	14
	Inconclusive	47	163	200	77

is $w_{XY} = w_X w_Y$. A flowchart of the classification of interactions is presented in Fig. 2 and a worked example is given in Box S1.

In many ecological studies, the response of the stressor treatment is measured as a mortality rate, denoted by m. The interaction measure is given by the same equation as for Multiple Predator Effects (MPE) and is calculated in terms of survivorship as $1 - m_{XY} = (1 - m_X)(1 - m_Y)$ representing additivity, or equivalently, no interaction, where m_{XY} is the mortality rate under the presence of two predators X and Y, m_X is the mortality in the presence of just predator X and m_Y is the mortality in the presence of just predator Y (Sih *et al.* 1998). We introduce the BI model to assess the effects of drug combinations, and in so doing we show the BI model for drugs is exactly equivalent to MPE. Because these mortality rates directly relate to survival (or population growth) rates, we follow the drug literature and use the relative growth response (w) – which measures growth rate in the presence of stressor A compared to the growth rate in an environment with no stressors – to classify interactions. As such, we use a measure for net interactions used by the BI model that is called Deviation from Additivity (DA):

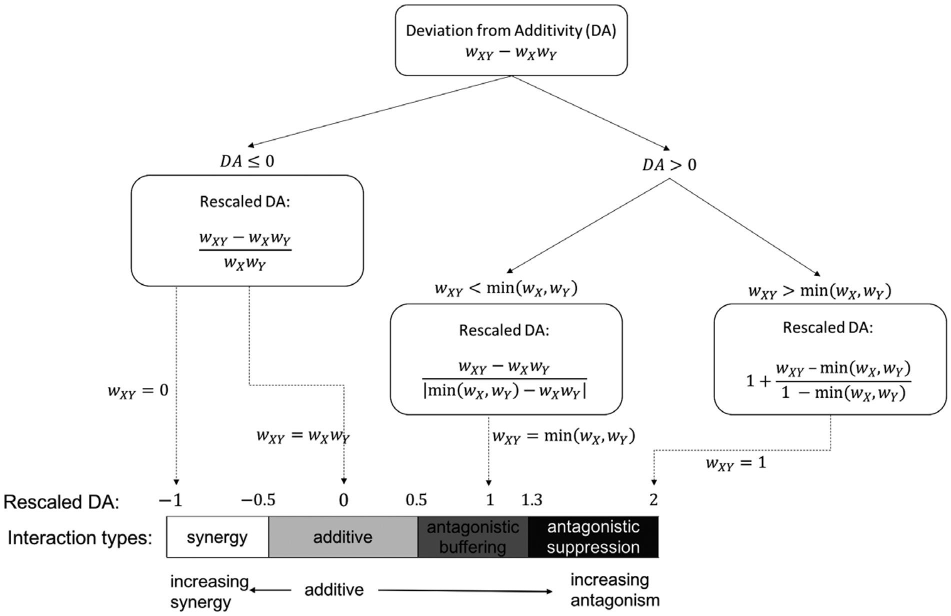

Figure 2 Overview of Classification of Interactions. Interaction classifications are done via the flow chart in the figure, where we start with the unscaled interaction metric (Deviation from Additivity) and apply the rescaling method to calculate interaction values and determine the interaction type. The construction of a rescaled measure with respect to the natural baselines – such as $w_{XY} = 0$ (lethal synergy), additive (no interaction) or $w_{XY} = 1$ (buffering) – yields a map of the interaction measure to distinct values (−1, 0, 1, 2 respectively). This together with the resulting distribution of interaction calculations over the entire data (Figure 3b) sharply delineates the boundaries across distinct interaction classes.

$$\mathrm{DA} = w_{XY} - w_X w_Y$$

Notably, additive models used in stressor interactions are often conducted by logarithmically transforming data, which transforms the additive null model into one version of a multiplicative null model (Tekin *et al.* 2018). Accordingly, deviations from the additive scenario of the Bliss Independence model measure the presence of interaction and the direction of the deviation defines the type of interaction. In this respect, sufficiently large negative and positive values of deviations suggest synergistic and antagonistic interactions.

Normalisation (Rescaling) method to enhance classification of interactions

To properly distinguish the interaction types and to interpret the magnitude of interactions, we applied a rescaling (normalisation) method that reliably identifies the interaction type and is proven to enhance the identification of interactions in drug (Yeh *et al.* 2006) and gene interaction (Sanjuán *et al.* 2004; Segre *et al.* 2005) studies. This is accomplished by quantifying the interaction strength relative to natural baselines of synergistic and antagonistic interactions.

As introduced by Segre and colleagues (Segre *et al.* 2005) and Yeh and colleagues (Yeh *et al.* 2006), the interaction measure is calculated by the following formulas:

$$\widetilde{DA} = \frac{DA}{|\tilde{w}_{XY} - w_X w_Y|} = \frac{w_{XY} - w_X w_Y}{|\tilde{w}_{XY} - w_X w_Y|}$$

where $\tilde{w}_{XY} = 0$ when $w_{XY} \leq w_X w_Y$ and $\tilde{w}_{XY} = \min(w_X, w_Y)$ when $w_{XY} > w_X w_Y$. Here, $\tilde{w}_{XY}$ represents a baseline of a specific response of stressor combinations, where $\tilde{w}_{XY} = 0$ corresponds to extreme synergy (*i.e.* lethality), whereas $\tilde{w}_{XY} = \min(w_X, w_Y)$ indicates that one stressor completely masks/buffers the effect of another stressor and represents extreme antagonism. To account for these baseline cases where multiple stressors produce extreme effects, the following rescaling is used when $w_{XY} > \min(w_X, w_Y)$:

$$\widetilde{DA} = 1 + \frac{w_{XY} - \min(w_X, w_Y)}{1 - \min(w_X, w_Y)}$$

Using this rescaling, extremely synergistic interactions – lethal or nearly lethal – would yield values close to −1. Antagonistic buffering interactions – where there is not much of a difference between operating with the strongest single stressor or with all of the multiple stressors present – would yield values close to 1. And extremely antagonistic interactions – the complete recovery of the response to the response level of the control – would equal 2 (Tekin *et al.* 2016).

Additionally, it has been repeatedly shown that rescaling yields a multimodal distribution with clearly delineated boundaries between interaction classes (Segre *et al.* 2005; Yeh *et al.* 2006; Tekin *et al.* 2017). In comparison, unscaled interaction calculations result in a unimodal distribution that is centred around additivity. This unimodal distribution does not provide a practical way to determine the interaction type based on the interaction measure or the distribution (refer to Results and Fig. 3 for more details). Finally, we note that we identified interactions as 'inconclusive' when the normalisation factor $|\tilde{w}_{XY} - w_X w_Y|$ was equal to zero, consistent with previous studies (e.g. Beppler *et al.* 2017).

Thresholds (cut-off values) to determine interaction type

Based on the cut-off values defined in other studies (Segre *et al.* 2005; Yeh *et al.* 2006; Tekin *et al.* 2016), expectations of interaction types (see above), and the resulting distribution of interaction calculations, we classified the interaction as synergistic when the rescaled DA measure was less than −0.5, antagonistic when it was greater than 0.5, and additive otherwise. Moreover, when the rescaled interaction measure exceeded 1.3, we identified the interaction as an extreme form of antagonism, typically referred to as suppression (Segre *et al.* 2005; Yeh *et al.* 2006; Tekin *et al.* 2016).

Uncertainty in the frequency of interaction

Because many studies did not give replicate measurements of stressor data, we relied mainly on the mean response values and thus estimated the uncertainty in the measurements by sampling across the studies in our meta-analysis. More precisely, we simulated the potential for variability by sampling across studies to estimate the uncertainty in the frequency of interactions. To do this, we used a nonparametric bootstrapping technique. We sampled the studies with replacement 10000 times and calculated the 95% confidence intervals from the resulting distributions.

RESULTS

In our study we obtained data from multi-stressor studies with quantitative response variables and assessed interaction types and interaction strengths of multi-stressor combinations using the RBI model. We further compared our findings with the conclusions reported in the original studies. We find that the unscaled measure of interactions yields a unimodal distribution around additivity (i.e. the peak is attained at Deviation from Additivity (DA) = 0), making it difficult to separate different interaction classes (Fig. 3a, see 'Normalisation (Rescaling) Method to Enhance Classification of Interactions'). In contrast, rescaling the interaction calculations resulted in a multimodal distribution with peaks occurring at the baselines of synergistic and antagonistic interactions (Fig. 3b) that are in exact agreement with expectations: peaks at – 1 (lethal synergy), 0 (additivity), 1 (antagonistic buffering) and 2 (extreme antagonism) (see 'Classification of Interactions'). This resulting distribution clearly delineates boundaries between distinct interaction classes, facilitating the classification of synergistic, additive and antagonistic cases. When rescaling is not applied to the interaction calculations, boundaries between these cases are ambiguous, demonstrating the significance of normalisation (rescaling) in the categorisation of interactions. The interaction types across our metadata, excluding the findings of inconclusive interaction type (see Materials and Methods), are distributed as follows: 19% synergy, 41% additivity and 40% antagonism.

We find that antagonism and additivity are the most observed interaction types using the Rescaled Bliss Independence

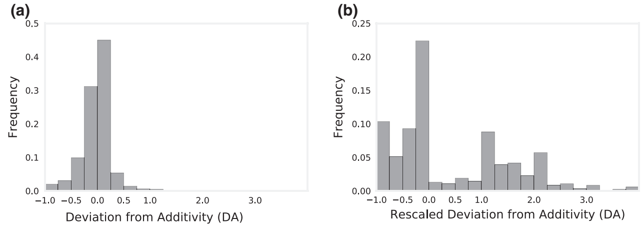

Figure 3 Unscaled Versus Rescaled Bliss Independence (RBI) Model Measures. (a) Frequency distribution of interaction measures across metadata does not yield clear distinctions between interaction types due to its unimodal characteristics. This is because weak single-stressor effects will often result in weak DA, which is misleading because this is a result of the single-stressor effect size and not the interaction itself. (b) Rescaled interaction measures eliminate dependence on the single-stressor effect sizes by defining the measure relative to the natural baselines of synergy and antagonism. This rescaling results in a multimodal distribution with peaks around −1 (extreme synergy), 0 (additivity), 1 (antagonistic buffering) and 2 (extreme antagonism) that allows us to tease apart synergistic, additive and antagonistic interactions. By definition (see section 'Classification of Interactions') and its resulting distribution across metadata, rescaling reveals the actual magnitude of interactions, and consequently, it enhances the identification of interaction type.

*framework, in strong contrast with many studies that find synergy is the most reported interaction type (*Fig. 4a*)*. Given the large discrepancies between the interaction findings in our study and the interaction findings reported by papers included in our meta-analysis, we also investigate the frequency of interaction types by RBI when the interaction is classified as synergistic, additive, antagonistic or inconclusive by studies examined in our paper (Fig. 4b). We see that only 29% of synergistic interactions from the original studies are confirmed as synergy in our analysis, and also that 17% of reported synergistic interactions are classified as antagonism, and 36% are classified as additive. Our RBI framework classifies 43% of additive (no-interaction) reports as additive, whereas the remaining ones are roughly evenly distributed across synergy, antagonism or inconclusive (17–21%). On the other hand, we find that when the interaction type is reported as antagonism, it is extremely unlikely to be classified as synergy within our RBI framework (4%), and that 50% of reported antagonism is classified as antagonistic by RBI (Fig. 4c).

Moreover, 62% of antagonism findings by RBI, spanning 170 of 840 data points (20% of data), are categorised as an extreme form of antagonism that is commonly referred to as suppression. Suppression represents the case where one stressor does not just mask the effect of the other, but actually reverses the effect of another stressor. We also observe that interaction types of 58% (487 out of 840) of the metadata are not reported, as represented by the 'inconclusive' bar in Fig. 4 (either interactions were not the particular focus of the study or no interaction type was reported or explicitly stated although authors looked for an interaction, see Fig. S3 for the number of data corresponding to each category).

Methods that do not rescale are more likely to miss antagonistic interactions that are smaller in magnitude. To check that this is consistent with our findings, we divided our antagonistic identification into subgroups from weak to strong (following Tekin *et al.* 2016). Specifically, strongly antagonistic interactions are > 1.3 by RBI, moderately antagonistic interactions are 1–1.3 by RBI, and weakly antagonistic interactions are 0.5–1 by RBI. Strongly antagonistic interactions match much better with the original studies than do weakly and moderately antagonistic interactions, consistent with expected failures of methods that lack rescaling. We found 23 interactions matching out of 170 total strongly antagonistic interactions (14%). The matching frequency was 2 of 80 (2.5%) for moderately antagonistic interactions and 1 of 28 (3.6%) for weakly antagonistic interactions. Synergistic interactions by RBI have an overall higher matching frequency in comparison to antagonistic interactions. Across all the different magnitudes of synergism (weak: -0.5 to -0.7 by RBI; moderate: -0.7 to -0.85; strong: -0.85 to -1), the matching frequency was fairly even: 19 of 36 weakly synergistic interactions match (53%), 11 of 21 moderately synergistic interactions match (52%) and 42 of 74 strongly synergistic interactions match (57%).

Finally, to see the effect of habitat classes on interaction types, we also separated the frequency bar charts by the three most common habitat classes: marine, freshwater and terrestrial, which comprise 96% of the metadata (Fig. 5). We did not find noteworthy differences in the prevalence of interaction types obtained by RBI between marine and freshwater habitats (Fig. 5). On the contrary, we observe that antagonism is slightly less prevalent in terrestrial habitats compared to marine and freshwater habitats (Fig. 5), though instances of each interaction type in terrestrial habitats are roughly similar (Fig. 5c).

DISCUSSION

We evaluated population-level response data for stressor interactions compiled from the literature over the last 25 years and conducted a systematic analysis of interaction patterns via a rescaled BI method – one of the most common interaction classification schemes used in drug–drug and gene–gene interactions. Although there has been a tendency towards studies reporting more synergies, as also pointed out previously by others (Darling & Côté 2008; Côté *et al.* 2016; Jackson *et al.* 2016), we actually observed more additivity and antagonism. Additivity is the most frequent interaction type (41% of metadata are classified as additive) and antagonism is nearly as frequent (40% of metadata are classified as antagonistic).

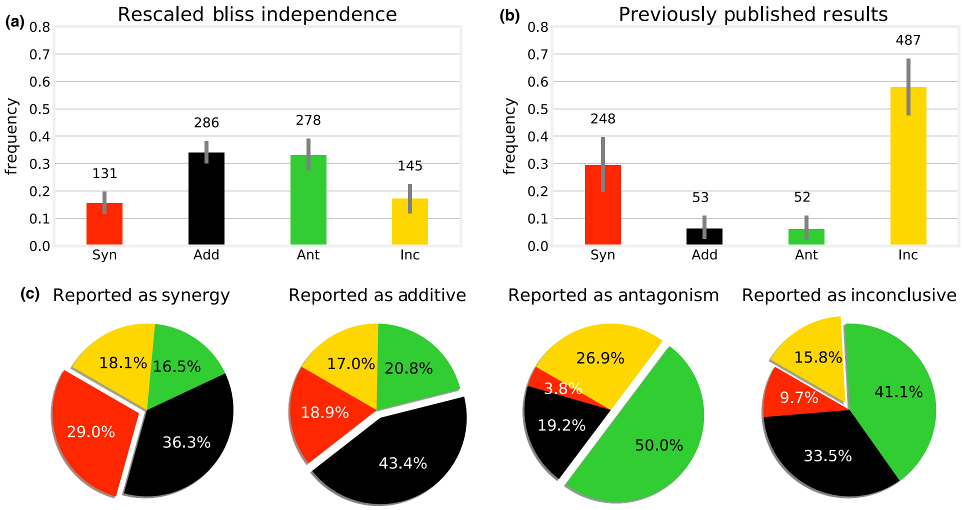

Figure 4 Comparison of Interaction Classes Obtained by Rescaled Bliss Independence (RBI) Framework and the Interaction Classes Reported in Studies. (a) The frequency of interaction types (Synergy, Additive, Antagonism, Inconclusive) are shown as bar graphs, with the actual counts corresponding to each interaction class shown at the top of each bar. Here, 'Inconclusive' cases under RBI correspond to cases for which the outcome is not obvious due to response values being indistinguishable, whereas 'Inconclusive' cases under previously published results correspond to cases for which no explicit interaction type is reported. (b) The reports of interactions are contrasted with the interactions found via the RBI model. The errors in the interaction measurements are estimated by sampling across different studies. The 95% CI for the frequency of the interactions are represented by error bars (see section 'Classification of Interactions'). (c) The frequency of each interaction type resulting from RBI is represented in separate pie charts for each interaction type identified by studies. See Table 1 for data.

Moreover, we see that our method frequently classifies previously synergistic findings as non-synergistic – either as antagonistic or as additive.

Due to the inherent limitations of methodologies (such as ANOVA) used by many previous studies, our findings add to a growing body of literature suggesting that it is extremely important to explore other interaction classification methods and to identify the method used based on more realistically motivated assumptions and hypotheses (e.g. Côté *et al.* 2016). When methods are not chosen in a way that can be consistently compared and evaluated, it is difficult to understand the implications of a multiple stressor study in the broader context of the field. Several researchers have recently begun to explore other interaction classification methods and to determine more nuanced ways to identify the best methods for specific situations.

For example in their meta-analysis of interactions in 171 studies in marine systems, Crain and colleagues considered that two stressors could both have negative effects on a response variable, both have positive effects, or have effects in different directions (Crain *et al.* 2008). When the stressors affected the response variable in opposite directions, synergy was defined as the situation when the net effect is more negative than the additive sum of the individual effects. As Piggott and colleagues point out, this method of defining makes sense only when the effect default or expected direction is negative, such as in the case of decreased survival rate or the decrease in any response variable (Piggott *et al.* 2015). After taking into account the magnitude and response direction of the net effect and interaction effect in their additive model, they reclassified interactions in the data from the Crain paper (Crain *et al.* 2008) in absolute terms. Analysing the data in this way, they found a slightly higher incidence of antagonism (43%) compared to the original Crain analysis (38%) (Piggott *et al.* 2015).

Although several models in both the ecology and drug literature have yielded a high frequency of antagonistic interactions across multiple stressor studies (Piggott *et al.* 2015; Côté *et al.* 2016; Jackson *et al.* 2016; Tekin *et al.* 2018), synergistic interactions appear to garner more attention and research than antagonistic ones. This is true even though the interest in synergy comes from opposite concerns with respect to conservation and human health. For ecological population studies, synergies are considered detrimental because they hasten the loss of threatened or endangered populations. For human health, stressor synergies are considered beneficial because they more efficiently kill bacteria (e.g. Chapin *et al.* 2000; Tong *et al.* 2004; Lorian 2005; Pan *et al.* 2006; Brook *et al.* 2008). More precisely, synergies always increase killing efficiency, but in some contexts that is considered a positive (drugs and health) and others a negative (species conservation).

In ecology, synergies pose some of the greatest dangers to populations of interest for conservation because they can lead

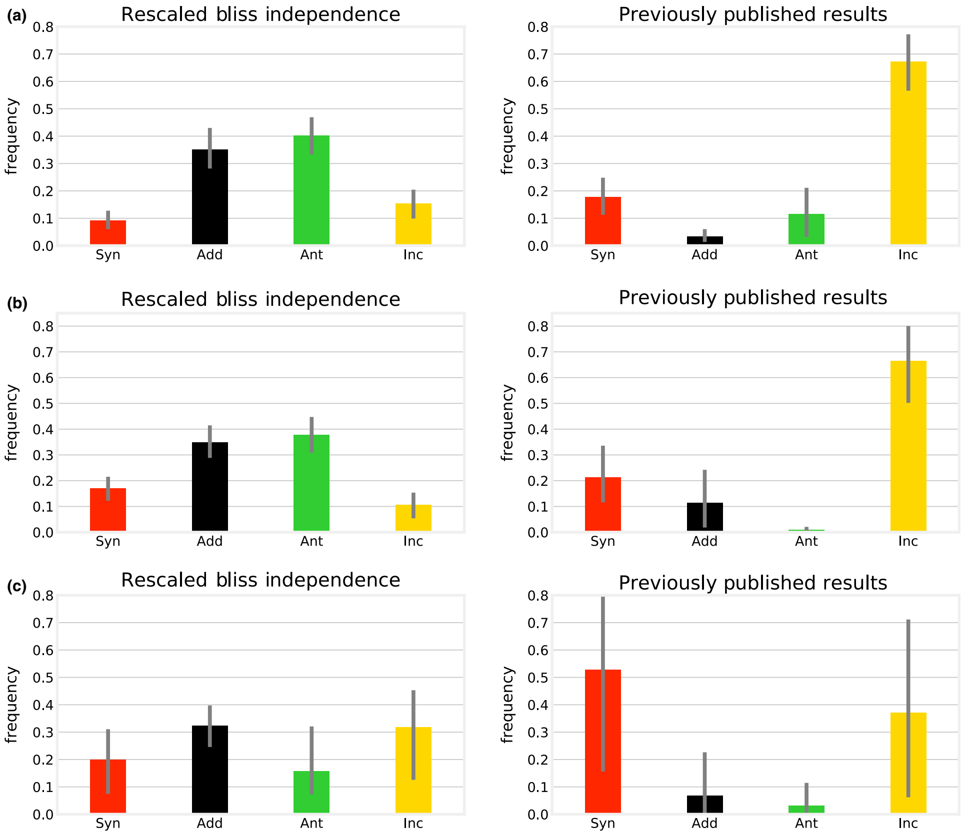

Figure 5 Comparison of Interaction Type Results by Freshwater, Marine and Terrestrial Habitat Classes. The frequency of interaction categorisations from data with freshwater, marine and terrestrial habitat classes are separated into distinct panels, respectively, panels a, b and c. The laboratory and estuary habitats are not included here as they only comprise 3.7% (31 out of 840) of metadata, and hence do not provide a sufficient number of data points for examining habitat effect. Interaction classification by Rescaled Bliss Independence (RBI) is fairly consistent between aquatic habitats and classifies relatively fewer antagonisms in terrestrial habitats. In contrast, interaction type frequencies substantially differ between marine, freshwater and terrestrial habitats in previously published results except for antagonistic interactions.

to population declines much more severe than an additive effect would predict. However, synergies also present the greatest opportunities. Indeed, Brown and colleagues (Brown *et al.* 2013) have shown that reducing a stressor within a synergistically interacting combination has the greatest benefit to ecosystem management. Hence, incorporating these outcomes is crucial for the management of populations and communities. In the clinic, the goal is to use synergy to eradicate harmful bacterial populations due to the greater killing efficiency of synergistic drugs.

From a basic science perspective, there is no reason to place primacy on synergism because antagonism in both drug–drug and ecological-stressor effects is found about as frequently as synergism according to large-scale studies in these fields: Drugs: 26% synergy, 37% antagonism (Yeh *et al.* 2006); Ecological stressors: 35% synergy, 42% antagonism (Darling & Côté 2008); Marine stressors: 36% synergy, 38% antagonism (Crain *et al.* 2008). Moreover, in our analysis we find 19% synergy and 40% antagonism, meaning that the higher rate of publication of presumed synergistic combinations likely arises from the biases and concerns discussed in the paragraph above and not because they occur more frequently across the space of all possible interactions.

The data used for our study partly overlap with the data used in other recent and remarkable meta-analyses that involved ecological interactions (Gruner *et al.* 2008; Harpole *et al.* 2011; Yue *et al.* 2017). Importantly, however, our present study differs in approach, results and sometimes questions. First, the measure of interactions in other studies is different in form from ours. All of these other recent papers use differences in slopes of lines or in log-ratios. Consequently, these measures are conceptually similar to ANOVA methods in that they can be converted into numerical values or equations for linear-space ANOVA (via difference in slopes) or log-space ANOVA (via differences in log-slopes that are equal to log-ratios).

Since comparisons to ANOVA methods are a major focus of this study and the results presented above, the conclusions we discuss apply equally well in comparing to the results of these previous meta-analyses. Furthermore, because log-ratios are used, all of the measures are normalised to the control treatment. We also always calculate and use relative fitnesses that are normalised with respect to the fitness of the no-stress (control) condition.

Second, and most importantly, the distribution of interactions is substantially different because of our specific type of rescaling, as explained above. The closest to our method is Yue *et al.* (2017) who employed a more advanced measure – Hedge's d – that relies on differences in linear slopes (analogous to one-way ANOVA) and includes a correction based on standard deviation for sample size. This correction is crucial because most of the studies included in the meta-analysis do in fact have small sample sizes. Nevertheless, this correction and the log-ratios themselves are fundamentally different than our rescaling method. Our rescaling prevents mis-categorisation of interactions that can arise from not accounting for single effects being either very small or very big. Indeed, earlier papers by us and others show that not including this kind of rescaling can completely change results because it is essentially comparing apples to oranges and leads to more interactions being identified as additive.

Third, some of these previous studies found and analysed over 600 interactions, whereas our more recent search found and included over 800 interactions. Despite the potential limitations of our keyword choice in finding every article on multiple-stressor interactions, this roughly 33% increase in the number of individual interaction studies also may change results. Fourth, Gruner *et al.* (2008) was primarily addressing the question of top-down versus bottom-up control, and Harpole *et al.* (2011) was looking for evidence of co-limitation. So, while measuring interactions was necessary for their studies, looking carefully at the distribution and types of interaction was not as essential, yet those are the direct goals of this present study.

Recent findings on higher order interactions in natural plant communities (Mayfield & Stouffer 2017), biodiversity of ecological communities (Bairey *et al.* 2016; Levine *et al.* 2017), epistasis and its effect on the variability of complex traits (Taylor & Ehrenreich 2015), as well as drug interactions (Beppler *et al.* 2016; Tekin *et al.* 2016), reveal the need to explore higher order interactions to better capture the characteristics of complex systems. This requires well-adapted interaction measures to higher order levels that are not simply based on pairwise effects, and the extension of the BI model (Bliss 1939) with rescaling (Segre *et al.* 2005) provides a potential path forward due to its ease of use and straightforward generalisation to characterise emergent interactions (Tekin *et al.* 2017). Future directions could entail broadening the analysis to studies with three or more stressors. While this study found only a few studies with three stressors, we may be able to find a greater number of studies with three or more stressors by examining published data beyond 25 years and expanding keywords across biological scales beyond the population-level.

In summary, we show how a newly introduced framework for drugs can also be used to study ecological interactions and perform a systematic analysis of pairwise interactions on metadata collected in the literature from the last 25 years. In doing so, we describe some of the drawbacks and limitations in using traditional methods of ANOVA and show that using an RBI framework leads to different conclusions than previous reports of interaction studies. When the underlying assumptions of ANOVA – such as for hidden replication that the variances are constant across different stressor treatments and thus implicitly that there are no interactions – are violated for the studied system, then the conclusions about interactions between different stressors may not be correct (Billick & Case 1994). Moreover, our findings are consistent with other recent reviews (Piggott *et al.* 2015; Côté *et al.* 2016; Jackson *et al.* 2016) as well as with our recent studies in drug interactions, indicating more antagonistic interactions than previously thought. Our study shows that choosing an appropriate framework for predicting interactions is essential to correctly find and categorise interactions, with broad and vital implications for a range of fields such as biodiversity, conservation strategies and human health.

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AUTHORSHIP

P.J.Y. and V.M.S. conceived of the project and designed the study. E.S.D., V.E. and N.S. collected data, and E.T., M.C.-L. and V.M.S devised modelling work and performed the analysis of data. All authors wrote and revised the manuscript.

DATA AVAILABILITY STATEMENT

Data are deposited in a public archive (https://doi.org/10.5068/D10D4Q).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Chapter Two: Supporting Information

How to generalize to three-stressor interactions

Here we present the formula to examine and rescale three-stressor interactions, following Beppler *et al*. 2016 and Tekin *et al.* 2016.

First, as noted in the Methods, Bliss Independence uses the idea of Deviation from Additivity, DA, where DA = $w_{XY} - w_X w_Y$ for two stressors. For three stressors, this can be extended to: $\mathrm{DA}_{\mathrm{X,Y,Z}} \equiv\ w_{\mathrm{XYZ}} - w_{\mathrm{X}} w_{\mathrm{Y}} w_{\mathrm{Z}}$.

In three-stressor combinations, we can obtain both a net (Deviation from Additivity) interaction, which is the total overall interaction, and an emergent interaction (E3), which isolates only the component of the interaction that results from the higher-order combination and not from any of the lower-order (pairwise) interactions (Beppler *et al.* 2016).

To obtain the emergent interaction for three stressors, there are three pairwise combinations, so subtracting all possible pairwise combinations yields:

$$\mathrm{E3} = \ \mathrm{DA}_{\mathrm{X,Y,Z}} - w_{\mathrm{X}}\ \mathrm{DA}_{\mathrm{Y,Z}} - w_{\mathrm{Y}}\ \mathrm{DA}_{\mathrm{X,Z}} - w_{\mathrm{Z}}\ \mathrm{DA}_{\mathrm{X,Y}}$$

Rewriting this equation solely in terms of the relative fitness gives:

$$\mathrm{E3} = w_{\mathrm{XYZ}} - w_{\mathrm{X}} w_{\mathrm{YZ}} - w_{\mathrm{Y}} w_{\mathrm{XZ}} - w_{\mathrm{Z}} w_{\mathrm{XY}} + 2 w_{\mathrm{X}} w_{\mathrm{Y}} w_{\mathrm{Z}}$$

We can rescale the E3 measure relative to the minimum of the fitness values attained by any single or pairwise components. The normalization factor would be:

$$|\min(w_{\mathrm{X}}, w_{\mathrm{Y}}, w_{\mathrm{Z}}, w_{\mathrm{XY}}, w_{\mathrm{XZ}}, w_{\mathrm{YZ}}) - w_{\mathrm{X}} w_{\mathrm{YZ}} - w_{\mathrm{Y}} w_{\mathrm{XZ}} - w_{\mathrm{Z}} w_{\mathrm{XY}} + 2 w_{\mathrm{X}} w_{\mathrm{Y}} w_{\mathrm{Z}}|$$

Thus, the new rescaled E3 measure would be:

$$[\mathrm{E3}]_{\mathrm{R}} = \frac{w_{\mathrm{XYZ}} - w_{\mathrm{X}} w_{\mathrm{YZ}} - w_{\mathrm{Y}} w_{\mathrm{XZ}} - w_{\mathrm{Z}} w_{\mathrm{XY}} + 2 w_{\mathrm{X}} w_{\mathrm{Y}} w_{\mathrm{Z}}}{|\min(w_{\mathrm{X}}, w_{\mathrm{Y}}, w_{\mathrm{Z}}, w_{\mathrm{XY}}, w_{\mathrm{XZ}}, w_{\mathrm{YZ}}) - w_{\mathrm{X}} w_{\mathrm{YZ}} - w_{\mathrm{Y}} w_{\mathrm{XZ}} - w_{\mathrm{Z}} w_{\mathrm{XY}} + 2 w_{\mathrm{X}} w_{\mathrm{Y}} w_{\mathrm{Z}}|}$$

Box S1. Calculation of Rescaled Bliss Independence (RBI) Interaction Measures

To illustrate the calculations necessary for obtaining the rescaled Bliss interaction measures, we work through examples of interactions. When the survival of a living organism is measured to be 70% (compared to the unstressed condition) under stressor x and 50% under stressor y, this corresponds to $w_x = 0.70$ and $w_y = 0.50$. When both stressors are present, the expected survival (assuming no interaction) is then $w_{xy} = w_x w_y = 0.35$. The rescaled value DA as a function of observed w_{xy}—survival under the stressor pair—is shown in Figure S4. Some specific example values are worked out below.

Synergy

Suppose we measure that $w_{xy} = 0.15$. In this case we have $DA_{xy} = w_{xy} - w_x w_y = 0.15 - 0.35 = -0.20$. The negative sign indicates that the observed growth is less than expected when there is no interaction. Following Figure 2, the corresponding rescaled interaction is $\widetilde{DA}_{xy} = \frac{w_{xy}-w_x w_y}{|0-w_x w_y|} = \frac{0.15-0.35}{0.35} = -0.57$. To see the difference between the rescaled and non-rescaled DA metrics, suppose that for a different stressor z we measure $w_z = 0.4$ and $w_{yz} = 0.00$. This would give $DA_{yz} = 0.00 - (0.50)(0.40) = -0.20$, the same unscaled DA value as for stressors (x, y). However, the stressor pair $y\ z$ is lethal to the organism so this is a much stronger synergy (in fact, the maximum possible synergy between two stressors). In contrast, the rescaled interaction values are $\widetilde{DA}_{yz} = -1.00 < -0.57 = \widetilde{DA}_{xy}$, correctly being classified as complete synergistic lethality and giving a larger deviation from 0 for the stronger interaction.

Antagonistic buffering

When $w_{xy} = 0.45$, then $DA_{xy} = 0.45 - 0.35 = 0.10$. Since $w_x w_y < w_{xy} < \min(w_x, w_y) = 0.50$ we are in the *antagonistic buffering* case. This means that, while less effective than expected, the stressor combination still reduces survival compared to both single stressors. The corresponding rescaled DA value is $\widetilde{DA}_{xy} = \frac{w_{xy}-w_x w_y}{|\min(w_x,w_y)-w_x w_y|} = \frac{0.10}{0.15} = \frac{2}{3} \approx 0.67$. This rescaled number means the strength of the interaction is 67% of the way between additivity and complete antagonistic buffering—when one stressor completely buffers or masks the effect of the other.

Antagonistic suppression

While less common than antagonistic buffering, in certain cases adding a second stressor can actually improve the survival of an organism relative to at least one of the single stressors (i.e., $w_{xy} > \min(w_x, w_y)$). This corresponds to *antagonistic suppression*. For example, when $w_{xy} = 0.60$, we get $DA_{xy} = 0.60 - 0.35 = 0.25$. The corresponding rescaled interaction is $\widetilde{DA}_{xy} = 1 + \frac{w_{xy}-\min(w_x,w_y)}{|1-\min(w_x,w_y)|} = 1 + \frac{0.10}{0.50} = 1.20$.

Additivity

Strictly speaking, an interaction is additive only when the survival is *exactly* as expected (i.e. if $w_{xy} = w_x w_y = 0.35$), corresponding to $DA_{xy} = \widetilde{DA}_{xy} = 0$. However, in practice one does not typically wish to consider interactions as non-additive when there are only minor deviations from this prediction. As opposed to evaluating a point null hypothesis (e.g. $\widetilde{DA}_{xy} = 0$) in a similar way to ANOVA, we propose evaluating the interval null hypothesis $|\widetilde{DA}_{xy}| < \widetilde{DA}_{crit}$, where $\widetilde{DA}_{crit}$ is the minimum strength for an interaction to be considered a synergy or antagonism of interest (rather than an additive interaction).

In this paper, we follow other work (Segre *et al.* 2005; Yeh *et al.* 2006; Beppler *et al.* 2016; Tekin *et al.* 2016) and categorize interactions where $|\widetilde{DA}_{xy}| < 0.5$ as additive. This choice is informed by the modes and minima of the distribution of interactions. For the analysis conducted here, we calculated interactions based only on the published mean effects because we do not have access to the raw data of the individual studies. In practice, one can also test an interval null hypothesis statistically and get a p-value through bootstrapping with access to the raw data (e.g. see Cruz-Loya *et al.* 2019 for an example involving temperature and antibiotic stress).

Table S1. List of papers analyzed. Habitat class, species, stressors, type of species response, summary of interaction types reported, and summary of interaction types by Rescaled Bliss Independence (RBI) framework are given.

habitat	source	species	stressors	responses (units reported)	interaction reported	interaction by Rescaled Bliss Independence (RBI)
Estuary	Little *et al.* 2000	*Menidia berylina*	petroleum and UV-B	mortality (%)	Synergy: 5	Additive: 1, Antagonism: 1, Synergy: 3
	Peachey 2005	*Callinectes sapidus, Menippe adina, Panopeus herbstii, Libinia dubia*	fluoranthene or pyrene and Artificial UV or Solar UV	mortality (%)	Synergy: 24	Additive: 6, Antagonism: 3, Synergy: 15
Freshwater	Aalto & Pulkkinen 2013	*Daphnia magna*	Phosphorus limitation, parasite infection	survival (%) and growth (µg)	None: 2	Antagonism: 2
	Alton *et al.* 2010	*Limnodynastes peronii*	UV-B and perceived predation	survival (%)	Synergy: 1	Additive: 1
	Amburgey *et al.* 2016	*Pseudacris maculata*	hydration and predation	survival (%)	None: 1	Antagonism: 1
	Arce-Funck *et al.* 2018	*Gammus fossarum*	alder or maple leaf litter with phosphorus conditioning or no conditioning and cadmium in water	survival (proportion)	None: 7	Additive: 3, Antagonism: 4
	Baud & Beck 2005	*Pseudacris crucifer*	UV and copper	survival (%)	Synergy: 2	Additive: 1, Antagonism: 1
	Bezirci *et al.* 2012	*Daphnia pulex*	salinity and kairomone	survival (%)	Antagonism: 29	Additive: 2, Antagonism: 13, None: 13, Synergy: 1
	Boone & Semlitsch 2003	*Rana catesbiana*	carbaryl and crayfish, sunfish, or newts	survival (%)	Antagonism: 6	Additive: 4, Antagonism: 2
	Boone *et al.* 2005	*Rana clamitans*	nitrate and carbaryl	survival (%)	Antagonism: 2	Additive: 1, Antagonism: 1
	Boone & Bridges-Britton 2006	*Hyla versicolor*	nitrate and atrazine	survival (%)	None: 1	Antagonism: 1
	Boone *et al.* 2007	*Rana catesbeiana, Ambystoma maculatum*	carbaryl and bluegill	survival (%)	None: 2	Antagonism: 1, None: 1
	Bridges *et al.* 2004	*Rana pipiens*	pesticides and SPMD	survival (%)	None: 1	Antagonism: 1
	Brown *et al.* 2013	*Anaxyrus woodhousii*	Bd and triclosan	survival (%)	Additive: 1	Antagonism: 1
	Buck *et al.* 2015	*Rana cascadae*	Bd and zooplankton	survival (%)	None: 1	Additive: 1
	Buser *et al.* 2012	*Daphnia magna*	*Pasteuria ramosa* and diazinon	survival (%)	None: 1	Additive: 1
	Coopman *et al.* 2014	*Daphnia magna*	WBD infection and microcystis	survival (%)	None: 5	Antagonism: 5
	Dastis & Derry 2016	*Leptodiaptomus minutus*	Copepod pond source and pH	survival (number)	None: 1	Antagonism: 1
	de Beeck *et al.* 2016	*Culex pipiens*	natural kairomones of *N. maculate* and Bti	mortality (log-transformed raw number)	Synergy: 1	Additive: 1
	de Beeck *et al.* 2018b	*Ischnura pumilio, Ischnura elegans*	chlorpyrifos and temperature	mortality (proportion)	None: 2	Additive: 1, Antagonism: 1
	de Coninck *et al.* 2013	*Daphnia magna*	parasite and carbaryl	survival (%)	Synergy: 2	Additive: 2

	Delnat *et al.* 2019a	*Culex pipiens*	temperature and CPF	survival (%)	Additive: 1, Synergy: 1	Additive: 2
	Dietrich *et al.* 2014	*Aeromonas salmonicida*	temperature and malathion	survival (%)	None: 1	Additive: 1
	Dinh *et al.* 2016	*Coenagrion puella*	heat and starvation	survival (%)	Synergy: 1	Antagonism: 1
	Engert *et al.* 2013	*Moina macrocopa*	temperature and humic substance	survival (days) and growth (mm)	None: 24, Synergy: 8	Additive: 3, Antagonism: 19, None: 10
	Gabor *et al.* 2018	*Osteopilus septentrionalis*	metyraponeand and Bd	survival (days)	None: 1	Antagonism: 1
	Gahl *et al.* 2011	*Lithobates sylvaticus*	salinity and kairomone	mortality (%) and growth (cm)	None: 8	Additive: 1, Antagonism: 7
	Gomez-Mestre *et al.* 2006	*Bufo americanus*	jelly coat and mold	survival (%)	None: 1	Synergy: 1
	Gorokhova *et al.* 2010	*Monoporeia affinis*	Hypoxia or normoxia and combination of contaminants	mortality (%)	None: 4	Additive: 2, Antagonism: 2
	Green *et al.* 2019	*Lithobates clamitans*	temperature and chloride	survival (proportion)	None: 6	Additive: 6
	Hallman & Brooks 2015	*Lithobates sphenocephalus, Hyla chrysoscelis*	BCCU and day:night temperature	survival (%)	None: 14	Additive: 7, None: 5, Synergy: 2
	Hani *et al.* 2019	*Gasterosteus aculeatus*	temperature and Cd exposure	offspring mortality (%)	None: 1	Additive: 1
	Hanlon & Relyea 2013	*Lithobates clamitans*	waterbug and malathion or endosulfan	survival (%)	None: 4	Antagonism: 4
	Hanlon & Parris 2014	*Hyla versicolor*	Bd and Roundup or Sevin	survival (%)	None: 2	Antagonism: 2
	Heine-Fuster *et al.* 2017	*Brachionus calyciflorus*	kairomone and metamidophos	growth (rate of population increase per day)	None: 2	Additive: 2
	Hesse *et al.* 2012	*Daphnia magna*	fish or triops kairomone and metschnikowia infection	survival (days) and growth (µm)	None: 5, Synergy: 9	Additive: 12, Antagonism: 2
	Heye *et al.* 2019	*Chironomus riparius*	food limitation and carbamazapine	mortality (%)	Additive: 1	Antagonism: 1
	Isaza *et al.* 2018	*Cherax destructor*	pH and nitrate	survival (%)	None: 2	Additive: 1, Antagonism: 1
	Jansen *et al.* 2011	*Daphnia magna*	carbaryl and parasites	mortality (%)	Additive: 1	Additive: 1
	Janssens & Stoks 2013	*Enallagma cyathigerum*	glyphosate and predator cue	growth (rate per day)	None: 1	Additive: 1
	Kelly *et al.* 2010	*Galaxias anomalus*	glyphosate and *Telogaster opisthorchis*	survival (%)	Synergy: 1	Additive: 1
	Kerby & Sih 2015	*Rana boylii, Pseudacris regilla*	pesticide exposure and predator cue	survival (proportion)	None: 2	Additive: 2
	Kern *et al.* 2015	*Limnodynastes peronii*	temperature and UVR exposure	survival (%)	None: 2	Additive: 2
	Kimberly & Salice 2014	*Physa pomilia*	pH and temperature	mortality (proportion mortality at 14 days)	None: 4	Additive: 1, Synergy: 3
	Kimberly & Salice 2013	*Physa pomilia*	temperature and cadmium exposure	survival (%)	None: 10	Additive: 2, None: 7, Synergy: 1
	Leduc *et al.* 2016	*Lithobates pipiens*	copper and nickel	survival (%)	Synergy: 5	Antagonism: 4, None: 1
	Li *et al.* 2012	*Hyalella azteca*	phosphorous and copper	growth (mm)	None: 2	Antagonism: 2
	Long *et al.* 1995	*Rana pipiens*	pH and UV	survival (%)	Synergy: 4	Additive: 3, Antagonism: 1
	Loureiro *et al.* 2015	*Daphnia galeata*	NaCl and temperature	survival (min)	Synergy: 1	Additive: 1
	Macaulay *et al.* 2020	*Coloburiscus humeralis*	temperature and imidaclorid	survival (%)	Synergy: 10	Additive: 6, Synergy: 4
	Macías *et al.* 2007	*Rana perezi, Bufo bufo*	$N\text{-}NO_2$ and UV-B	mortality (%)	Synergy: 8	Antagonism: 4, None: 4

Manek *et al.* 2014	*Pimephales promelas*	cadmium and UVR exposure	mortality (%)	None: 1	Additive: 1
Mari *et al.* 2016	*Salvelinus alpinus*	warm temperature and sediment	survival (%)	None: 4, Synergy: 1	Additive: 2, Synergy: 3
Metts *et al.* 2005	*Ambystoma maculatum*	temperature and acidification	survival (%) and growth (mm per day)	None: 6	Additive: 2, Antagonism: 2, None: 1, Synergy: 1
Mothersill *et al.* 2007	*Salmo salar*	cadmium and aluminum	survival (%)	Synergy: 1	Synergy: 1
Mothersill *et al.* 2014	*Salmo salar*	heavy metal contamination and UV-B	survival (%)	Synergy: 1	Synergy: 1
Ortiz-Santaliestra *et al.* 2010	*Pelophylax perezi*	salinity and ammonium	mortality (%)	None: 4, Synergy: 4	Additive: 4, Antagonism: 2, None: 1, Synergy: 1
Ortiz-Santaliestra *et al.* 2011	*Pelobates cultripes*	ammonium and predator presence	mortality (%)	None: 8	Additive: 3, Antagonism: 5
Pandolfo *et al.* 2010	*Ligumia recta, Potamilus alatus, Lampsilis powellii*	temperature and copper	survival (%)	None: 18	Additive: 3, Antagonism: 11, None: 1, Synergy: 3
Pauley *et al.* 2015	*Hyla versicolor*	mosquito bits, torpedoes, or dunks and predator presence	survival (%)	None: 3	Additive: 1, Synergy: 2
Pestana *et al.* 2009	*Chironomus riparius*	imidacloprid, predation risk	growth (mm)	None: 4	Additive: 1, Antagonism: 3
Philippe *et al.* 2018a	*Nothobranchius furzeri*	temperature and cadmium	survival (%)	None: 2	Additive: 1, Synergy: 1
Philippe *et al.* 2018b	*Nothobranchius furzeri*	temperature and cadmium	survival (%)	Additive: 3	Additive: 3
Philippe *et al.* 2019	*Nothobranchius furzeri*	temperature and 3,4-DCA	survival (%)	None: 2	Antagonism: 2
Plautz *et al.* 2013	*Biomphalaria glabrata*	predator cue and malathion or cadmium	survival (%)	None: 2	Additive: 1, Antagonism: 1
Poore *et al.* 2013	*Peramphithoe parmerong*	pH and temperature	survival (number)	None: 2	Additive: 1, Synergy: 1
Relyea 2003	*Rana sylvatica, Rana catesbiana, Hyla versicolor, Bufo americanus*	carbaryl and predation	mortality (%)	Antagonism: 5, Synergy: 7	Additive: 1, Antagonism: 5, None: 5, Synergy: 1
Relyea *et al.* 2005	*Lithobates sphenocephalus, Hyla versicolor, Anaxyrus americanus*	pesticide and predation	survival (%)	None: 16	Additive: 3, Antagonism: 6, None: 5, Synergy: 2
Reyes *et al.* 2015	*Daphnia magna*	age at exposure to deltamethrin and low food	survival (%)	None: 3	Additive: 3
Roe *et al.* 2006	*Ambystoma talpoideum*	contaminated sediment and high larval density	mortality (%)	Additive: 2	Additive: 1, Antagonism: 1
Rogell *et al.* 2009	*Bufo calamita*	salinity and temperature	survival (%)	None: 2	Additive: 2
Rohr *et al.* 2013	*Osteopilus septentrionalis*	atrazine and Bd	mortality (%)	None: 1	Additive: 1
Rumrill *et al.* 2016	*Anaxyrus terrestris*	coper and predator	survival (%)	None: 1	Antagonism: 1
Saari *et al.* 2018	*Pimephales promelas*	dissolved oxygen and diltiazem	survival (%)	None: 8	Additive: 1, Antagonism: 1, None: 2, Synergy: 4
Sandland & Carmosini 2006	*Physa gyrina*	atrazine and predation	survival (%)	None: 1	Additive: 1
Sievers *et al.* 2018	*Limnodynastes tasmaniensis*	copper and imidaclorid	mortality (proportion)	None: 4	Additive: 4

	Smith *et al.* 2011	*Bufo americanus, Rana sylvatica*	malathion and nitrate	survival (%) and growth (mm)	Additive: 3, None: 12	Additive: 7, Antagonism: 7, None: 1
	Smith *et al.* 2015	*Oncorhynchus mykiss*	radiation and aluminum	survival (%)	None: 2	Antagonism: 2
	Suhett *et al.* 2011	*Moina macrocopa*	salt and humic substance	growth (mm)	Antagonism: 2	Additive: 1, Antagonism: 1
	Tüzün & Stoks 2017	*Coenagrion puella*	esfenvalerate and hatching period	survival (%)	None: 2	Additive: 2
	Taylor *et al.* 2016	*Daphnia pulex-pulicaria*	copper and dissolved organic carbon	survival (%)	None: 8	Antagonism: 6, None: 2
	Teffer *et al.* 2019	*Oncorhynchus kisutch*	temperature and handling stress (gill net or biopsy)	mortality (%)	None: 3, Additive: 1	Additive: 3, Synergy: 1
	Teplitsky *et al.* 2007	*Rana arvalis*	acidity and predator presence	growth (g)	None: 2	Additive: 1, Synergy: 1
	Vandenbrouck *et al.* 2011	*Daphnia magna*	temperature and nickel	growth (% of control)	None: 8	Antagonism: 8
	Verheyen & Stoks 2019	*Ischnura elegans*	daily temperature and chlorpyrifos	mortality (%)	None: 2	Additive: 2
	Whittington & Walsh 2015	*Daphnia lumholtzi*	predator presence and cyanobacteria	growth (mm^2)	None: 3	Additive: 1, Antagonism: 2
	Wood & Welch 2015	*Anaxyrus terrestris*	brackish water and carbaryl, glyphosate, or atrazine	survival (proportion)	None: 3	Additive: 1, Antagonism: 2
Laboratory	Ankomah *et al.* 2013	*Escherichia coli*	TOB and TET	growth by death (rate)	None: 1	Antagonism: 1
	Friman & Buckling 2013	*Pseudomonas fluorescens*	phage and protist	growth (rate)	None: 1	Additive: 1
Marine	Anderson *et al.* 1998	*Crassostrea virginica*	TBT and hypoxia	mortality (%)	Synergy: 1	Additive: 1
	Anthony *et al.* 2007	*Acropora intermedia*	temperature and light	survival (%)	None: 1	Additive: 1
	Bible *et al.* 2017	*Ostrea lurida*	salinity and temperature	mortality (proportion)	None: 8, Synergy: 4	Additive: 8, Antagonism: 1, Synergy: 3
	Blake & Duffy 2010	*Gammarus mucronatus, Elasmopus levis*	heat and reduced salinity	growth (mg)	Antagonism: 1, None: 1	Antagonism: 2
	Bogan *et al.* 2019	*Crepidula fornicate*	pH and low food culture	survival (proportion)	Synergy: 3	Additive: 1, Antagonism: 1, Synergy: 1
	Bonsdorff *et al.* 1995	*Macoma balthica*	predation and disturbance	survival (%)	Synergy: 2	Additive: 2
	Braid *et al.* 2005	*Haliotis rufescens*	temperature and WS-LRP exposure	mortality (number)	Synergy: 1	Additive: 1
	Bundy *et al.* 2003	*Fundulus heteroclitus, Crassostrea virginica*	nutrient addition and trace element addition	growth (%, mg, proportion)	None: 11	Additive: 6, Antagonism: 5
	Carreja *et al.* 2016	*Taliepus dentatus*	low UV-B and temperature	mortality (%)	None: 2	Antagonism: 2
	Ceccherelli *et al.* 2018	*Posidonia oceanica*	nutrients and burial	survival (%)	Additive: 2, Synergy: 2	Additive: 2, Antagonism: 1, Synergy: 1
	Cleveland *et al.* 2000	*Mysidopsis bahia*	TPH exposure and solar radiation	mortality (%)	Synergy: 10	Additive: 5, Antagonism: 3, Synergy: 2
	Cumbo *et al.* 2013	*Pocillopora damicornis*	temperature and acidification	mortality (%)	None: 1	Antagonism: 1
	Davis *et al.* 2018a	Rockfish (*Sebastes* spp)	hypoxia and high pCO$_2$	mortality (%)	None: 2	Antagonism: 2
	Deschaseaux *et al.* 2010	*Bembicium nanum, Dolabrifera brazier, Siphonaria denticulate*	temperature and salinity	mortality (%)	None: 6	Additive: 2, Antagonism: 3, Synergy: 1

Dineshram *et al.* 2016	*Crassostrea gigas*	temperature and reduced salinity	survival (%)	None: 1	Additive: 1
Duquesne & Liess 2003	*Paramoera walker*	heavy metals and UV-B	mortality (%)	Synergy: 1	Additive: 1
Fabricius *et al.* 2013	*Montipora tuberculosa, Acropora millepora*	temperature and nutrients	survival (%)	None: 8	Additive: 1, Antagonism: 1, None: 2, Synergy: 4
Firth & Williams 2009	*Cellana toreuma*	temperature and salinity	mortality (%)	None: 4	Additive: 1, Antagonism: 2, None: 1
Gaitán-Espitia *et al.* 2014	*Macrocystis pyrifera*	temperature and pCO_s	mortality (%)	None: 1	Additive: 1
Glaspie *et al.* 2018	*Saccostrea glomerate*	chronic acidification and episodic acidification	mortality (proportion)	None: 1	Synergy: 1
Gobler *et al.* 2018	*Menidia beryllina*	temperature or 20% less optimal diet and pCO_2	survival (%)	None: 5	Additive: 1, Antagonism: 2, Synergy: 2
Holmer *et al.* 2011	*Halophila ovalis*	alga and temperature	mortality (% per day) and growth (mg per apex per day)	Additive: 2, None: 4, Synergy: 5	Additive: 4, Antagonism: 3, None: 2, Synergy: 2
Jansson *et al.* 2015	*Macoma balthica*	low O_2 and high pH	survival (%)	None: 1	Antagonism: 1
Kahn & Durako 2006	*Thalassia testudinum*	salinity and NH_4^+	survival (days)	None: 14	Additive: 6, Antagonism: 7, None: 1
Kawai & Tokeshi 2007	*Septifer virgatus*	thermal period and disturbance period	mortality (%)	Additive: 6	Additive: 3, None: 1, Synergy: 2
Kwok & Leung 2005	*Tigriopus japonicus*	copper or temperature and salinity	mortality (%)	None: 11	Additive: 2, Antagonism: 4, Synergy: 5
Lenihan *et al.* 2003	*Nototanais dimorphus, Spiophanes tcherniai, Heterophoxus videns, Austrosignum grande, Eudorella splendida, Sterichinus neumayeri, Monoculodes demissus*	total organic carbon and copper	growth (abundance)	None: 27	Additive: 5, Antagonism: 13, None: 4, Synergy: 5
Liess *et al.* 2001	*Paramoera walker*	copper and UV-B	survival (number)	Synergy: 4	Additive: 3, Synergy: 1
Macías *et al.* 2007	*Rana perezi*	$N\text{-}NO_2$ and UV-B	mortality (%)	Synergy: 4	Antagonism: 4
Marques *et al.* 2017	*Amphistegina gibbosa*	copper and pH	mortality (%)	None: 9	Additive: 3, Antagonism: 6
Olsen *et al.* 2014	*Dictyota menstrualis*	heat and alga	survival (%)	Synergy: 2	Additive: 1, Antagonism: 1
Pestana *et al.* 2010	*Daphnia magna*	imidacloprid and predation	growth (rate by day)	None: 4	Additive: 2, Antagonism: 1, None: 1
Prosser *et al.* 2011	*Danio rerio*	phenanthrene and mycobacterium infection	survival (%)	None: 2	Antagonism: 2
Przeslawski *et al.* 2005	*Siphonaria denticulata, Bembicium nanum, Dolabrifera brazier,*	temperature and salinity	mortality (%)	Synergy: 6	Additive: 4, Antagonism: 1, Synergy: 1
Ragagnin *et al.* 2018	*Pagurus criniticornis*	pH and photoperiod	mortality (proportion)	Synergy: 1	Additive: 1

	Renegar & Riegl 2005	*Acropora cervicornis*	nutrients and CO_2	growth (mg per day)	Additive: 1, Antagonism: 1, None: 4	Additive: 2, Antagonism: 3, Synergy: 1
	Salo & Pedersen 2014	*Zostera marina*	salinity and temperature	mortality (%)	Synergy: 4	Additive: 1, Antagonism: 2, None: 1
	Serrano *et al.* 2018	*Porites astreoides*	temperature and NO_3	mortality (%)	None: 1	Antagonism: 1
	Speights *et al.* 2017	*Crassostrea virginica*	temperature and CO_2	survival (proportion)	None: 3	Antagonism: 3
	Staton *et al.* 2002	*Microarthridion littorale*	salinity and pesticide exposure	survival (%)	None: 2	Antagonism: 2
	Steevens *et al.* 1999	*Lytechinus variegatus*	phenanthrene or benzo[a]pyrene and UV-B	survival (%)	Additive: 16	Additive: 5, Antagonism: 1, None: 5, Synergy: 5
	Swiney *et al.* 2017	*Paralithodes camtschaticus*	pH and temperature	survival (%)	None: 2	Antagonism: 1, Synergy: 1
	Thyrring *et al.* 2015	*Mytilus edulis*	temperature and lead	survival (%)	None: 20	Additive: 4, Antagonism: 7, None: 7, Synergy: 2
	Vaz-Pinto *et al.* 2013	*Sargassum muticum*	CO_2 and temperature	survival (%)	None: 1	Additive: 1
Terrestrial	Chu & Chow 2002	*Caenorhabditis elegans*	cadmium and nickel, zinc, or copper	mortality (%)	Synergy: 3	Additive: 1, None: 1, Synergy: 1
	Dżugan *et al.* 2012	*Gallus gallus domesticus*	cadmium and zinc	mortality (%)	Antagonism: 6	Antagonism: 6
	Gan *et al.* 2016	*Mus musculus*	[3-BrPA] and 4-OHT	mortality (%)	None: 2	Additive: 2
	Højer *et al.* 2001	*Folsomia candida*	nonylphenol and humidity	survival (%)	Synergy: 30	Additive: 11, Antagonism: 7, Synergy: 12
	Holliday *et al.* 2009	*Malaclemys terrapin*	salinity and PCB	growth (g)	None: 12	Additive: 8, Antagonism: 1, None: 3
	Jayawardena *et al.* 2016	*Polypedates cruciger*	cercariae and chlorpyrifos, dimethoate, glyphosate, or propanil	mortality (%)	None: 4	Antagonism: 4
	Jensen *et al.* 2009	*Dendrobaena octaedra*	nonylphenol and temperature	survival (%)	Synergy: 30	Additive: 5, Antagonism: 1, None: 15, Synergy: 9
	Kramarz *et al.* 2007	*Helix aspersa*	cadmium exposure and nematode infection	mortality (%)	None: 48	Additive: 18, None: 29, Synergy: 1
	Long *et al.* 2009	*Lumbricus rubellus*	moisture level and fluoranthene	survival (%)	Additive: 12	Additive: 5, Antagonism: 4, None: 3
	Pringle *et al.* 2016	*Acacia drepanolobium*	herbivory and soil type or ants	mortality (%) and survival (%)	Additive: 1, None: 1	Antagonism: 1, Synergy: 1
	Schmidt *et al.* 2014	*Folsomia candida*	chemical activity of phenanthrene and water activity	mortality (%)	Synergy: 36	Additive: 11, Antagonism: 2, None: 10, Synergy: 13
	Slotsbo *et al.* 2009	*Folsomia candida*	temperature and mercury	survival (%)	Synergy: 1	Synergy: 1
	Ushakova 2003	*Spirorbis spirorbis, Circeus spirillum*	temperature and salinity	mortality (%)	None: 4	Antagonism: 4
	Yu *et al.* 2015	*Xenopus laevis*	pesticide and UV-B	mortality (%)	Synergy: 1	Additive: 1

Table S2: List of three-stressor interactions found in literature. Habitat class, species, stressors, and type of response measured.

habitat	source	species	stressors	responses
Freshwater	Piggott *et al.* 2015	*Fragilaria ungeriana, Achnanthidium minutissimum, Navicula cryptotenella, Gomphonema parvulum, Nitzschia palea, Cocconeis placentula, Cymbella kappii, Navicula cryptocephala, Fragilaria vaucheriae, Encyonema minutum,*	temperature, sediment, nutrient	growth
	de Beek *et al.* 2018a	*Ischnura elegans*	temperature, CPF, density	mortality
	Hasenbein *et al.* 2018	*Hyalella azteca*	salinity, temperature, bifenthrin	survival
	Davis *et al.* 2018b	*Agapetus fuscipes, Silo pallipes*	low sediment, phosphorus, nitrogen	growth
	Elbrecht *et al.* 2016	*Chaetopteryx villosa*	sediment, reduced flow, nutrient addition	growth
	Bruder *et al.* 2017	*Salmo trutta*	slow flow velocity, low nutrient enrichment, constant DCD	survival
	Delnat *et al.* 2019b	*Culex pipiens*	DTV-7, CPF, *Bacillus thuringiensis israelensis*	mortality
Marine	Blake & Duffy 2016	*Zostera marina*	shade, temperature, grazers	growth
	Vasquez *et al.* 2015	*Limulus polyphemus*	temperature, salinity, ambient O_2	survival
	Cheng *et al.* 2015	*Ostrea lurida*	hypoxia, salinity, temperature	survival
	Vasquez *et al.* 2017	*Limulus polyphemus*	salinity, temperature, oxygen	survival
	Li *et al.* 2017	*Thalassiosira weissflogii*	CO_2, nutrient, PAR intensity	growth
	Büscher *et al.* 2017	*Lophelia pertusa*	low food, temperature, pCO_2	mortality
	Gobler *et al.* 2018	*Menidia beryllina*	diet, CO_2, temperature	survival
	Dineshram *et al.* 2016	*Crassostrea gigas*	temperature, salinity, pH	survival
	Caronni *et al.* 2017	*Chrysophaeum taylorii*	nutrient enrichment, mechanical disturbance, hydrodynamic stress	growth
Terrestrial	Morgado *et al.* 2016	*Porcellionidespru-inosus*	chlopyrifos, soil, soil moisture level	survival
	Janssens *et al.* 2017	*Lestes viridis*	egg temperature, larval temperature, previous esfenvalerate concentration	survival

Table S3. Number of Replicates in the Interaction Studies Examined. Of the 840 interaction studies examined here, the majority (68%) had 1-6 replicates.

No. replicates per treatment	No. of interactions
1-3	223
4-6	352
7-9	51
10-12	60
13-15	6
16-18	32
19-21	26
21+	13
Unequal replicates	42
Unknown or unclear	35

Supporting Information Figures

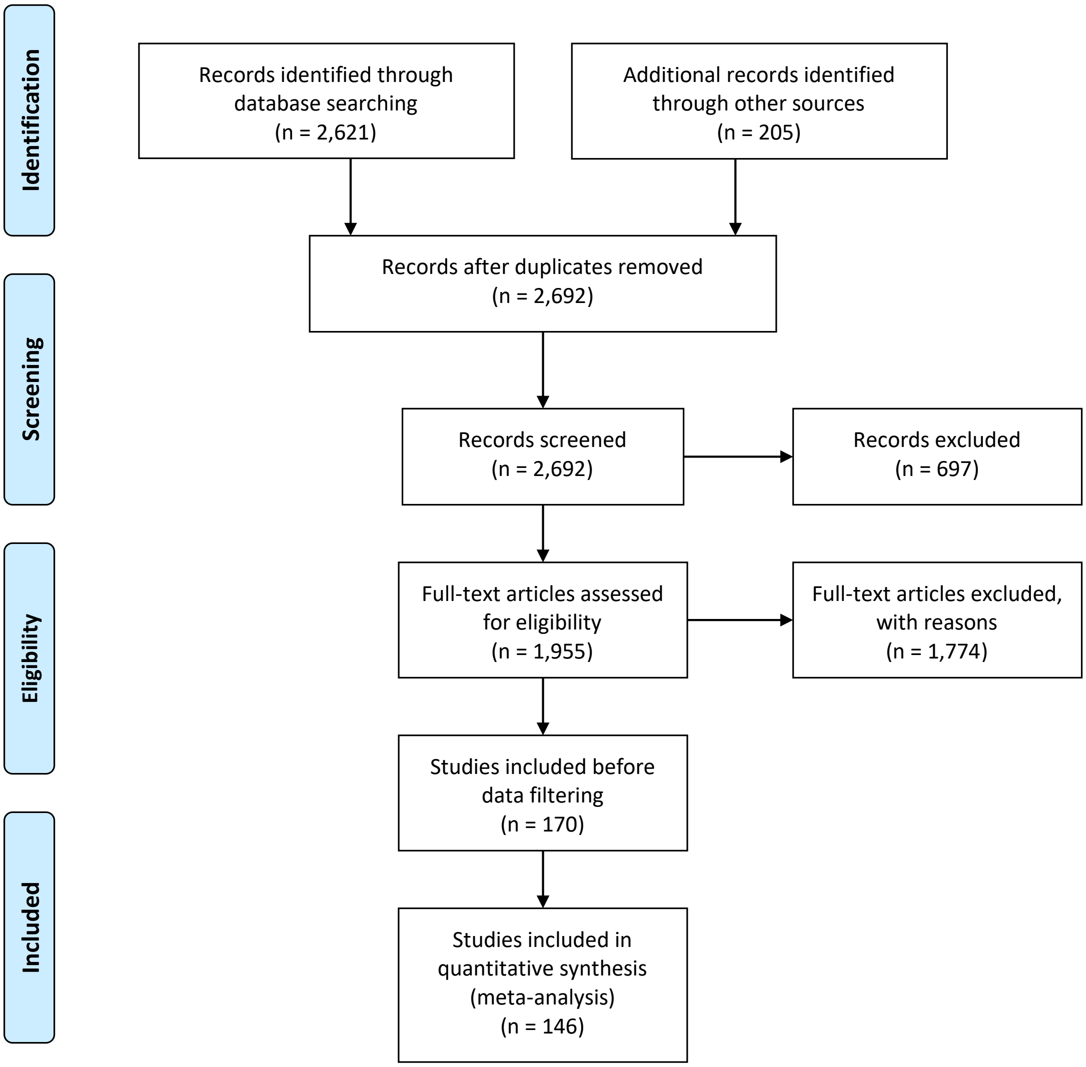

Figure S1. PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) (Moher *et al.* 2009) Flow Diagram. A total of 2,692 unique studies (records) were narrowed to 146 papers with 840 total data entries that were included in our quantitative synthesis.

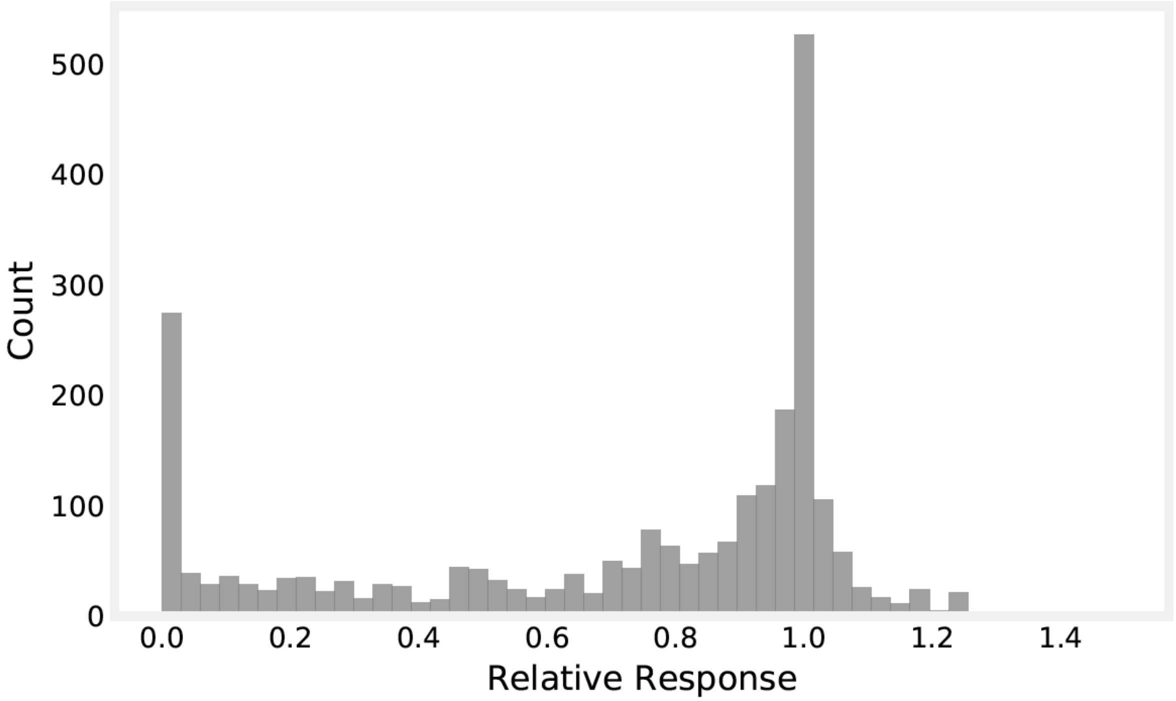

Figure S2. Distribution of relative response values across meta-data included in our study. Refer to Materials and Methods for details.

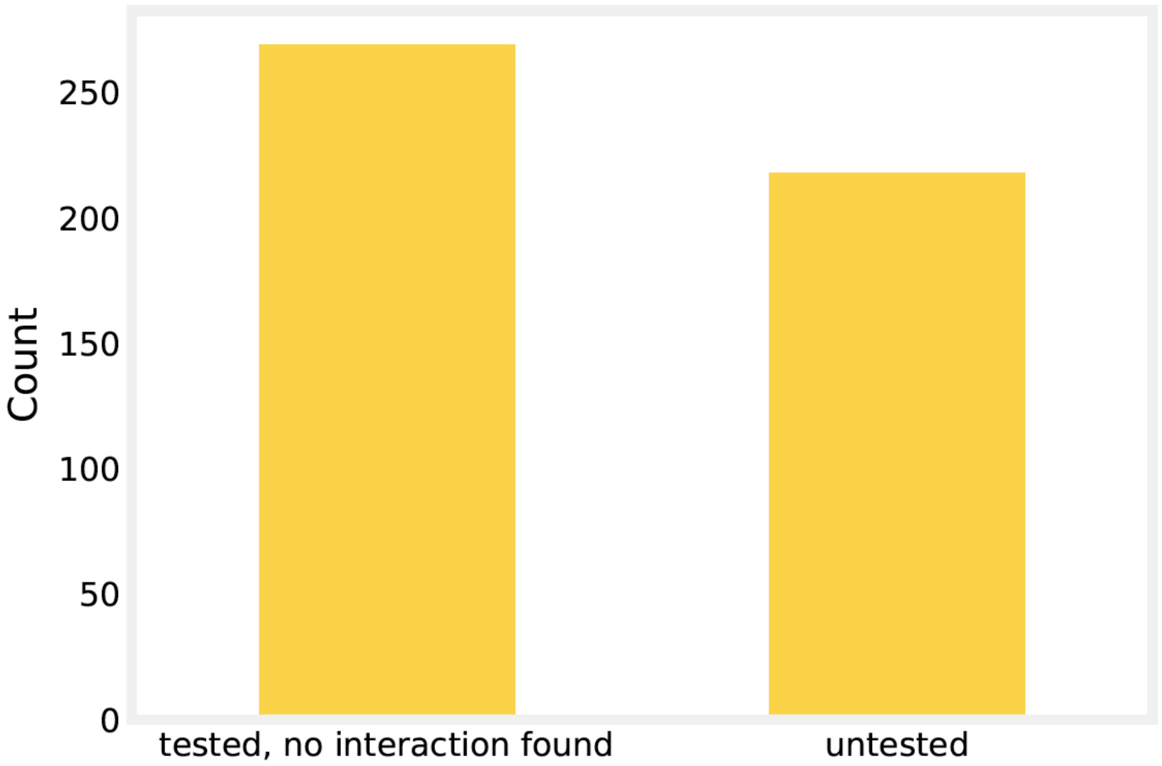

Figure S3. Inconclusive reports of interaction types across metadata. The metadata that is labeled as "inconclusive" are separated into two groups: 1) "tested, no interaction found", indicating that the authors looked for an interaction but ultimately did not find one or did not explicitly state a conclusive interaction type, 2) "untested", meaning that categorization of interactions was not the particular focus of the study, and hence it is not explored and reported in the study. Many studies did not focus on interactions and thus did not report interactions in their study. The number of data corresponding to each group among 487 data points is represented as a bar graph.

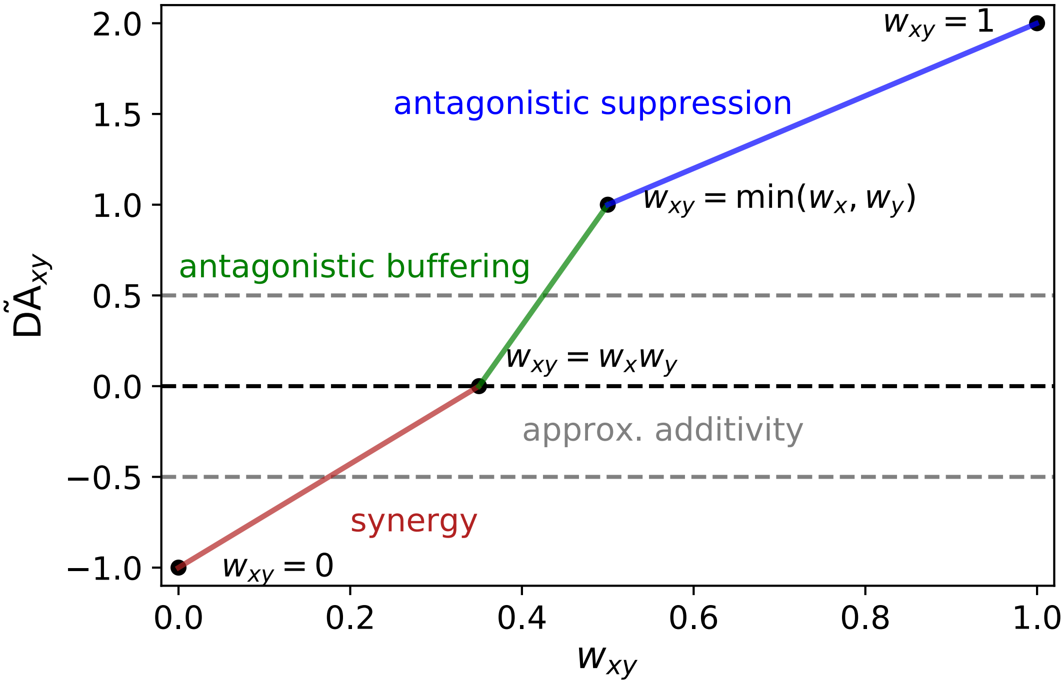

Figure S4. Rescaled Bliss Interaction (RBI) metric. Here we show an example of how the rescaled DA interaction metric changes as a function of w_{xy}, the survival proportion when both stressors are present. In this example, the survival proportions for the individual stressors are $w_x = 0.7$ and $w_y = 0.5$. The case when the observed survival is equal to the expected survival under a null model of independence ($w_{xy} = w_x w_y$) corresponds to $\widetilde{DA}_{xy} = 0$. Small deviations from these predictions are considered approximately additive interactions (gray dotted lines). When the observed survival is less than expected under the null model, the rescaled interaction is synergistic (red line) and $\widetilde{DA}_{xy} < 0$. The minimum possible value is -1 and corresponds to the stressor combination being lethal ($w_{xy} = 0$). When the observed growth is greater than expected when there is no interaction, the interaction is antagonistic (green and blue lines). Antagonistic buffering (green line, $0 < \widetilde{DA}_{xy} \leq 1$) corresponds to cases where the combined stressors reduce growth more than the single strongest stressor ($w_x w_y < w_{xy} \leq \min(w_x, w_y)$). Antagonistic suppression (blue line, $\widetilde{DA}_{xy} > 1$) corresponds to the case where a single stressor reduces survival more than the combined stressors.

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Meta-analysis of three-stressor combinations on population-level fitness reveal substantial higher-order interactions

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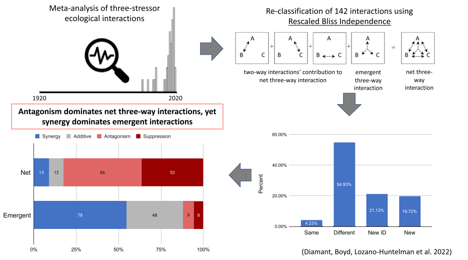
HIGHLIGHTS

- We used a novel interaction framework, the Rescaled Bliss Independence (RBI).
- RBI quantifies multiple aspects of higher-order interactions.
- RBI does not rely on restrictive assumptions that can lead to misidentification.
- Through meta-analysis, 96 % of net 3-way interactions were newly or re-classified.
- RBI identified emergent interactions, which only occur with 3+ stressors.

GRAPHICAL ABSTRACT



(Diamant, Boyd, Lozano-Huntelman et al. 2022)

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ABSTRACT

Although natural populations are typically subjected to multiple stressors, most past research has focused on single-stressor and two-stressor interactions, with little attention paid to higher-order interactions among three or more stressors. However, higher-order interactions increasingly appear to be widespread. Consequently, we used a recently introduced and improved framework to re-analyze higher-order ecological interactions. We conducted a literature review of the last 100 years (1920–2020) and reanalyzed 142 ecological three-stressor interactions on species' populations from 38 published papers; the vast majority of these studies were from the past 10 years. We found that 95.8 % ($n = 136$) of the three-stressor combinations had either not been categorized before or resulted in different interactions than previously reported. We also found substantial levels of emergent properties—interactions that are not due to strong pairwise interactions within the combination but rather uniquely due to all three stressors being combined. Calculating net interactions—the overall accounting for all possible interactions within a combination including the emergent and all pairwise interactions—we found that the most prevalent interaction type is antagonism, corresponding to a smaller than expected effect based on single stressor effects. In contrast, for emergent interactions, the most prevalent interaction type is synergistic, resulting in a larger than expected effect based on single stressor effects. Additionally, we found that hidden suppressive interactions—where a pairwise interaction is suppressed by a third stressor—are found in the majority of combinations (74 %). Collectively, understanding multiple stressor interactions through applying an appropriate framework is crucial for answering fundamental questions in ecology and has implications for conservation biology and population management. Crucially, identifying emergent properties can reveal hidden suppressive interactions that could be particularly important for the ecological management of at-risk populations.

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1. Introduction

Individuals in natural populations almost always face multiple stressors that affect their ability to survive and to find food, shelter, mates, and safety (Blaustein and Kiesecker, 2002; Côté et al., 2016). These stressors include changes to biological or environmental factors that can result in unfavorable responses within a population (Vinebrooke et al., 2004), further leading to unfavorable responses across entire ecological systems (Jackson et al., 2021, 2016). Over the past century, ecological stressors such as climate change, pollution, and habitat destruction have adversely affected natural systems, contributing to biodiversity loss and a continuing threat to populations and ecosystems (Butchart et al., 2010; Didham et al., 2007; Halpern et al., 2015). These stressors rarely occur in isolation. Instead, they often co-occur with other stressors, raising the possibility of interactions and potentially changing the overall impact on populations (Côté et al., 2016; Crain et al., 2008). Therefore, there is a great need to properly assess and predict stressor interactions to mitigate their cumulative effects.

When the combined impact of two stressors is equal to the amount of the individual effects in isolation, the interaction type is defined as an additive interaction, or no interaction (Bliss, 1939; Loewe, 1953; Folt et al., 1999; Yeh et al., 2006; Piggott et al., 2015c; Jackson et al., 2016). Alternatively, two stressors could interact synergistically—increasing the overall effects—or antagonistically—decreasing the overall effects (Bliss, 1939; Loewe, 1953; Folt et al., 1999; Yeh et al., 2006; Piggott et al., 2015c). For example, synergistic interactions were observed when the combined effects of high temperatures and low pH decreased calcification (the production of shells and plates) in certain marine animals when compared to the individual effects of each stressor (Rodolfo-Metalpa et al., 2011). On the other hand, antagonistic interactions were found in coral (*Pocillopora meandrina*) microbiome response to multiple stressor interactions (Maher et al., 2019). Specifically, increased temperature and coral scarring both decreased the abundance of the dominant taxon (*Endozoicimonacae*) in coral microbiomes. However, the combined stressor effect led to a lower magnitude response than predicted if there were no interactions between the stressors (Maher et al., 2019). Notably, an extreme form of antagonism is termed suppression—one stressor reverses another stressor's effects; e.g., the combined effect is weaker than expected by each or both of the stressors' effects (Yeh et al., 2006; Chait et al., 2007; Yeh et al., 2009; Singh and Yeh, 2017). An extreme form of suppression, also known as “reversals” (Jackson et al., 2016), refers to a combination of stressors leading to opposite effects than expected by additivity—e.g., both stressors individually decreasing growth but in combination increasing growth. For example, the combined effect of carbaryl and nitrate decreased green frog (*Rana clamitans*) tadpole growth despite their individual positive effects on tadpole growth (Boone et al., 2005).

Pairwise interactions—the effects of two stressors in combination compared to individual effects—have been well studied in the ecological literature. Empirical work on pairwise stressor interactions (Hesse et al., 2012; Cramp et al., 2014; Van Praet et al., 2014; Sniegula et al., 2017; Delnat et al., 2019), literature reviews, and meta-analyses (Darling and Côté, 2008; Crain et al., 2008; Ban et al., 2014; Matthaei and Lange, 2015; Piggott et al., 2015c; Côté et al., 2016; Jackson et al., 2016; Villar-Argaiz et al., 2018; Tekin et al., 2020) have revealed their substantial influence across biological systems and scales. Yet, there are likely more than two stressors acting on all, or almost all, wild populations. In fact, multiple stressor interactions are more frequent than previously thought, despite having received less attention (Beppler et al., 2016; Tekin et al., 2018a).

When studying interactions, higher-order combinations—defined here as a combination of three or more stressors—have historically been ignored (Pomerantz, 1981) despite their importance in ecological communities (Billick and Case, 1994; Levine et al., 2017). The assumptions that have been used to justify this include (1) paired interactions or single-stressor effects provide the main effects, so one only needs to worry about paired interactions or single effects; higher-order interactions, therefore, provide negligible effects (Pomerantz, 1981; Wootton, 1994; Ban and Alder, 2008; Wood et al., 2012; Wood, 2016); (2) higher-order interactions are complex and depend on accurate, specific parameters and underlying null models that are often not available for effective and reliable testing (Billick and Case, 1994; Thompson et al., 2018); and, (3) from an experimental standpoint, the collection of higher-order interactions, whether in the lab or the field, can be onerous, time-consuming, and logistically difficult as increasing stressor combinations could theoretically lead to an exponentially large number of experiments, which has given rise to research on approximating multi-stressor responses from single pairs (Billick and Case, 1994; Côté et al., 2016; Wood, 2016; Zimmer et al., 2016; though see: Levine et al., 2017).

In recent years, however, multiple stressor interactions have received more theoretical and experimental attention. These include studies across scales of biological organization—from the individual (Piggott et al., 2015a; Bruder et al., 2017; Hunn et al., 2019) to the population (Bruder et al., 2017; Macaulay et al., 2021b) to the community level (Piggott et al., 2015b; Piggott et al., 2015d; Winkworth et al., 2015; Bruder et al., 2016; Elbrecht et al., 2016; Salis et al., 2017; Beermann et al., 2018; Davis et al., 2018; Salis et al., 2019; Juvigny-Khenafou et al., 2020; Juvigny-Khenafou et al., 2021a; Juvigny-Khenafou et al., 2021b; Macaulay et al., 2021a)—and across time (Macaulay et al., 2021c).

A recently introduced framework to examine interactions was used specifically to analyze both pairwise and higher-order interactions (Tekin et al., 2018b, 2020). This framework, the Rescaled Bliss Independence (RBI), was originally drawn from the pharmacology and microbiology fields (Beppler et al., 2016; Tekin et al., 2016). The RBI has several key advantages compared to the most-used method to examine interactions in ecology, ANOVA. First, ANOVA incorporates several assumptions that are often violated or not tested (Text A.1). Second, RBI enables direct comparisons of interaction effects from absolute to relative fitness. Third, the framework allows for straightforward generalization from pairwise to higher-order interactions while keeping the ability to rescale interaction terms—that is, to normalize interaction values relative to a natural baseline, much like the way we typically measure fitness as relative fitness rather than absolute fitness (see Text A.2 for details). Finally, and crucially, the framework enables the identification of emergent properties—that is, what interactions arise that are the result of all three stressors together, rather than just being the dominant effect due to a pairwise interaction dominating the landscape of multiple stressors.

Emergent properties only arise from three or more stressors; there are no emergent properties in two-stressor interactions because the interaction between the two stressors is what emerges from the combination of the stressors. However, in three or more stressor combinations, the interaction from three stressors could be coming primarily from a strong two-stressor interaction—a non-emergent interaction—or the interaction could be the result of all three stressors together—an emergent interaction (Beppler et al., 2016). Together, pairwise and emergent interactions constitute the observed net three-way interaction (Fig. 1A). For example, imagine ecological stressors A, B, and C all are impacting the growth of a population X. Let's say that the interaction among all three stressors together is synergistic. But this three-stressor interaction could arise primarily from a strong synergistic pairwise interaction between two of the three stressors, say A and B, which would obscure all other interactions (described in Fig. 1B). This would be a case of a non-emergent interaction. Or this three-stressor interaction could need all three stressors present to show a synergistic interaction, which would be an emergent interaction (Beppler et al., 2016; Tekin et al., 2016). Thus, it is imperative to quantify both pairwise and three-stressor combinations to determine the nature of population X's response to stressors A, B, and C.

The importance of identifying emergent properties lies in our ability to understand the impacts of specific stressors, even when most populations experience multiple stressors in combination. In fields such as conservation ecology or climate change biology, there is often an emphasis on conserving and bolstering endangered and threatened populations by mitigating at least one of the stressors affecting population survival and growth (Brown et al., 2013). However, if we do not clearly understand how stressors interact, we could be mitigating the wrong stressors, or at least, not the optimal

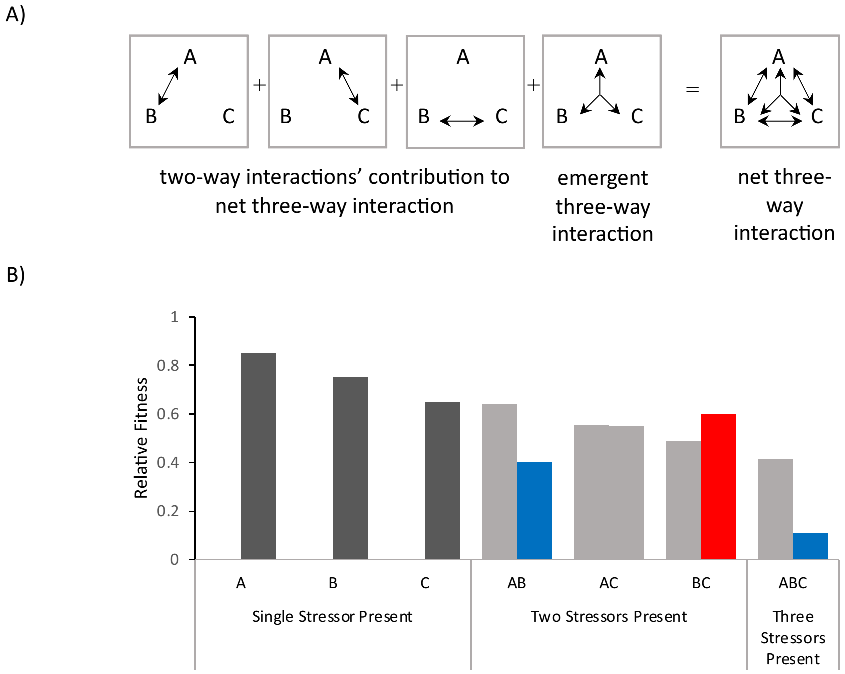

Fig. 1. The components needed to assess three-stressor interactions. A) Net three-way interactions are composed of lower-order two-way interactions between given stressors (A, B, and C) and the higher-order emergent three-way interaction that is only quantifiable with all three stressors present and when all two-way interactions are known. Together, these compose the net three-way interaction. Fig. 1 is partially adapted from Tekin et al. (2018b). B) An example of the contribution of two-way interactions on net three-way interactions. Here, single stressors all decrease relative fitness. In two-stressor and three-stressor interactions, light gray represents the additive expectation based on single-stressor effects on fitness. Blue represents synergistic interactions and red represents antagonistic interactions. In this example, the strong synergistic two-stressor interaction between A and B, rather than an emergent interaction, overshadows the additive interaction between A and C and the antagonistic interaction between B and C. This leads to a synergistic net three-way interaction between A, B, and C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

stressors. A striking example of the importance of emergent interactions can be seen in the field of pharmacology, where the combination of three antibiotic stressors trimethoprim, streptomycin, and erythromycin have a synergistic effect, efficiently reducing bacterial population size. However, if one of the drug stressors (for example, erythromycin) is removed from this combination the overall killing efficiency actually *increases* (Beppler et al., 2017). This results in the population of concern, the bacteria, decreasing more when there are only two stressors, rather than three (Beppler et al., 2017). This is an example of a critical emergent interaction.

In much the same way, understanding multiple stressors and their emergent, higher-order, effects could be crucial for understanding how best to conserve species and populations. In an ecological example, pairwise interactions on stream macroinvertebrate family richness vary across environmental gradients, from synergistic to additive to antagonistic (Kefford et al., 2022), pointing to the importance of understanding how ecological interactions themselves can be affected by a third factor.

Unlike ANOVA and similar approaches, the Rescaled Bliss Independence framework is conducive to rescaling the interaction measure (e.g., normalization), which allows for more easily measuring and identifying the strength of an interaction. Like absolute versus relative fitness, a rescaled interaction value is normalized by a natural baseline (e.g., lethality). Rescaling results in a multimodal distribution with clearer cut-offs around values. Therefore, interaction types are more easily distinguishable (Segrè et al., 2005; Tekin et al., 2016, 2020). Without the rescaling step, raw interaction values can be exactly the same even though they may represent different interaction types, leading to incorrect interpretations (Tekin et al., 2016, 2017). Therefore, rescaling is critical to comparing interaction measures and identifying interaction types.

Recently, three critical issues have been identified when evaluating interaction terms within linear regression models—a common statistical method used to test for interactions (Duncan and Kefford, 2021). First, a function space (e.g., linear, logarithmic, square root) must be chosen within which to perform the regressions. Depending on this choice of space (referred to as scale by Duncan and Kefford, 2021), interactions may exist in one analysis but not another. Choice of space is equivalent to different choices of null models that may be based on multiplicative (e.g., growth under stressor A = 0.5, growth under stressor B = 0.5, so the null additive model predicts growth under stressors A and B = 0.5*0.5 = 0.25) or additive assumptions (e.g., growth under stressor A = 1–0.5 = 0.5, growth under stressor B = 1–0.5 = 0.5, so the null additive model predicts growth under stressors A and B = 1–0.5 - 0.5 = 0). In addition, nonlinearities can introduce issues in terms of identifying interactions, and there are two distinct ways this can happen, corresponding to the second and third issues. The second issue is that single-stressors could be correlated, and if a single-stressor has nonlinear effects on the response variable such as growth, then these correlations can induce an apparent but artifactual interaction in terms of the regressions models because the partial derivatives will not be constant. Third, the interaction terms themselves might not be linear across a range of stressor intensities. Specifically, this means that the interaction term should not be just the product of the two linear stressors but instead, at least one of the stressors should be multiplied in a nonlinear form.

For our methods and results in this paper, the RBI is not limited by the issues raised above, primarily because RBI is not based on linear regression models. For the first issue, the RBI uses a multiplicative null model (Bliss independence (Bliss, 1939)) for the given level of biological interest: population fitness. Because relative fitness must vary between 0 and 1 and can be considered as a percentage, a multiplicative null model is appropriate. This null model would need to possibly be adapted if other response variables at other biological levels are considered. For the second and third issues in terms of nonlinearities, it is important to recognize that the RBI does not model continuous independent variables; instead, it uses a single data point for each stressor or combination of stressors. Moreover, in terms of the second issue and effects of correlations, our analysis here explicitly relies on controlled full-factorial experimental designs. Because the experiments are controlled and cover a full combinatorial range, the stressors are varied independently such that no correlations are present. In contrast, measurements based on field conditions and naturally occurring climate may have essential correlations that need to be included and that are crucial for devising management strategies. Third, because the RBI relies on single data points and does not require a single interaction type for any set of stressors, it does not assume a linear or non-linear response at all. That is, the same stressors can be classified as having different types of interactions when different amounts of each stressor are used. For example, stressor combinations at low concentrations might yield synergistic interactions, yet might yield antagonistic interactions at high concentrations. If an interaction measure is desired that covers the range of values for stressors and can account for nonlinearities in single-stressor responses, Loewe's additivity is a natural choice (Loewe, 1953). However, this method cannot be rescaled and cannot examine emergent properties, making it ill-suited for testing for stressor interactions at higher orders as we do here.

Here we conduct a literature search from January 1920–November 2020, finding 3+ stressor interaction studies mostly from the last 10 years. We re-analyze stressor interactions using the new Rescaled Bliss Independence (RBI) framework (Tekin et al., 2016), recently applied to ecological studies to identify two-stressor interactions (Tekin et al., 2020). For simplicity, we define "stressors" as factors that affect population growth or fitness. While most of these "stressors" decrease population growth or fitness, a few stressors in the studies examined here actually increase population growth and/or fitness. We aim to obtain a more detailed, accurate, and complete understanding of higher-order ecological stressor interactions. Specifically, we use this framework to reanalyze the data from previously published papers (that used traditional methods i.e., ANOVA, General Linear Model, or log-logistic) that measure three-stressor interactions and all the lower-order interactions: all three pairwise combinations of stressors and all three single-stressors. We ask three questions: (1) How well does this new framework match previously published interaction results? (2) How often do emergent properties appear in higher-order ecological interactions? (3) Can we find patterns of emergent properties—that is, for example, do they primarily occur in synergistic interactions or antagonistic interactions? Based on prior findings in drug-bacteria systems (Tekin et al., 2018a), we hypothesized that (1) The new framework will yield different results than previously published interaction results; (2) We will find a substantial number of emergent properties from higher-order interactions; and (3) We will find more emergent antagonistic interactions than emergent synergistic interactions.

2. Methods

2.1. Study selection and criteria

We conducted a literature search using the *Web of Science* database to select the studies included in our analysis. We searched one hundred years of published literature, from January 1920–November 2020. As multi-stressor papers increased in prevalence over the last few years, we acknowledge that there have been more papers published since the completion of the search. Nonetheless, this search has collected a substantial number of studies for us to evaluate broad trends in data and demonstrate how the RBI can be applied to three stressor combinations. We used the following key terms included in the papers' keywords, title, and/or abstract: "multiple stressors," "multiple antagonism," "multiple synergy," "multiple disturbance," "multifactor," "additions," "indirect interactions," and "stressors" (Fig. A.1). Then, we further filtered the search results by selecting the following specific topic categories to reflect our interest in ecological studies: agriculture dairy animal science, biodiversity conservation, biology, biotechnology applied microbiology, ecology, environmental sciences, evolutionary biology, genetics heredity, marine freshwater biology, microbiology, and zoology.

We removed repeated records in the search so that there was only a single record per paper present. We only selected papers that measured growth, mortality, and/or survival at the population level for a specific species. We restricted our analysis to one level of biological organization so that the results are more generalizable to the level itself and not confounded by potential differences across scales (e.g., Simmons et al., 2021). Additionally, the RBI has only been used on fitness metrics using established equations based on relative fitness. While the RBI might be theoretically applicable to other scales of biological organization, for the sake of testing and demonstrating the RBI on ecological systems, we restricted our analysis based on previous studies. Next, we examined the remaining papers to determine the presence of the following criteria: the study had (1) three individual stressors and a full multi-factorial design was implemented, (2) quantitative response variables, and (3) explicit control treatments. From the remaining papers, we extracted growth at the population level (e.g., growth rate), mortality, and survival data from figures and tables from each of the qualifying studies. We used these variables as proxies for population-level growth. We included the following: stressor type, stressor units, responses for individual stressors, responses for combinations of stressors, responses for control variables, sample size, species of the organism tested, species' habitat at the life stage the population was studied (i.e., estuary, freshwater, marine, or terrestrial), and the interaction type between stressors determined by the original authors (i.e., additive, synergistic, or antagonistic). If a study did not specify if there was an interaction among the three stressors, we determined whether the authors specifically sought to investigate an interaction. Additionally, if a study reported that there was no interaction among the stressors—but the authors explicitly sought to investigate an interaction—we classified the interaction as additive because additivity is the null hypothesis when testing for interactions. Most of the quantitative responses from each study reflected mean values generated from raw values by the authors, often summarized from tables or figures provided in the studies. Other quantitative responses were directly obtained from raw data. We recorded the latest time point as the response value if mean or raw data were presented as a time series. Importantly, these factors could enhance or inhibit growth.

Finally, we filtered out combinations that would not work with the RBI framework. Specifically, RBI only works with uniform factors (e.g., all inhibitors or all enhancers of growth). Therefore, combinations that affected populations in opposite directions (e.g., a stressor decreasing population size and one increasing population size) were removed. Combinations that had a positive control value of zero could not be analyzed and were removed from the dataset. Combinations that had a lethal single stressor or a single stressor with no effect were assessed separately. In instances where one or more of the single stressors was lethal, we could only accurately identify the presence of a net suppressive interaction, and not an emergent effect. In this case, distinguishing additive, synergistic, or antagonistic interactions is not possible since a population cannot exhibit growth or survival less than zero. However, if the population demonstrates growth or survival in the presence of the three stressors combined, we could determine a suppressive interaction when at least one of the individual stressors is lethal. Additionally, there were cases where a single stressor had no effect. This can be problematic because it is unclear if the single stressor in combination with additional stressors has any effect or if we only see the effects of the additional stressors. Since we can only identify synergistic combinations under these circumstances, those cases were not included in our analysis. This step filtered our data from 396 unique stressor combinations to 142 unique stressor combinations. Many of the papers compared multiple combinations of three stressors at different quantities or scales. In total, the 142 stressor combinations came from 38 papers that met the requirements needed for our study (Table A.1).

2.2. Data analysis

The RBI framework has previously been used to examine drug interactions and pairwise stressor interactions by relying on Bliss Independence as the additive model to determine if there is an interaction between stressors on a population (Tekin et al., 2016; Beppler et al., 2016; Tekin et al., 2018a, 2020). We applied this framework to ecological studies exploring the impact of three stressors in each environment. Within this framework, there are a total of seven possible measurements one can take among the three stressors (stressor A, stressor B, and stressor C) acting simultaneously. These are: (1) the effects of A alone, (2) the effects of B alone, (3) the effects of C alone, (4) the pairwise effects of A and B by themselves, (5) the pairwise effects of A and C by themselves, (6) the pairwise effects of B and C by themselves, and (7) the effects of all three stressors A, B, and C together. The net interaction—termed deviation from additivity (*DA*) (Eq. (1))—occurs when we remove the effects of the individual stressors from consideration. Removing the result of the pairwise interactions produces the emergent effects (*E3*) (Eq. (2)). Further, we can rewrite Eq. (2) to only reflect relative fitness effects (Eq. (3)).

$$DA = w_{ABC} - w_A w_B w_C \tag{1}$$

$$E3 = w_{ABC} - DA_{AB} w_C - DA_{AC} w_B - DA_{BC} w_A - w_A w_B w_C \tag{2}$$

$$E3 = w_{ABC} - w_{AB} w_C - w_{AC} w_B - w_{BC} w_A + 2 w_A w_B w_C \tag{3}$$

Here, w refers to fitness relative to the control, non-stressed treatment. A, B, and C referring to each of the three stressors. Subscripts refer to conditions when those stressors are individually (e.g., w_A) or concurrently (e.g., w_{AB}) present. Upon calculating these initial interactions, rescaling methods and cutoff values used by Tekin et al. (2018b) were used to further investigate and identify interactions. Rescaling the interaction values creates a trimodal distribution allowing for easier identification of net and emergent interaction types (see Text A.2). The cutoff values at -0.5 and 0.5 allow for clear identification of interaction types (see Fig. 2 of Tekin et al., 2016). After rescaling (see Text A.2), both net and emergent interaction values below -0.5 were synergistic, values between -0.5 and 0.5 were additive, and values above 0.5 were considered antagonistic (where values above 1.3 were considered suppressive). For more information about rescaling and details on how to determine interactions for combinations where all stressors increase growth, see Text A.2 and A.3. We also include more information about RBI in Box 1.

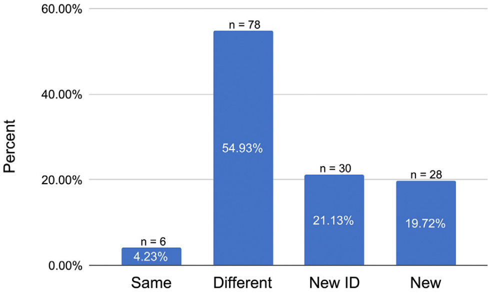

Fig. 2. Total three-stressor interactions identified by Rescaled Bliss Independence (RBI) contrasted to previously published results. Over 40 % of the total three-stressor interactions examined were untested and are therefore "new" interactions (19.72 %) or were tested in the original study, but the interaction type was not specified or classified by the original study and are therefore "new ID" interactions (21.13 %) that are now newly classified using RBI. Only 4.23 % of interactions analyzed with RBI remained the "same" as previously published results; 54.93 % resulted in a "different" interaction than previously reported.

The framework described above requires that all single stressors have a non-lethal effect on the populations' relative fitness, i.e., the whole population does not die in the single stressor treatment ($0 < w \neq 1$). This is because if a single stressor is completely lethal to the entire population ($w = 0$) or if a single stressor has no effect ($w = 1$) we would not be able to identify all interaction types. For example, if the use of one stressor results in complete lethality one cannot determine if a combination with that stressor interacts synergistically or additively if the combination also results in complete lethality. Similarly, if a single stressor appears to have no effect ($w = 1$) there is no way to distinguish if that stressor interacts at all with the system and is relevant or if the other stressor in the combination acts additively. This framework also requires relative fitness to be calculated with reference to a positive control (the growth of the population under no stressor present).

While estimating uncertainty and within-treatment variation would be useful, there are challenges for this in the context of this meta-analysis. First, many papers do not provide the data needed to make this possible. Arguably, we could use bootstrapping to estimate probabilistic bounds on how measured proportions within habitat classes could change if we were to sample different studies, which is what we had done with a previous paper (Tekin et al., 2020). However, this paper is using substantially fewer data due to the relative novelty of 3+ factorial experiments. Second, many papers do not provide the data needed to estimate error within individual experiments, and they lack the replicate number needed to obtain reliable error estimates (Fig. A.2)—which is often challenging for the scale of full-factorial experiments. Thus, this analysis provides a coarse overview of differences between our methods and those originally used, though there may be some error in the proportions of interaction types themselves. We hope researchers can increasingly conduct experiments with substantial replicate numbers in order to calculate within-treatment variance.

3. Results

For our analysis, we collected data from multi-stressor ecological studies published over the last 100 years, with most of these studies being published in the past 10 years as interest has increased and methods have improved (Fig. A.3). We subsequently applied the RBI framework to reanalyze three-stressor interaction data derived from those studies. These data included populations from unicellular and multicellular species across habitats, and from both biological, chemical, and physical stressors (summarized in Table 1). Our findings were then compared to those from the original studies. We found new net interactions that were previously unidentified by the original authors and net interactions that we reclassified based on our methods (Fig. 2). Of the 142 interactions, 19.7 % ($n = 28$) were interactions that were previously untested (e.g., experiments were conducted but no statistical analysis on the interactions themselves were reported) but classified as an interaction using RBI. We classified 21.1 % ($n = 30$) of previously tested but unspecified interactions (e.g., an interaction was found using statistical analyses by previous authors though the type of interaction was not explicitly stated). Only 4.2 % ($n = 6$) of the total interactions reanalyzed by RBI were re-classified with the same interaction type previously reported in the original studies while 54.9 % ($n = 78$) were different from what was previously reported. Collectively, 95.8 % of interactions were newly classified or reclassified by RBI.

Of the combinations that resulted in the same interaction type when applying both the original method described in the published studies and the RBI, 83 % ($n = 5$) were additive, 17 % ($n = 1$) were synergistic, and none were antagonistic (Table 2). Among the interactions reclassified by the RBI, 67 % ($n = 51$) were previously published additive interactions, all of which

Box 1

Determining interactions of higher-order combinations using Rescaled Bliss Independence (RBI) framework.

The RBI framework is based on Bliss independence (Bliss, 1939) and is used to quantify synergistic, additive, and antagonistic interactions. Bliss independence assumes that the relative effect of each stressor at a set concentration are independent of each other and is therefore used to define additivity. Positive or negative deviations from this additivity are then considered to be antagonistic or synergistic, respectively. For example, if two stressors, stressor A and stressor B, each inhibit growth by 50 % alone the combination of stressors AB would result in the expected inhibition of growth to be 75 % (0.75 = 1–0.5 × 0.5). If the expected inhibition of growth was observed when stressors A and B were used in combination, the interaction would be considered additive. There are other approaches that can be used to measure the interactions of a combination, such as Loewe Additivity (Loewe, 1953) that, unlike RBI, assumes that a stressor cannot interact with itself. However, the RBI framework was chosen for its unique ability to determine an emergent interaction (described below) that quantifies how much of the interaction is emerging from having all three stressors in combination, rather than from a strong interaction from a 2-stressor combination (Beppler et al., 2016; Tekin et al., 2017).

In a multi-stressor combination, several factors can contribute to the overall effect of the combination. The first factors to consider are the effects of each stressor alone. The next set of factors are the effects of each of the smaller sub-sets of interactions that interact additively with the other stressors (or combination of stressors) in the mix. For example, when examining three-stressor interactions, two stressors may have a strong interaction with each other that does not involve the third stressor yet the third stressor still contributes its own individual effects. The final factor is the highest order emergent effect, the effect of the interaction between all stressors present in the combination. All of these factors are used to describe the interactions of a three-stressor combination. Two main types of interactions can be described: (1) the overall effect, which is also termed the net effect or deviation from additivity (DA), and (2) the emergent effect that only occurs when all three stressors are present. Both of these described are in detail below.

The net deviation from additivity, DA, is determined by only removing the fitness effects contributed by each stressor alone from the overall fitness effect assuming Bliss independence. Once the net DA is calculated, one can subtract the additive contributions of each stressor and also the effects of all lower-order interactions, leaving the emergent effect. To find the net deviation from additivity for three stressor combinations, we remove the additive effects of each stressor. This framework has been used to examine 2-, 3-, 4-, and 5-stressor combinations but can also be expanded to *N* number of stressors. Below is how DA is determined in terms of relative fitness $\left(w_A = \frac{\text{fitness when exposed to stressor A}}{\text{fitness in control/unstressed setting}}\right)$ for a 3-stressor combination.

$$DA_{ABC} = w_{ABC} - w_A w_B w_C$$

After the initial interaction value is determined, a rescaling process is used to better distinguish between interaction types (Tekin et al., 2017). This step is crucial and analogous to rescaling fitness from absolute to relative fitness. For rescaling, when the DA is synergistic, one rescales to the lethal case. This is because when measuring growth, it is not possible to be deader than dead. If the interaction was not synergistic then it was normalized to the minimum fitness of an individual stressor within the deviation from additivity formulas. Below is an example of rescaling the DA with a 3-stressor combination.

$$DA_{rescaled} = \frac{DA_{ABC}}{|\min(w_A, w_B, w_C) - w_A w_B w_C|}$$

Emergent interactions are only present in higher-order combinations. For example, when considering all possible stressor effects that can occur within a single 3-stressor combination, there are a total of seven effects: three individual effects and four possible interactions (Fig. 1A). First, all three individual stressors have their effect. These effects are accounted for when we are determining the deviation from additivity. Next, the three pairwise interactions between two stressors can interact with the remaining third individual stressor. And finally, there is the emergent effect, which is the interaction that is strictly because all three stressors are in combination and does not occur in any of the single- or pairwise-stressor effects. Similar to the DA calculations, the emergent calculations (*E3*) remove the effects of the single stressors but then also removes the effects of the pairwise interaction and only leaves the effects that are uniquely due to the 3-stressor combination.

$$E3 = \overbrace{DA_{ABC}}^{\substack{\text{removal of}\\ \text{single stressor}\\ \text{effects}}} \quad \underbrace{- w_A DA_{BC} - w_B DA_{AC} - w_C DA_{B,C}}_{\text{removal of pairwise interaction effects}}$$

This equation can also be written solely in terms of fitness effects:

$$E3 = w_{ABC} - w_{AB} w_C - w_{AC} w_B - w_{BC} w_A + 2 w_A w_B w_C$$

The emergent interactions are then rescaled similarly to the DA values (Tekin et al., 2018b). For more information about the rescaling process please refer to Text A.2. For more information on the modifications to the RBI framework and rescaling to account for stressors that increased relative fitness rather than decrease relative fitness, please refer to Text A.3.

were reassigned as antagonistic (Fig. 3). The remaining interactions reclassified by RBI were synergistic and reclassified as antagonism (32 %, $n = 25$) or additive (0.013 %, $n = 1$). We found that 91 % ($n = 51$) of additive and 96 % ($n = 25$) of synergistic net interactions were reclassified as antagonistic net interactions using RBI (Table 2). No previously identified interaction type ($n = 77$) was newly re-classified as synergy, but 4 % ($n = 1$) of interactions reported as synergy were confirmed using RBI (Table 2, Fig. 3).

We also examined the frequency of interaction types among net and emergent three-stressor combinations (Fig. 4). Of the net interactions identified, we found that antagonism was the most prevalent interaction type at 82 % ($n = 117$). Of the antagonistic interactions, 44 % ($n = 52$) were

Table 1
Description of organisms and stressors used in the included experiments. Counts per category are presented and represent unique stressor combinations in the final filtered dataset.

Habitat class			
Estuary	Freshwater	Marine	Terrestrial
4	60	73	5
Species class			
Unicellular		Multicellular	
13		129	
Stressor class			
Chemical	Biological	Thermal	Other physical
215	59	91	61

suppressive. The remaining net properties were composed of 9 % ($n = 13$) synergistic and 8 % ($n = 12$) additive interactions (Fig. 4A). Of the emergent interactions, we found that synergy and additivity were the leading interaction types across emergent properties–accounting for 55 % ($n = 78$) and 33 % ($n = 47$), respectively (Fig. 4A). Antagonism accounted for 12 % ($n = 17$) of emergent interactions and of the antagonistic interactions, of which 47 % ($n = 8$) were suppressive (Fig. 4A).

When comparing the frequency of interaction types among the net and emergent properties, we found that there was almost four times as many instances of additivity in emergent interactions (emergent: $n = 47$, net: $n = 12$) (Fig. 4A). There were also substantially fewer instances of emergent antagonistic interactions ($n = 17$), including fewer suppressive interactions ($n = 8$) among emergent when compared to net properties ($n = 117$ total antagonism, including $n = 52$ suppressive interactions). We also observed more synergistic emergent interactions (emergent: $n = 78$) than synergistic net interactions (net: $n = 13$). Across all combinations, 74 % ($n = 105$) were found to have instances of "hidden suppression" where a pairwise combination is suppressed by the presence of a third stressor (e.g., better fitness with three stressors than with two stressors for negative stressor combinations). These interactions are only present in 3+ stressor interaction combinations. We then examined if there was a correlation between the net and emergent interactions. We did not find a significant correlation after performing a Spearman's correlation ($p = 0.98$) (Fig. 4B). The comparison of the interactions' distributions can be seen in Fig. 5.

4. Discussion

The implications of finding higher-order interactions extend beyond basic science. There is growing awareness that stressor interactions are crucial for population management and response predictions across systems. Pairwise interactions have received plenty of attention and have been the subject of many studies. In contrast, higher-order and emergent interactions in 3+ stressor systems—which almost certainly present a more accurate representation of what natural populations face and will continue to face—remain less understood. Properly identifying these interactions is critical for managing ecological stressors (Brown et al., 2013; Piggott et al., 2015c). Using Rescaled Bliss Independence, rather than traditional ANOVA or GLM methods, we were able to identify these emergent interactions—disentangling which and how individual and pairwise stressor effects modulate each other to produce the combined observed interaction. The finding that most emergent interactions are synergistic or additive rather than antagonistic, while the majority of combinations also exhibit hidden suppression, suggests that we need to identify *which* stressors are involved in a given system and what the impact may be if a given stressor is removed or decreased.

Table 2
Comparison counts for each net interaction type originally reported in previously published results and how they were re-classified using Rescaled Bliss Independence (RBI). "Inconclusive" interactions under "Previously Published Results" correspond to cases for which no explicit interaction type is reported or investigated.

Net interactions		Rescaled Bliss Independence (RBI)		
		Synergy	Additive	Antagonism
Previously Published Results	Synergy	1	1	25
	Additive	0	5	51
	Antagonism	0	0	1
	Inconclusive	12	6	40

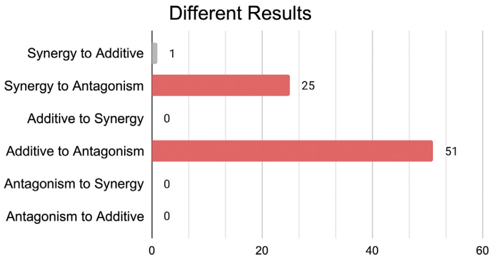

Fig. 3. Traditional methods have difficulty identifying antagonistic three-stressor interactions. Each bar shows the number of combinations that demonstrated a change from the previously published interaction type to the type of interaction with RBI.

4.1. Differences in interaction classification using RBI

In this study, we surveyed ecological literature published between 1920 and 2020 that examined the effect of three stressors simultaneously on population mortality, survival, or growth at the population-level of a given species. After re-analyzing data from previously published results using a newly introduced framework, the RBI, we identified 142 three-way interactions. We hypothesized that RBI would lead to differences in classification in comparison to the original studies. We found that only 4.2 % of the results generated by RBI matched those in the original studies, meaning that nearly 96 % of interactions were classified as new (either unspecified or not investigated by the previous authors) or different interaction types (Fig. 2). RBI has several advantages over commonly-used ANOVA methods. ANOVA relies on restrictive assumptions that often are not met by most studies (Tekin et al., 2020). Furthermore, RBI can rescale interaction terms to relative baselines, which means interactions can be placed into context much like relative fitness, which typically yields much more useful information than absolute fitness. In addition, and crucially, RBI can identify emergent properties. RBI can more accurately and reliably identify interactions, raising concern for the strong mismatch between our results and those originally published. While we were unable to calculate error bounds given the nature of the data used in this meta-analysis, the mismatched pattern we found is still striking even if there may be a degree of error therein driven by within-treatment variation.

Our results show that methods used in the original studies may have difficulty identifying antagonistic interactions (Fig. 3). When comparing our findings using the RBI framework to those of the original findings, we found that interactions were more often reclassified as antagonisms than synergy, and antagonisms made up most interaction types (82 %). Furthermore, there were no instances of both RBI and original methods classifying combinations as antagonistic. In one example, an interaction was classified as synergistic using restricted maximum likelihood ANOVA methods when assessing the combined effects of UV-radiation, water temperature, and salinity stress on mollusk embryos (Przeslawski et al., 2005). However, during our reanalysis, we reclassified the interaction as antagonistic. Similar results were observed when using RBI to reanalyze work on pesticide combinations and food limitation in *Daphnia magna* (Shahid et al., 2019), and a combination of abiotic and biotic stressors in a seagrass (*Zostera noltei*)

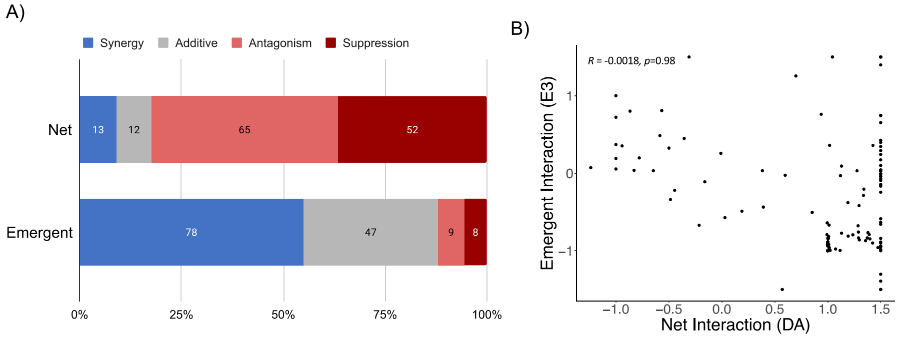

Fig. 4. The composition of the net and emergent three-stressor interactions using the RBI method. In each panel, gray represents additivity, blue represents synergism, and red represents antagonism. A darkening red illustrates an intensifying antagonism (e.g., antagonism ➔ suppression). Panel A) demonstrates the composition of the net and emergent interactions, respectively. Panel B) shows no significant correlation between the net and emergent interaction values (Spearman correlation, $\rho = -0.0018$, $p = 0.98$). For reference, net interactions represent the deviation from additivity (DA), which describes the overall effect of all interactions occurring within a combination. Emergent interactions (E3) are the effects uniquely due to all three stressors being in combination. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Vieira et al., 2020). Traditional ANOVA and log-logistic methods initially classified these combinations as synergy, but using RBI they were reclassified as antagonism.

In contrast to our findings, some prior studies observed more synergistic net interactions in comparison to antagonistic interactions when examining three-stressor interactions, even if antagonism was more common in two-stressor combinations (Crain et al., 2008; Maher et al., 2019). Our study examined nearly three times as many interactions as Crain et al. (2008) ($n = 142$ and $n = 48$ respectively). Maher and colleagues (Maher et al., 2019) found that synergies dominate three-stressor interactions in the coral microbiome using GLM and LMM models rather than RBI, which could explain the finding of synergistic interactions. This study also focused on a different biological scale—the microbiome *community* rather than a unique population's fitness.

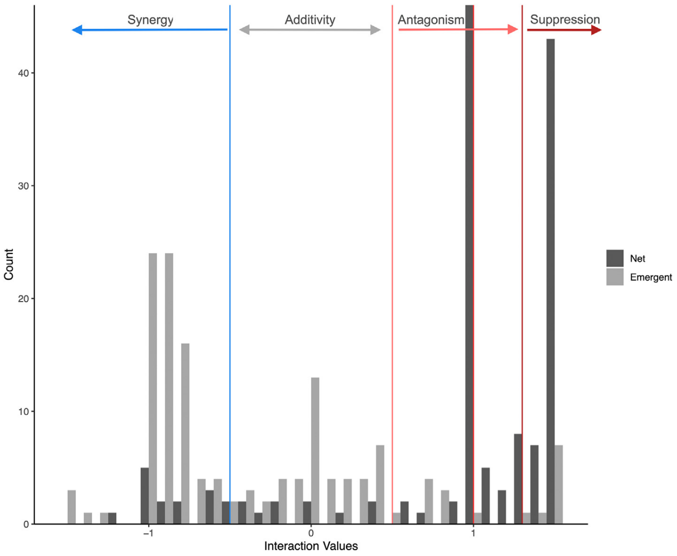

Fig. 5. The distribution of interaction values of both net and emergent three-stressor interactions. The distribution of net (DA) and emergent (E3) values. Cut-off values for each interaction type are as follows: synergy is less than −0.5, additivity is between −0.5 and 0.5, antagonism is above 0.5, and suppression is above 1.3. Rescaled values are distributed across multimodal peaks. Thus, rescaling aids in the identification of interaction types through the use of these peaks. More details on the justification for these cut-offs can be found in the Methods. For reference, net interactions represent deviation from additivity (DA) that is calculated by removing the expected combined single-stressor effects from the overall effects. Emergent interactions (E3) are deviations from the expectations of the single-stressor effects and the combined pairwise effects. Emergent interactions are solely due to all three stressors being in combination together.

4.2. Prevalence of emergent interactions

Higher-order interactions involving three or more stressors and emergent properties that arise from higher-order combinations are still poorly understood (Tekin et al., 2018b, 2017). We asked how often emergent properties appear in higher-order ecological stressor interactions. We hypothesized that there would be a substantial number of emergent properties from higher-order interactions. We found that hidden suppression, a type of emergent effect, occurs 74 % of the time, suggesting that emergent properties are common among higher-order ecological interactions. Moreover, we demonstrated that the RBI framework can identify higher-order emergent interactions that are overlooked or not explicitly explored when using traditional methods. For example, when using a general linearized model (GLM) to investigate the effect of pH, temperature, and oxygen availability on moon jellyfish, additive interactions were reported (Algueró-Muñiz et al., 2016). However, using RBI, we did not confirm the original authors' conclusion and instead found antagonistic net interactions, and we also identified synergistic emergent interactions.

4.3. Pattern of emergent interactions

Our data also demonstrate that emergent properties persist across all interaction types—synergy, additivity, and antagonism. Our hypothesis —that emergent antagonistic interactions would be more prevalent than synergistic ones—was not supported. We demonstrated that synergy and additivity are the leading effects across emergent interactions—accounting for 55 % and 33 % of the total interaction types identified in our study, respectively (Fig. 4A). Among the 1.2 % of emergent antagonistic interactions we identified by RBI, 47 % of them were characterized as suppressive (where one stressor reverses another stressor's effects) (Fig. 4). From a very different field, that of microbiology, antibiotic-combination studies reveal that higher-order emergent interactions were most often antagonistic than synergistic (Beppler et al., 2016; Tekin et al., 2018a). By identifying emergent interaction types, we can determine the combined effects of specific factors on populations in complex habitats that are subjected to multiple stressors at any given time.

4.4. Comparing three-stressor to two-stressor interactions

Although our study addresses three-stressor interactions, our results are comparable to the previously mentioned two-stressor interaction studies in that additivity and antagonism were also found to be the most prevalent interaction types in a reanalysis of two-way interactions using RBI from ecological studies within the past 25 years (Tekin et al., 2020). In the two-stressor studies, 41 % ($n = 286$ of 840) of interactions were identified as additivity and 40 % ($n = 278$ of 840) as antagonism (Tekin et al., 2020). Interestingly, those results correspond well with previous reports that were not using RBI (Darling and Côté, 2008; Côté et al., 2016; Jackson et al., 2016). This provides support for the idea that synergy may be overemphasized in the literature and that antagonism may occur more often than previously thought (Darling and Côté, 2008), at least for net interactions. Synergy has been overemphasized in other biological disciplines, including research on antibiotic resistance (Singh and Yeh, 2017).

Historically, whether three-stressor interactions exist and, if they do, to what extent they affect natural populations and ecosystems has been a subject of debate since the 1960s (e.g., Vandermeer, 1969; Pomerantz, 1981; Abrams, 1983; Billick and Case, 1994). At the population level, one major limitation in understanding these interactions is determining an applicable and generalizable model. By applying RBI, we were able to properly assess three-stressor interactions and determine that not only do emergent properties exist across biological systems but that they are also relatively common. Thus, a population's response cannot necessarily be predicted by assuming additivity across stressors.

4.5. Future work and implications of higher-order interactions

Further work still needs to be done to scale from population dynamics to community and ecosystem functioning across time (Côté et al., 2016; Brooks and Crowe, 2019; Jackson et al., 2021). Beyond the scale of single-species populations, interactions between species and resources (Coyte et al., 2015; Butler and O'Dwyer, 2020) and higher-order interactions between species (Kelsic et al., 2015; Grilli et al., 2017) have been shown to be important in modeling stability in ecological communities. Additionally, evolution in response to multiple stressor interactions and the fitness landscapes they form could influence adaptive dynamics (Ogbunugafor et al., 2016), population outcomes (Venturelli et al., 2015), and therefore broader eco-evolutionary dynamics. Indeed, understanding selection can help determine trajectories that populations may traverse while adapting to given stressors (Toprak et al., 2012).

Crucially, identifying emergent properties can reveal hidden suppressive interactions (i.e., suppressive interactions that only occur among higher-order interactions). These hidden suppressive interactions could be particularly important for the ecological management of at-risk populations. In a three-stressor combination, the addition of a third stressor may suppress a two-stressor interaction. For example, when examining the combined effects of acidification, drought, and warming, the interaction between drought and acidification is suppressed by the effect of higher temperature on plankton producer biomass resulting in more biomass with all three stressors than with two (Christensen et al., 2006). Such an observation may be important for incorporating necessary mitigation strategies. In this example, alleviating acidification would result in lower biomass because it would undo the suppression of the interaction between warming and drought. Instead, mitigating warming and drought would be a better strategy if the goal were to increase biomass. If the stressor interactions are not clearly understood or identified, the wrong stressor could be mitigated. These hidden suppressive interactions are common: the majority (74 %) of our re-analyzed stressor combinations revealed hidden suppression between a pairwise interaction and a third stressor. Hidden suppression is also prevalent in bacterial response to multiple drug combinations (Lozano-Huntelman et al., 2021).

Throughout the ecological literature, investigations of three-stressor interactions involving biotic and environmental stressors remain scarce. We completed a thorough literature search of thousands of relevant results and found that only 0.2 % of research articles from 1990 onwards examining three ecological stressors qualified for our meta-analysis. Comparatively, when exploring pairwise interactions across the ecological literature, Tekin et al. (2020) found that nearly 8 % of search results were applicable. Nevertheless, over the last decade, studies investigating higher-order interactions across disciplines have increased dramatically (Fig. A.3). Most of the studies that qualified for re-analysis here occurred within the past five years. Indeed, in the two years since this search, 3+ stressor studies have continued to be published, adding to the growth of this important and timely field (e.g., Juvigny-Khenafou et al., 2021a; Juvigny-Khenafou et al., 2021b; Macaulay et al., 2021a, 2021b, 2021c). The recent increase in higher-order interaction studies highlights how crucial it is that we extend our research beyond pairwise interactions to examine the effect of stressors more accurately in combination.

5. Conclusion

In conclusion, we show that this new RBI framework can be generalized from pairwise interactions to three or more stressors to examine how multiple stressors interact. RBI can distinguish between net and emergent interactions, providing greater insight into complex biological systems. From a basic science perspective, predicting higher-order interactions is essential to understanding how the combined effects of multiple stressors interact and impact diverse biological systems. From a conservation perspective, multiple stressor interactions can influence the population size of species of concern. To understand how stressor effects occur, we must be able to better characterize them to understand how stressors interact with each other to determine the net effect. For example, if a sensitive population is facing multiple stressors and one stressor is mitigated, that mitigation might lead to unforeseen circumstances due to emergent interactions. If that stressor was interacting antagonistically with the other stressors, the mitigation might lead to population decline. On the other hand, if that stressor was interacting synergistically, that mitigation might be beneficial for the population's conservation. As we show here, there are substantial numbers of higher-order interactions, and removing one stressor does not necessarily help the growth of a population of concern. Additionally, identifying emergent properties can reveal hidden suppressive interactions, where adding a third stressor can suppress a two-stressor interaction. Here we show that emergent hidden suppressive interactions are common and identifying these interactions could particularly be important for ecological management of at-risk populations. For example, in a population at risk due to the interaction between two stressors, having a third stressor that suppresses this interaction may help mitigate further loss. Thus, understanding these complex patterns in higher-order interactions can be crucial to choosing the correct stressors to mitigate.

CRediT authorship contribution statement

Eleanor S. Diamant: Visualization, Writing – original draft, Writing – review & editing. **Sada Boyd:** Writing – original draft, Writing – review & editing. **Natalie Ann Lozano-Huntelman:** Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Vivien Enriquez:** Data curation, Investigation, Writing – review & editing. **Alexis R. Kim:** Data curation, Investigation, Writing – review & editing. **Van M. Savage:** Conceptualization, Funding acquisition, Methodology, Writing – review & editing. **Pamela J. Yeh:** Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Data availability

Datasets are available in Dryad. DOI: doi:https://doi.org/10.5068/D1W10G

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Pre-print statement

This manuscript has been made available on a preprint server (Diamant et al., 2022).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.161163.

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Chapter Three: Supporting Information

Text A.1. Issues with using ANOVA (ANalysis Of VAriance) for classifying interaction types

The limitations and potential false inferences of applying ANOVA to test for and classify interactions are fourfold. **First**, variance in response measures within and across all treatments is determined by the number of experimental replicates, which are often limited in stressor-combination studies. This then leads to poor estimates of higher-order moments (e.g., kurtosis and skewness), potentially leading to inaccurate results. **Second**, though hidden replication (e.g., assuming variance is constant across treatments) is often used to justify applying ANOVA in these scenarios, hidden replication rests on the assumption that there is *no interaction* between variables (Welham et al., 2014). Thus, employing hidden replication when testing for interaction often invalidates the findings themselves. Further, non-linear pairwise interactions require carefully chosen data transformations when the assumptions of ANOVA are otherwise not violated (Pomerantz, 1981; Billick and Case, 1994; Gotelli et al., 1999). It is particularly important to transform the underlying additive model to a multiplicative model when stressors have large effects on populations (Segrè et al., 2005; Tekin et al., 2018a). **Third**, ANOVA assumes Gaussian or parametric distributions when comparing variances between treatments. Therefore, a large number of replicates per treatment would be necessary to accurately assess the variance of non-normal distributions and subsequently reconstruct ANOVA based on the non-parametric null model. **Fourth**, ANOVA methods do not allow for rescaling. Rescaling or normalization, relative to control baselines (e.g., population fitness with no stressors present), is often necessary to classify interaction types because different interaction types may result in similar unscaled values. Additionally, rescaling results in multimodal distribution of interaction values, aiding interaction classifications (Figure 5) (Segrè et al., 2005; Tekin et al., 2018b, 2020).

Text A.2. Mathematics of Rescaling Bliss Independence with Multiple Stressors that Inhibit Growth.

Once interaction values are calculated as described in the methods it may be hard to distinguish a cut-off value for each type of interaction. To do this rescaling is needed to transform a unimodal distribution of the raw interaction scores to become trimodal allowing for clearer distinctions of truly antagonistic and synergistic interactions. For this study, we follow protocols developed by Beppler *et al.* (2016) and Tekin *et al*. (2016) to rescale. For both net and emergent synergistic interactions, we rescale normalized to a lethal case because when measuring growth rates, relative fitness can't be below zero.

$$DA_{rescaled} = \frac{DA}{|\mathbf{0} - w_A w_B|} \qquad DA_{rescaled} = \frac{DA}{|\mathbf{0} - w_A w_B w_C|}$$

Where DA refers to deviation from additivity, as described in the main text, and *w* refers to the population-level relative fitness for each stressor or combination of stressors in the subscript (A, B, and/or C). When rescaling occurs for non-synergistic net interactions the interaction value is normalized to the minimum of the single stressor effects.

$$DA_{rescaled} = \frac{DA}{|\min(w_A, w_B) - w_A w_B|} \qquad DA_{rescaled} = \frac{DA}{|\min(w_A, w_B, w_C) - w_A w_B w_C|}$$

When rescaling occurs for non-synergistic emergent interactions, we chose to normalize the interaction value to the minimum of the pairwise interactions. Tekin *et al*. (2016) recommend this normalization option because it may be more biologically relevant than other options.

$$E3_{rescaled} = \frac{E3}{|\min(w_A DA_{BC}, w_C DA_{AC}, w_C DA_{AB}) - w_A DA_{BC} - w_B, DA_{AC} - w_C DA_{AB}|}$$

Text A.3. Mathematics of Rescaling Bliss Independence with Stressors That Increase Growth

For combinations that only had stressors that increased growth, we adapted the protocols developed by Beppler *et al.* (2016) and Tekin *et al*. (2016) to rescale. For the initial net (DA) and emergent (E3) interactions, the signs were reversed to keep synergistic interactions negative values and antagonistic interactions positive values, following the equations below.

$$DA = w_A w_B w_C - w_{ABC}$$

$$E3 = w_{AB} w_C + w_{AC} w_B + w_{BC} w_A - 2 w_A w_B w_C - w_{ABC}$$

When rescaling synergistic interactions, the interaction value is normalized to the maximum positive value because there is no upper limit to what a synergistic combination of promoters can be. Ideally this maximal value would be infinity, however this is not practical. To estimate this maximal value, we used twice the amount of the relative fitness highest-order combination.

$$DA_{rescaled} = \frac{DA}{|2w_{AB} - w_A w_B|} \qquad DA_{rescaled} = \frac{DA}{|2w_{ABC} - w_A w_B w_C|}$$

When rescaling occurs for non-synergistic net interactions, the interaction value is normalized to the maximum of the single stressor effects. This was done to keep with the same definition of rescaling to buffering, as described in Tekin *et al*. (2016): buffering normalizes to the most extreme of the single stressors.

$$DA_{rescaled} = \frac{DA}{|\max(w_A, w_B) - w_A w_B|} \qquad DA_{rescaled} = \frac{DA}{|\max(w_A, w_B, w_C) - w_A w_B w_C|}$$

When rescaling occurs for non-synergistic emergent interactions, we chose to normalize the interaction value to the maximum of the pairwise interactions. Tekin *et al*. (2016) recommend this normalization option because it may be more biologically relevant than other options. Again, we chose to use the maximum of the single and pairwise interactions, follow the definition of buffering which normalizes to the most extreme of the single stressors and lower-order combos interacting additively with the third stressor (Tekin *et al*., 2016). When all stressors inhibit growth the most extreme affect would result in the minimal amount of growth. Whereas, in combinations where combinations had stressors that all increased growth, the most extreme affect would result in the maximum amount of growth.

$$E3_{rescaled} = \frac{E3}{|\max(w_A DA_{BC}, w_C DA_{AC}, w_C DA_{AB}) - w_A DA_{BC} - w_B, DA_{AC} - w_C DA_{AB}|}$$

Table A.1. Research articles included in our study. The author(s), publication year, habitat type, species, unique stressors, response variable, net three-way interactions reported, interactions identified by RBI are provided, including the emergent and net three-way interactions, and total number of net interactions that are re-classified. Interactions are listed for each unique combination of stressors tested in each research article, sometimes including more than one combination per stressor. Data presented are after data filtering (n = 142 unique stressor combinations). Of note, original studies often also included two-way interactions, which we are not including nor re-analyzing here.

Habitat	Source	Species	Stressors	Responses (units reported)	Interaction reported (net)	Interaction by RBI (emergent)	Interaction by RBI (net)	Net interactions reclassified
Estuary	(Bazzano and Elmer, 2017)	*Spartina alterniflora*	silicon, *Fusarium palustre*, nitrogen	growth (g)	Interaction not specified: 2	Antagonism: 2	Antagonism: 2	Newly classified: 2
	(Gil et al., 2016)	*Porites rus*	nutrients, sediment, overfishing	survival (%)	Additive: 1	Synergy: 1	Antagonism: 1	Re-classified: 1
	(Gobler et al., 2018)	*Menidia berylina*	pCO_2, temperature, food limitation	growth (mm), survival (%)	Synergy: 1	Antagonism: 1	Synergy: 1	No change :1
Freshwater	(Buck et al., 2012)	*Rana cascadae*	carbaryl, *Batrachochytrium dendrobatidis, Pseudacris regilla*	growth (mg/d)	Additive: 1	Additive: 1	Antagonism: 1	Re-classified: 1
	(Davis et al., 2018)	*Agapetus fuscipes, Silo pallipes*	sediment level, phosphorus, nitrogen	growth (individuals/ mesocosm)	Interaction not specified: 6 Synergy: 6	Additive: 3, Antagonism: 4, Synergy: 5	Antagonism: 12	Newly classified: 6, re-classified: 6
	(De Coninck et al., 2013)	*Daphnia magna*	parasites, carbaryl, carbaryl pre-sensitivity	survival (%)	Interaction not specified: 1	Additive: 1	Synergy: 1	Newly classified: 1
	(Elbrecht et al., 2016)	*Ceratopogonidae*	sediment, flow, nutrients	growth (number of individuals)	Additive: 1	Additive: 1	Antagonism: 1	Re-classified: 1
	(Hasenbein et al., 2018)	*Hyalella azteca*	salinity, temperature, bifenthrin	survival (%)	Interaction not tested: 3	Additive: 1, Antagonism: 2	Antagonism: 1, Synergy: 2	Newly classified: 3
	(Hatch and Blaustein, 2000)	*Rana cascadae*	pH, nitrate, UVB	survival (%)	Additive: 4	Additive: 4	Additive: 2, Antagonism: 2	No change: 2, reclassified: 2
	(Hintz et al., 2019)	*Physella acuta, Helisoma trivolvis*	nutrients, predator presence, non-invasive snails	growth (g)	Additive: 1, Interaction not specified: 1	Additive: 1, Synergy: 1	Antagonism: 2	Newly classified: 1, re-classified: 1
	(Houde et al., 2019)	*Oncorhynchus tshawytscha*	salinity, temperature, hypoxia	survival (count)	Interaction not specified: 2	Additive: 2	Antagonism: 2	Newly classified: 2
	(Manzi et al., 2020)	*Daphnia crustacea*	temperature, low food quality, parasite infection	growth (per capita rate of increase per day)	Additive: 2	Additive: 1, Synergy: 1	Additive: 2	No change: 2

	(Op de Beeck et al., 2018)	*Ischnura elegans*	temperature, CPF, density	survival (proportion intact dead larvae per mesocosm)	Interaction not tested: 2	Synergy: 2	Antagonism: 2	Newly classified: 2
	(Piggott et al., 2015)	*Gomphonema minutum, Encyonema minutum, Fragilaria vaucheriae, Gomphoneis minuta* var. *cassiae*	temperature, sediment, nutrients	growth (cells per cm^2 x1000)	Additive: 11	Additive: 5, Synergy: 6	Antagonism: 11	Re-classified: 11
	(Reitsema et al., 2020)	*Berula erecta*	CO_2, nutrients, flow velocity	growth (average number)	Additive: 1	Synergy: 1	Antagonism: 1	Re-classified: 1
	(Relyea, 2006)	*Rana clamitans*	predator presence, high pH, high carbaryl	survival (%)	Additive: 1	Additive: 1	Antagonism: 1	Re-classified: 1
	(Shahid et al., 2019)	*Daphnia magna*	food limitation, Esfenvalerate, Prochloraz	survival (%)	Interaction not tested: 5, Synergy: 10,	Synergy: 15	Antagonism: 15	Newly classified: 5, re-classified: 10
	(Chen et al., 2004)	*Spartina maritima*	nutrient availability, inundation, soil type	survival (%)	Interaction not tested: 2	Synergy: 2	Antagonism: 2	Newly classified: 2
Marine	(Algueró-Muñiz et al., 2016)	*Aurelia aurita*	pH, temperature, oxygen availability	survival (%)	Additive: 10	Synergy: 10	Antagonism: 10	Re-classified: 10
	(Andrew et al., 2019)	*Phaeocystis antarctica*	temperature, light, iron	growth (µ)	Interaction not specified: 1	Additive: 1	Additive: 1	Newly classified: 1
	(Armitage and Fong, 2006)	*Cerithidea californica*	nutrient, predation, snail size	Survival (%)	Additive: 1	Additive: 1	Antagonism: 1	No change 1
	(Büscher et al., 2017)	*Lophelia pertusa*	elevated CO_2, temperature, low food availability	survival (% per day)	Antagonism: 1	Synergy: 1	Antagonism: 1	No change: 1
	(Dineshram et al., 2016)	*Crassostrea gigas*	temperature, reduced salinity, pH	survival (%)	Additive: 1	Additive: 1	Additive: 1	No change: 1
	(Gamain et al., 2018)	*Zostera noltei*	temperature, pesticide mixture, copper	growth rate (µ)	Interaction not specified: 1	Additive: 1	Synergy: 1	Newly classified: 1
	(Gobler et al., 2018)	*Menidia beryllina*	diet, pCO_2, temperature	survival (%)	Synergy: 1	Additive: 1	Antagonism: 1	Re-classified: 1
	(Gusha et al., 2019)	*Pseudodiaptomus hessei picophytoplankton*	temperature, nutrients, grazing	Biomass (chl-a mg L -1)	Interaction not specified: 7	Additive: 2, Antagonism: 2, Synergy: 3	Antagonism: 7	Newly classified: 7
	(Hoadley et al., 2016)	*Symbiodinium trenchii*	temperature, nutrients, pCO_s	growth (cm^2)	Additive: 1	Additive: 1	Antagonism: 1	Re-classified: 1
	(Johnson et al., 2018)	*Padina boryana*	nutrient, sediment, herbivory	biomass (%)	Additive: 2	Synergy: 2	Antagonism: 2	Re-classified: 2

	(Oliver et al., 2019)	*Crassostrea gigas*	Imidacloprid, handling, air exposure	survival (%)	Additive: 1	Synergy: 1	Antagonism: 1	Re-classified: 1
	(Przeslawski et al., 2005)	*Dolabrifera brazieri, Bembicium nanum, Siphonaria denticulata*	temperature, salinity, light	survival (proportion)	Additive: 16, Synergy: 8	Synergy: 24	Antagonism: 24	Re-classified: 24
	(Vasquez et al., 2015b)	*Limulus polyphemus*	temperature, salinity, oxygen	survival (%)	Interaction not tested: 8	Additive: 6, Antagonism: 2	Additive: 1, Antagonism: 4, Synergy: 3	Re-classified: 8
	(Vasquez et al., 2015a)	*Limulus polyphemus*	temperature, oxygen, H_2S	survival (%)	Interaction not tested: 1	Additive: 1	Additive: 1	Re-classified: 1
	(Vasquez et al., 2017)	*Limulus polyphemus*	temperature, salinity, oxygen	survival (%)	Interaction not specified: 12	Additive: 9, Antagonism: 3	Additive: 3, Antagonism: 5, Synergy: 4	Newly classified: 12
	(Vieira et al., 2020)	*Zostera noltei*	nutrients, sediment, density	growth (shoot density)	Synergy: 1	Synergy: 1	Additive: 1	Re-classified: 1
Terrestrial	(Janssens et al., 2017)	*Lestes viridis*	egg temperature, larval temperature, previous esfenvalerate concentration	survival (%)	Interaction not tested: 1	Additive: 1	Antagonism: 1	Newly classified: 1
	(McKinney and Cleland, 2014)	*Amsinckia tessellata*	exotic vs native origins, water availability, fine root addition	growth (g/pot)	Interaction not specified: 1	Antagonism: 1	Antagonism: 1	Newly classified: 1
	(Stevens and Gowing, 2014)	*Anthoxanthum odoratum*	clipping, *Plantago lanceolata*, *Prunella vulgaris*	growth (g)	Interaction not specified: 2	Antagonism: 1, Synergy: 1	Antagonism: 1, Synergy: 1	Newly classified: 2
	(Wilsey, 1996)	*Stipa occidentalis*	CO_2, clipping, urea treatment	growth (g/plot)	Additive: 1	Synergy: 1	Antagonism: 1	Re-classified: 1

PRISMA 2009 Flow Diagram

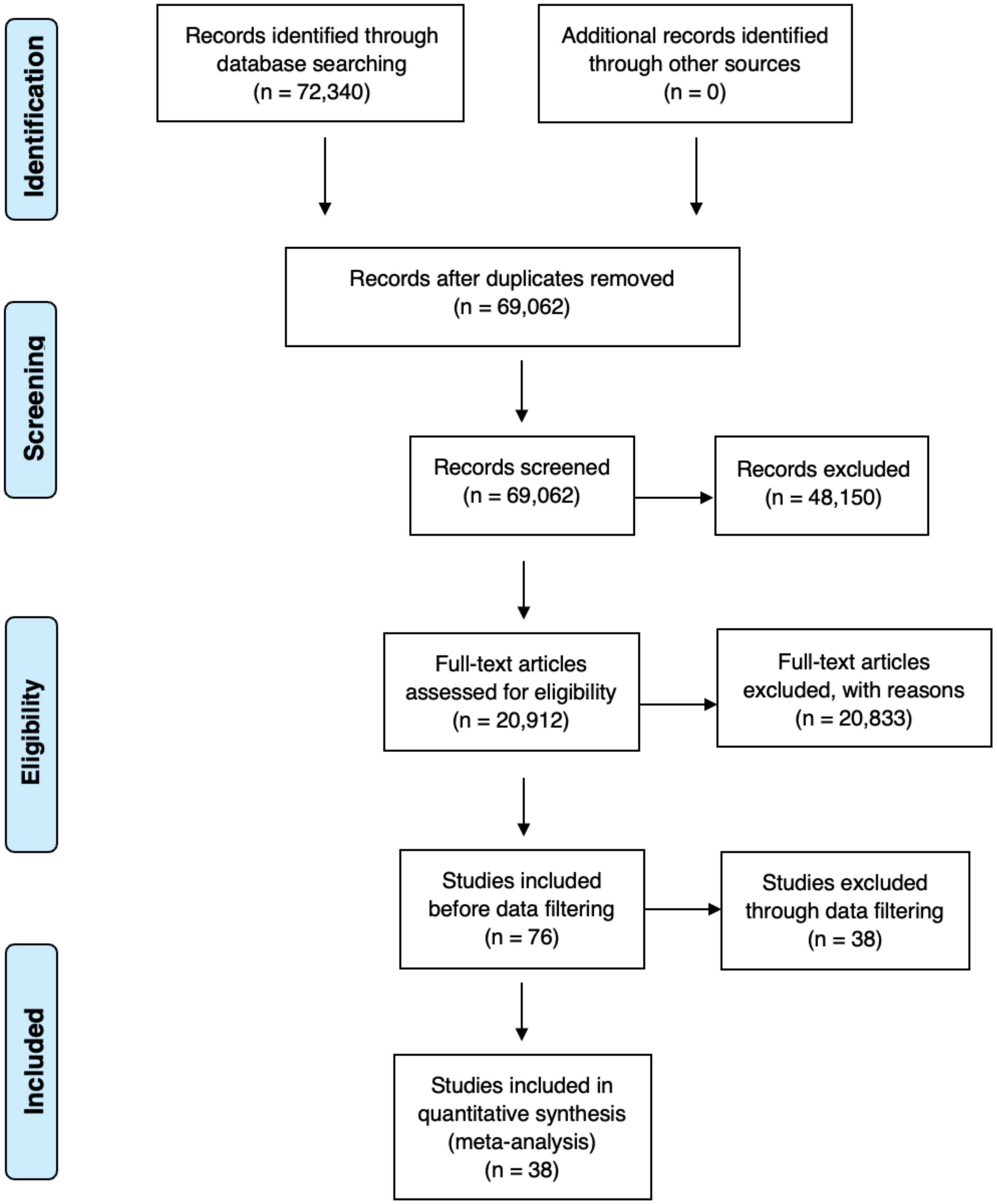

Figure A.1. PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) (Moher et al., 2009) Flow Diagram. Using the *Web of Science* database, 38 out of 20,912 studies (records) were identified and included in our meta-analysis, resulting in 142 unique interactions.

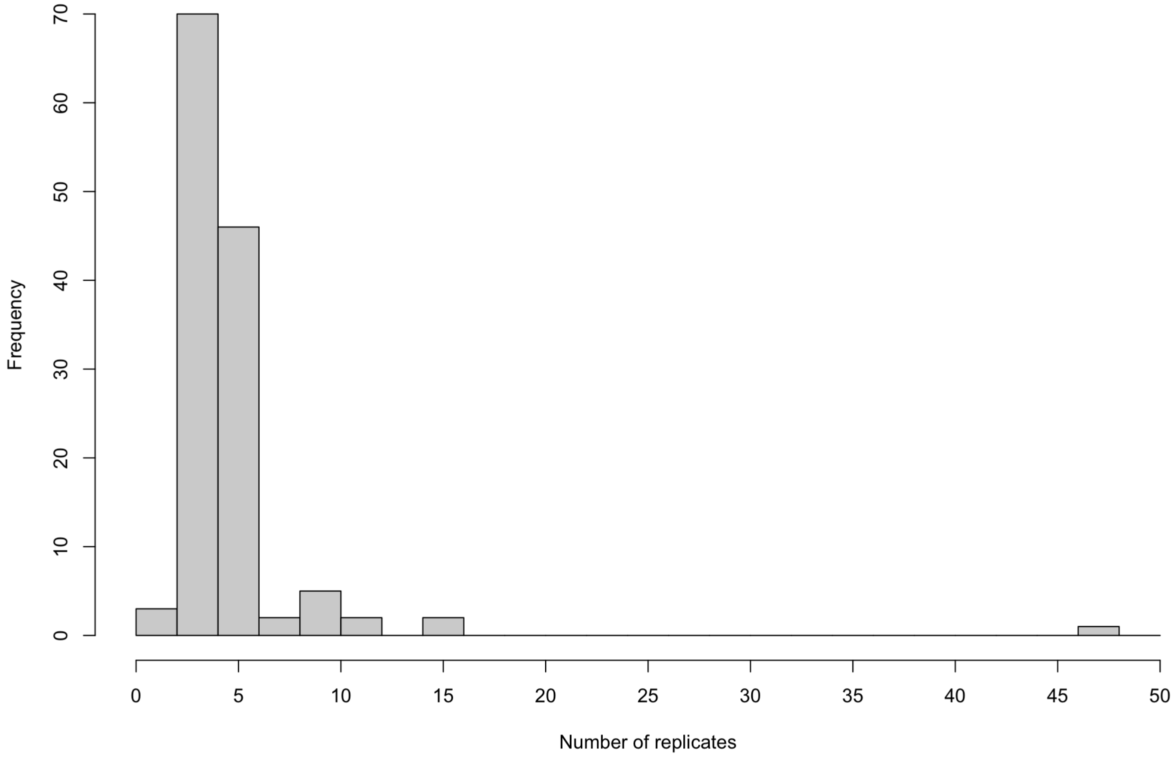

Figure A.2. The average number of replicates per treatment for each qualifying three-stressor combinations that examined growth, mortality, or survival at the population level between January 1920-November 2020. The majority of stressor combinations were tested with ≤ 5 replicates (*n* = 87). In the case that replicate numbers varied between treatment, the average replicate number is reported. A handful of studies (*n* = 11) tested survival with a bimodal distribution and thus each individual was considered a replicate. Most of these studies tested 50+ individuals and thus are excluded from this histogram to highlight the distribution of the most common experimental design.

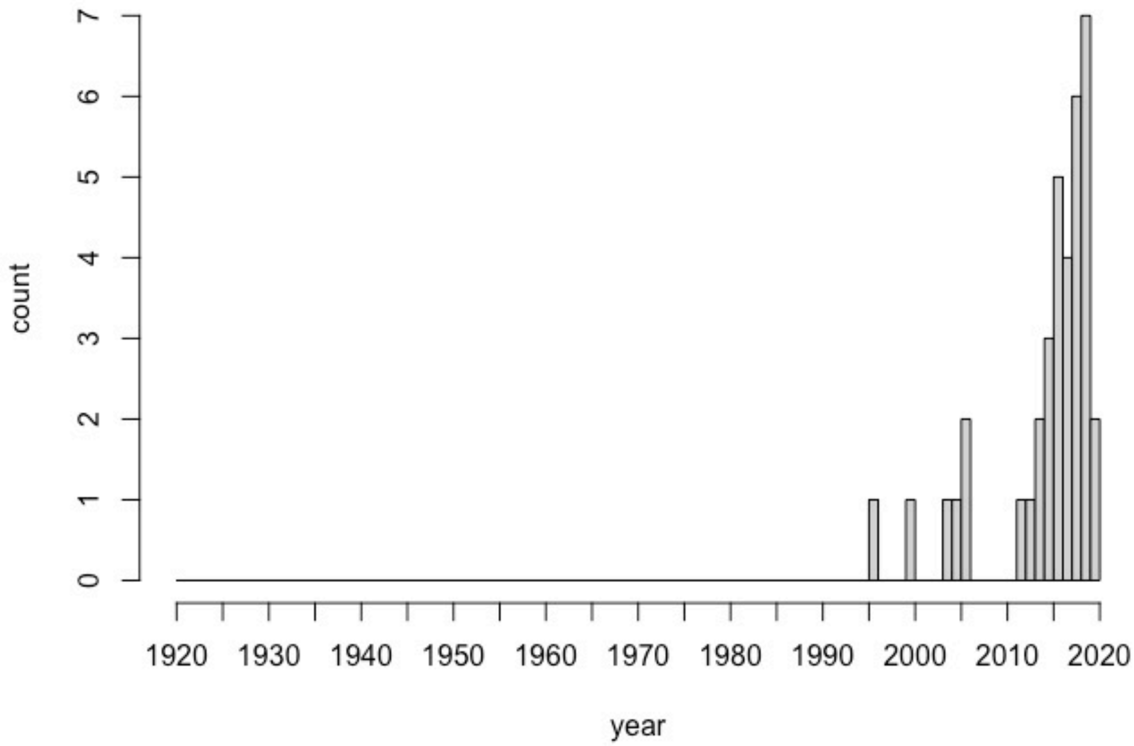

Figure A.3. The number of qualifying three-stressor studies that examined growth, mortality, or survival at the population level between January 1920-November 2020, by year. Across a 100-year timespan, we identified 38 unique papers that were conducted in a factorial design that fit our data quality requirements needed for RBI. Most qualifying studies were conducted from 2016 to 2020 (*n* = 24).

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Chapter Four: Complex patterns of convergence and non-convergence in multiple populations of an urban bird species[1]

Abstract

Urbanization presents a natural evolution experiment because selection pressures in cities can be strongly mismatched with those found in species' historic habitats. However, some species have managed to adapt and even thrive in these novel conditions. When a species persists across multiple cities, a fundamental question arises: do we see convergent evolution? By testing if and how convergent evolution happens across multiple urban populations, we can assess the repeatability of evolution and predictability of population response to anthropogenic change. Here, we examine variation within and across multiple populations of a songbird, the dark-eyed junco (*Junco hyemalis*). We measured juncos in three cities and three non-urban populations in Southern California to determine whether morphological variation relates to differences in environmental conditions across cities or vary between urban areas regardless of urban selective pressures. Bill shape diverged across urban populations, whereby Los Angeles and Santa Barbara juncos had shorter, deeper bills, but San Diego juncos did not. On the other hand, body size decreased with the built environment, regardless of the population. Southern Californian urban juncos exhibit both convergent and non-convergent morphological evolution. Studying multiple urban populations can help us determine the predictability of evolutionary response to novel environments.

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[1] This chapter is in preparation for submission with the following author order: Eleanor S. Diamant and Pamela J. Yeh

Introduction

Urbanization drastically changes a species' natural habitat, creating novel selection pressures when compared with a given species' evolutionary history and environmental tolerance (Bonier et al. 2007; Smith and Bernatchez 2008; Smith et al. 2008, 2014; Sol et al. 2014; Johnson and Munshi-South 2017; Rivkin et al. 2019). As cities expand (Seto et al. 2012; Simkin et al. 2022; UN 2019), a fundamental question is whether we are able to predict how animals evolve in urban habitats. One way to assess this is to evaluate the possibility of convergent evolution by comparing different cities. Determining population response to urbanization provides an arena to study adaptation-in-action (Diamond 1986; Palumbi 2001; Yeh and Price 2004; Hendry et al. 2008; Pergams and Lacy 2008; Marnocha et al. 2011; Donihue and Lambert 2015; Alberti et al. 2017a; Campbell-Staton et al. 2020), as urban populations are a model for testing basic eco-evolutionary questions (Diamond 1986; Hahs and Evans 2015; Wong and Candolin 2015; Alberti et al. 2017a). Most notably, we can assess if similar large-scale landscape shifts result in similar responses, or if stochasticity shapes divergent evolutionary outcomes despite inferred similar selective pressures.

While the field of urban evolution has developed substantially over the past couple of decades, its degree of predictability remains an outstanding question (Perrier et al. 2018; Rivkin et al. 2019; Miles et al. 2021). Yet, exploring the repeatability or predictability of evolutionary response provides an *in situ* natural experiment to test the role of contingency in evolution, a fundamental and open question in biology (Gould 1989; Blount et al. 2018). Most research has focused on single urban–non-urban pairs or urban-rural gradients (e.g., Johnson and Munshi-South 2017) to understand population response to broad urban characteristics, such as heat, altered food resources, species interactions, and anthropogenic disturbance (Shochat et al. 2006). The few studies on species across multiple cities have found mixed results. For example, parallel shifts in response to urban stressors have been found in urban *Anolis* lizards (Campbell-Staton et al. 2020) and in eastern gray squirrels (*Sciurus carolinsis*) (Gibbs et al. 2019), yet a combination of parallel and non-parallel responses were found in acorn ants (*Temnothorax curvispinosus*) (Diamond et al. 2018) and in great tits (*Parus major*) (Salmón et al. 2021; Caizergues et al. 2022). Responses to urbanization in European blackbirds (*Tardus merula*)—a common and successful urban colonist—morphologically vary across region and trait (Evans et al. 2009). These findings suggest that broad differences in urban and non-urban environments can shape evolution within urban environments. However, the repeatability of evolution in single urban–non-urban pairs may not be a given (Miller et al. 2018; Rivkin et al. 2019). Populations may vary repeatably in response to similar selection pressures in different cities (Santangelo et al. 2022). Non-repeatable responses may also occur due to more stochastic, less predictable factors such as founder effects, non-adaptive non-directional evolution after colonization, differential selection pressures due to differences in community composition and competition therefrom, socioecological heterogeneity across cities, or social learning and innovation in some but not all urban populations, and subsequent eco-evolutionary feedbacks within cities (Miles et al. 2019; Schell et al. 2020; Des Roches et al. 2021; Caspi et al. 2022; Verrelli et al. 2022).

Evolutionary response in combination with or in the form of phenotypic plasticity drives differences in urban phenotypes within and across species (Miranda et al. 2013; Alberti 2015; Miranda 2017; Campbell-Staton et al. 2020; Caspi et al. 2022; Mascarenhas et al. 2022). Morphological evolution that underlies behavioral shifts is one such avenue to explore the repeatability of evolutionary outcomes driven by selection pressures on behavior. While morphological evolution has been documented in a few species across cities (reviewed in Alberti et al. 2017b), broad patterns have been challenging to infer. For example, wing loading has evolved in human-modified landscapes likely in response to differential selection on flight behavior in different habitat structures in one species (Bitton and Graham 2015) and vehicular traffic in another (Brown and Bomberger Brown 2013). Avian bills are particularly relevant for teasing apart the impact of behavior and abiotic stressors on the repeatability of evolution. Bill shape has shifted amongst a few species in urban environments in association with differences in resources and therefore feeding performance (Badyaev et al. 2008; De León et al. 2011; Berthouly-Salazar et al. 2012; Bosse et al. 2017). On the other hand, selection on higher minimum song frequency, a trait many bird populations repeatedly exhibits in response to urban noise (Slabbekoorn and den Boer-Visser 2006; Potvin et al. 2011), can constrain evolution as it selects for shorter bills (Giraudeau et al. 2014). Simultaneously, bills are used for thermoregulation (Symonds and Tattersall 2010; Greenberg et al. 2012; Tattersall et al. 2016; LaBarbera et al. 2020); the urban heat island effect has been associated with larger bills in Northern cardinals (*Cardinalis cardinalis*) in multiple cities (Miller et al. 2018).

The dark-eyed junco (*Junco hyemalis*) (hereafter: “junco”), a common North American passerine, has successfully colonized several cities in Southern California (Atwell et al. 2016; NestWatch 2021; Lehman 2022) and can be a model system for understanding urban evolutionary and non-evolutionary responses in wild birds (Atwell et al. 2016). In the early 1980s, a junco breeding population established in San Diego County (Yeh and Price 2004). A second population established in Los Angeles, likely in the late 1990’s or 2000’s (Atwell et al.

2016). A breeding population has also been observed in Santa Barbara, CA, where they were absent in residential areas before the 1970s (Lehman 2022). The San Diego population differed in morphological traits in comparison to a non-urban mountain pair: San Diego juncos had shorter wings and tails in comparison to local mountain non-urban birds (Rasner et al. 2004) and a reduced white tail patch, a sexually-selected trait (Yeh 2004; Price et al. 2008). Behaviorally, Los Angeles and San Diego juncos have repeatedly demonstrated adaptive phenotypic plasticity in urban nesting behavior by nesting above-ground and on artificial surfaces, as well as producing more clutches per season than non-urban populations (Yeh and Price 2004; Yeh et al. 2007; Bressler et al. 2020). They have also repeatedly exhibited less fearfulness to humans in comparison to non-urban birds (Atwell et al. 2012; Diamant et al. 2023). Yet urban song does not show repeated differences; San Diego birds, unlike Los Angeles birds, show an increased song frequency likely in response to urban noise (Slabbekoorn et al. 2007; Newman et al. 2008; Reichard et al. 2018; Wong et al. 2022). Only San Diego has been tested for morphological shifts, which likely represent evolutionary divergence based on common garden experiments in juncos (Rasner et al. 2004). Of note, the San Diego population is likely a result of genetic input from two different subspecies, one of which exists in the local mountains to the east, and the other of which breeds in Northern Californian coastal environments (Friis et al. 2022), Nonetheless, traits that correspond to urban or coastal characteristics in San Diego are strikingly different than local non-urban birds in the Laguna mountains.

Here, we asked if dark-eyed junco morphology is associated with abiotic factors and landscape change across three geographically distinct urban populations across Southern California’s coast in comparison to non-urban populations, or if morphology varies between cities non-repeatedly.

We hypothesize that morphological changes will be associated with relevant urban factors, regardless of the city. More specifically we ask if:

i. Wing size and loading is associated with built cover and differs from non-urban habitats repeatedly across cities. Because smaller bird species are associated with cities and higher wing loading is beneficial in heterogenous environments, we expect differences in wing shape and size to be associated with built cover.

ii. Bill shape differs similarly across cities, and if differences relate to climate and/or built cover. We hypothesize that larger bills will be associated with urban areas, as has been found in other birds. If the urban heat island effect underlies shifts in bill shape, we expect a positive association between bill surface area and temperature regardless of population. If differential resources in urban (vs non-urban) areas drives selection on bill morphology, we expect bill length to depth ratio ("bill ratio") to differ with the built-up environment and for bill size to vary between urban and non-urban sites regardless of temperature.

We tested these hypotheses by testing for parallel shifts across cities while accounting for relevant environmental variables.

Methods

Study system

We conducted field work across southern California (Figure 4-1), focusing on coastal Southern Californian cities and neighboring mountains. To compare across cities, we sampled juncos in Santa Barbara County ("Santa Barbara"), Los Angeles County ("Los Angeles"), and San Diego County ("San Diego"). For all three urban sites, we sampled juncos at the local campuses of the

University of California (University of California Santa Barbara (UCSB), Los Angeles (UCLA), and San Diego (UCSD), respectively). Each of these sites is broadly similar in level of urbanization, characterized by a combination of green spaces and large buildings, with heavy but seasonal human traffic. All urban sites are coastal (between 0.06 – 9.43 km from ocean), low elevation areas experiencing a Mediterranean climate. We compared these locations to non-urban mountain sites that might be indicative of their historic non-urban breeding range (Allen et al. 2016). The mountain sampling sites were the Santa Monica Mountains, the Angeles Forest in the San Gabriel Mountains, and the UC James Reserve in the San Jacinto Mountains (Figure 4-1).

Field methods

We captured adult juncos using targeted mist netting (mesh size of 30mm, between 1-3 nets at once) over a five-year span, between January and July 2018-2022 and between January and March 2023 in the morning (0630-1100). Banding began at each site following the onset of singing and territorial behavior by male juncos. In Los Angeles and San Diego, this typically occurs in mid to late January and in Santa Barbara in mid-February. Juncos were lured to the net using audio playbacks of regional junco song. We conducted targeted mist netting across each site, setting up nets across multiple banding locations where juncos were present and singing. We regularly moved nets if birds were either banded or not responding within 30-45 minutes. Male juncos primarily respond, though females are often also captured during the breeding season. Each individual was sexed by plumage and/or primary sex characteristics (cloacal protrusion and/or brood patch), aged by molt limit, scored for fat content, and checked for active molt (Pyle 1997). We measured the following morphological traits: unflattened wing chord (wing length; measurement described in (Ralph et al. 1993), tail length from the posterior end of the uropygial gland to the posterior tip of the longest rectrix, tarsus length, bill length from the distal edge of the nares to the tip of the upper mandible, bill depth and width at the posterior edge of the nares, and weight on a calibrated and tared precision pocket scale. Multiple researchers measured juncos over the course of the project, with two primary individuals (ESD and WAM) who trained all others on these measurements. Error for the primary researchers is presented in the supplementary material.

In total, we measured 356 individuals in Los Angeles, 29 in Santa Barbara, 41 in San Diego, and 67 across non-urban mountain sites (Table 4-1). All animals were cared for and tested according to institutional guideline and approval (IACUC #ARC-2018-007).

Statistical Analyses

Ecological differences between urban and non-urban populations

We characterized each banding location by measuring and extracting the built-up index (Valdiviezo-N et al. 2018) in a 50m radius around the coordinates of each bird's capture location using Landsat-8 images (courtesy of the U.S. Geological Survey) and QGIS (QGIS.org 2020). All three cities have a higher built cover than non-urban mountain sites (Figure 4-2). We then conducted an ANOVA on these data to determine if the built-up index differed between urban and non-urban sites.

To determine if micro-climate differed between sites, we obtained 30-year monthly average temperature, maximum temperature, and precipitation (1991-2020) at an 800 m resolution (PRISM Climate Group 2014) through the package *prism* (Hart and Bell 2015). The average for each variable was calculated across an 800 m x 800 m grid using the packages *raster* (Hijmans and van Etten 2020), *sf* (Pebesma 2018), and *stars* (Pebesma and Bivand 2023). We intersected banding location with the raster dataset to obtain averaged climate at the capture location for each bird. As temperature variables were correlated with each other, we used maximum temperature (tmax) in our analyses because temperature extremes have previously been found to be relevant for understanding thermoregulation by junco bills (LaBarbera et al. 2020). Because variance within each group were not equal, we ran a Wilcoxon signed rank test to determine if sites differed in tmax.

Morphological differences across and between populations

We conducted multiple linear mixed models (LMMs), each with a different dependent morphological variable, to test hypotheses on morphological shifts in response to urbanization and whether shifts repeat in difference cities.

We calculated wing loading by dividing weight (g) by wing length (mm). We considered wing loading as a dependent variable and sex, built-up index, population (UCLA, UCSD, UCSB, and non-urban), and measurer as independent variables. We also considered wing length—which, in the absence of wing loading differences, acts as a proxy for body size—as a dependent variable and sex, built-up index, population, and measurer as independent variables.

To assess hypotheses on bill size and shape being driven by microclimate, resources, or a combination thereof, we considered three bill variables: bill length (mm) as a proxy for size, bill length to depth ratio, and bill surface area (mm^2). We estimated total bill surface area as:

$$\frac{(bill\ width + bill\ depth)}{4} \times bill\ length$$ (Greenberg et al. 2012).

To determine relative bill surface area, tarsus length was used as a proxy for body size; bill surface area was divided by tarsus length. While we did not assess thermal differences between bills directly, thermal differences have been assessed in a closely related species, the song sparrow (*Melospiza melodia*) (Greenberg et al. 2012). Since then, associations between temperature, humidity, and bill surface area have been found in juncos (LaBarbera et al. 2020) and urbanized Northern cardinals (Miller et al. 2018). For bill length, which may be under selection based on surface area and foraging, we included sex, tmax, precipitation, built-up index, population, and measurer as independent variables. For bill ratio, we considered sex, built-up index, population, and measurer as independent variables. For bill surface area, we considered sex, tmax, precipitation, population, and measurer as independent variables.

To determine the best-fit model for each independent variable, we conducted an automated model selection with the *glmulti* package (Calcagno and Mazancourt 2010). From this, we examined models with AIC <2 from the lowest value. As the measurer was an important variable in nearly all models, we included that as a random effect in our models. We then calculated AIC values considering the other relevant fixed effects and present the models with the lowest AIC. In the case that multiple models were within 2 AIC from each other, we removed variables with variable inflation factor ≥ 2.5 and chose the simpler model if model reduction did not result in removing a significant variable.

Subsequently, the following models were selected for analysis:

1. **Wing loading** as the response variable, sex and built-up index as fixed effects, and measurer as a random effect.
2. **Wing length** as the response variable, sex and built-up index as fixed effects, and measurer as a random effect.
3. **Bill length** as the response variable, tmax, sex, and population as fixed effects, and measurer as a random effect.
4. **Bill ratio** as the response variable, population as a fixed effect, and measurer as a random effect.
5. **Bill surface area by tarsus length** as the response variable, tmax and population as fixed effects, and measurer as a random effect.

LMMs were analyzed with the package *lme4* (Bates et al. 2015) and *car* (Fox and Weisberg 2019). For instances where population was a fixed effect, we calculated z -scores and p-values for within-model pairwise contrasts using the *multcomp* package (Hothorn et al. 2022). All analyses were conducted using R v. 4.1.3 (R Core team 2020).

Results

Ecological differences between populations

All UC campuses had a higher built-up index than non-urban sites ($p < 0.0001$ for all comparisons with non-urban populations) but had similar built-up index amongst themselves ($0.45 \leq p \leq 0.78$); Figure 4-2A). All UC campuses differed from each other in tmax ($p < 0.0001$ for all urban pairwise comparisons), but only UCSB differed in tmax from the non-urban group ($p < 0.0001$; other comparisons $0.47 \leq p \leq 0.60$) (Figure 4-2B).

Morphological differences across and between populations

Collectively, patterns varied between traits—some traits varied between populations regardless of abiotic variation, while others were better explained by ecological variation regardless of abiotic variation. These results are summarized in Figure 4-3.

Wing loading was not significantly associated with built-up index ($X^2 = 2.28$, $p = 0.131$). Population was not in the best fit model. Wing size, a proxy for body size, varied significantly with built cover ($X^2 = 5.26$, $p = 0.022$) such that banding locations with more built cover, regardless of population, were occupied by birds with shorter wings (Figure 4-4).

Bill length differed between populations ($X^2 = 77.4$, $p < 0.0001$), and with regards to tmax ($X^2 = 8.54$, $p = 0.003$). Locations with higher maximum temperatures were occupied by birds with longer bills. However, urban areas did not have consistent differences in bill length (Figure 4-5A). UCLA juncos had shorter bills than non-urban juncos and other urban junco populations (UCLA-nonurban contrast: $z = -8.53$, $p < 0.001$; UCLA-UCSB contrast: $z = -0.49$, $p < 0.001$; UCLA-UCSD contrast: $z = -2.98$; $p = 0.014$), despite occupying locations with the highest maximum temperatures across urban sites (Figure 4-2B). All other comparisons were not significant ($1.02 \leq z \leq 2.18$; $0.12 \leq p \leq 0.73$). The built-up index was not in the best fit model.

Bill surface area exhibited similar patterns as bill length; surface area was higher relative to body size in locations with higher maximum temperature ($X^2 = 4.08$, $p = 0.043$) and bill surface area relative to body varied between populations ($X^2 = 44.61$, $p < 0.0001$). Patterns between urban areas did not correspond to temperature differences between cities (Figure 4-5B); UCLA juncos had a significantly lower bill surface area in comparison to non-urban juncos ($z = -6.32$, $p < 0.001$) and UCSB ($z = -3.49$; $p = 0.002$); as well as marginally lower bill surface area in comparison to UCSD ($z = -2.46$; $p = 0.06$). All other comparisons were not significant ($0.297 \leq z \leq 1.37$; $0.50 \leq z \leq 0.99$). The built-up index was not in the best fit model.

Bill ratio differed between populations ($X^2 = 50.9$, $p < 0.0001$) (Figure 4-5C), however the built-up index did not explain these differences. UCLA and UCSB juncos had a lower bill ratio in comparison with non-urban juncos ($z = -6.68$, $p < 0.001$; and $z = -3.33$, $p = 0.005$ respectively). In addition, UCLA juncos had a lower bill ratio in comparison with UCSD juncos ($z = -3.18$, $p = 0.007$). UCSD did not significantly differ in comparison with non-urban juncos ($z = -0.96$, $p = 0.76$). Results for all models are presented in the supplement (Tables S4-2 and S4-3).

Discussion

Our hypotheses that urban areas would exhibit convergent morphologies in response to similar ecological shifts was not broadly supported; rather, complex patterns emerged. Urban populations across Southern California exhibited non-convergent and convergent morphological differences across populations. Specifically, the degree of built cover was associated with smaller birds regardless of population, while bill size and shape differed between urban populations regardless of the degree of urbanization. Urban Los Angeles and urban Santa Barbara breeding junco populations exhibited shorter and relatively deeper bills than non-urban juncos, but urban San Diego breeding juncos did not. Variation in the variables we analyzed (temperature or the degree of built cover) between cities does not sufficiently explain the differences in beak morphology between cities, suggesting that similar large-scale ecological differences driven by anthropogenic structures and human disturbance do not necessarily lead to convergence. The variation between cities suggests that urban landscapes can facilitate intraspecific diversity despite their similar strong selective filters, while built cover might act as a filter on some but not all morphological traits (Devictor et al. 2008; Lokatis and Jeschke 2022).

Non-Convergence of Morphological Evolution

Urbanization provides a unique opportunity to assess the repeatability of evolution under strong selection because urban areas have been viewed as natural "replicates" of strong ecological change (Donihue and Lambert 2015; Alberti et al. 2017a); overarching differences between urban areas and their neighboring non-urban counterparts have consistently been found due to human disturbance, built cover, and associated stressors. Nonetheless, variation within and across cities complicate this notion. Studying populations across cities fundamentally allows us to test the role of contingency in evolution—i.e., are evolutionary trajectories predictable based on environmental conditions, or to what degree do randomness and incidental change drive unpredictable outcomes in evolutionary trajectories (Gould 1989). If evolution is deterministically driven by large-scale shifts in an organism's ecology rather than differences between cities and/or stochastic non-deterministic processes, then evolutionary response to urbanization would be directional and predictable. However, morphological divergence differs across cities in Southern California despite broad similarities in the level of the built environment across coastal cities. The differences we found between urban and non-urban populations suggest that evolution across urban environments might not be predictable. Thus, how populations might respond and adapt to urban environments might not be generalizable from studies in single cities, possibly due to differences within and across cities, and to more random or time-dependent differences between urban populations.

One potential non-adaptive cause of divergence across cities is a founder effect. While the San Diego juncos were initially an island population with a small founding population, the morphological differences first observed in this population in comparison to non-urban birds were driven by selection rather than drift (Rasner et al. 2004; Yeh 2004). It is unlikely that divergence in the Los Angeles population is driven by a founder effect because it is not an isolated population. We have had two instances of juncos captured at UCLA that have also been sighted or captured at peri-urban and non-urban locations. One individual recaptured at UCLA during the breeding season was initially banded at the Santa Susana Field Station at the foothills of the Santa Susana mountains, which connect to the Santa Monica mountains (dispersal distance of approximately 18 miles between capture sites) (Mark Osokow, pers. comm.). Another individual was color-banded by us as a nestling at UCLA and sighted within Griffith Park, an urban park that is over 4,000 acres in Los Angeles and connected to local mountains, ~8 miles from campus (Zelia Scott, pers. comm.). As such, there is likely active gene flow between local non-urban mountain populations and the Los Angeles urban population, which we assume has been the case throughout the history of colonization.

A lack of divergence in some urban populations could be due to gene flow, an absence of significant selection pressures, a lack of standing variation, a short time since colonization, selection not operating on a heritable trait, or differences in plasticity that could potentially prevent adaptive evolution in cities (Shochat et al. 2006; Caspi et al. 2022). Our results suggest that selection is likely driving morphological divergence across urban populations in size and bill shape upon colonization rather than divergence due to genetic drift. However, non-adaptive processes or sample size limitations might explain a lack of statistically significant divergence in Santa Barbara junco bill shape in comparison to other urban and non-urban populations.

Bill shape divergence

Los Angeles urban juncos differ in bill shape, with shorter and deeper bills, in comparison to non-urban and San Diego urban juncos. Santa Barbara birds are intermediate, as they are significantly different than non-urban juncos but not San Diego juncos. In addition, Los Angeles urban juncos had lower bill surface area than non-urban juncos and Santa Barbara juncos.

We tested the hypothesis that bill surface area is driven by selection on thermoregulation (Snow 1954; Symonds and Tattersall 2010; Greenberg et al. 2012; Tattersall et al. 2016), which was not supported by our data. If thermoregulation explained bill shape differences across cities, we would expect higher bill surface area in cities with higher temperatures. Conversely, we would expect lower bill surface area with low temperatures. We found that while temperature was broadly associated with larger bill surface area, significant population differences did not match this pattern. Instead, urban Los Angeles juncos had *lower* bill surface area despite Los Angeles having a *higher* average maximum temperatures relative to other urban sites (Figure 4-2B), contrary to findings in non-urban populations (LaBarbera et al. 2020). Juncos may be coping with urban heat through other forms of thermoregulation. While tarsii are also thermoregulatory organs, they seem less adaptive to temperature in smaller birds (Fröhlich et al. 2023). Feather melanism and differences in behavior at different temperatures also potentially impact heat absorption and conservation in juncos (Carr and Lima 2012; de Zwaan et al. 2017) and should be further explored in the urban context.

We next considered that selection on song traits could drive bill shape in the urban environment (Palacios and Tubaro 2000; Derryberry 2009; Giraudeau et al. 2014), although in the juncos this explanation is also unlikely because Los Angeles urban juncos do not sing at a higher frequency than non-urban juncos (Wong et al. 2022). Urban birds typically have higher minimum frequency to cope with urban noise (e.g. Slabbekoorn and Peet 2003; Badyaev et al. 2008; Derryberry et al. 2020), which is associated with less narrow bills (Giraudeau et al. 2014). San Diego juncos (Reichard et al. 2020), not Los Angeles juncos, sing at a higher frequency. Thus, the shift in bill shape is likely not due to selection on song.

A third explanation for the bill shape variation is that differences in foraging within and between cities due to the built environment could drive differences in bill shape. This explanation did not fit the differences we found between cities. All studied cities had higher built cover than non-urban areas, suggesting that Los Angeles juncos, and potentially Santa Barbara juncos, have uniquely diverged in their bill shape in the built environment.

If bill shape reflects selection on foraging strategies in built environments, then differences in behavioral innovation could explain divergent evolution across cities. Bill size and shape polymorphisms are often associated with variation in foraging niche across bird species (e.g., Smith 1987, 1990). Indeed, bill divergence in response to shifts in food availability and anthropogenic waste is well known in birds (Grant and Grant 2002). Selection from supplemental feeding on hard-shelled seeds in bird feeders has driven the evolution of longer bills in urban house finches (Badyaev et al. 2008) and great tits (Bosse et al. 2017). Los Angeles juncos have shorter rather than longer bills, suggesting that adaptation to supplemental feeding of hard shells, as is common in bird feeders, does not drive this shift. Instead, Los Angeles juncos might have learned to exploit other resources, like anthropogenic food waste, in the built environment while other urban junco populations have not.

Adaptive behavioral plasticity in resource exploitation amongst Los Angeles and Santa Barbara urban juncos, but not San Diego urban juncos, can explain their bill shape divergence and higher variation in comparison to other populations. Urban environments broadly exhibit differences in resource availability, trophic dynamics, and indirect supplemental feeding through exotic vegetation and human waste (reviewed in Shochat et al. 2006). Multiple bird species have been found to access and exploit anthropogenic food resources due to increased resource availability where many humans eat and/or have high human food refuse (Martin et al. 2010; Goldenberg et al. 2016; Stofberg et al. 2019). Red-winged starlings (*Onychognathus morio*) at the University of Cape Town campus in South Africa consume more anthropogenic food and gain more weight when human activity is higher but supplement their diets with more natural items when human activity is lower (Stofberg et al. 2019). Niche expansion was also noted in Darwin's finches (*Geospiza fortis*), where anthropogenic food flattened selection on bill shape in human-modified environments (De León et al. 2011). Therefore, there could be selection driven by exploiting anthropogenic food resources in the built environment, creating a niche the juncos have learned to exploit. Thus, adaptive behavioral plasticity might have facilitated rapid evolution (Ghalambor et al. 2007; Caspi et al. 2022) in the Los Angeles and potentially the Santa Barbara population.

What remains unclear is why this has not occurred in San Diego juncos. This could be a matter of chance (Ossola et al. 2021), where Los Angeles juncos just so happened to learn to exploit these resources, and potentially opportunity as Los Angeles is a denser city which may lead to more waste.

Together, these findings contribute to answering how repeatable evolutionary response is to urbanization and anthropogenic change (Johnson and Munshi-South 2017; Rivkin et al. 2019). There is some evidence that either convergent or divergent evolution can occur (Evans et al. 2009; Diamond et al. 2018; Campbell-Staton et al. 2020; Salmón et al. 2021; Caizergues et al. 2022; Santangelo et al. 2022), but these trajectories depend on the strength of similar stressors in urban environments and stochastic processes in the population's evolutionary history (Lambert et al. 2021). In the junco, some behavioral traits such as novel and above-ground nesting behavior (Bressler et al. 2020) and fear response (Diamant et al. 2023) converge across cities while others, like song (Wong et al. 2022), do not. Morphologically, body size and bill shape have different patterns of convergence and non-convergence between urban populations. Thus, both evolutionary and non-evolutionary processes could lead to independent outcomes and prevent trait homogenization across cities.

Conclusions

Contrary to our expectations, urban dark-eyed junco populations across Southern California show complex patterns of convergence and non-convergence. Most notably, Los Angeles juncos have longer and differently shaped bills in comparison to non-urban juncos and the long-established urban San Diego junco population. On the other hand, body size differs with respect to built cover regardless of population. These results indicate that non-convergent evolution occurs across cities in bill shape, but convergence might be occurring in body size. Thus, evolutionary response might not be generalizable across cities. These differences might be due to local behavioral innovations that did not occur in other cities. Further work should explore behavioral variation, and potential effects on diet and fitness, in response to fine-scale landscape differences within and between cities.

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Tables and Figures

Table 4-1. Sampling effort across sites by sex.

	Location			
	Los Angeles urban (UCLA)	San Diego urban (UCSD)	Santa Barbara urban (UCSB)	Non-urban Southern Californian mountains
Male	243	34	26	58
Female	113	7	3	9

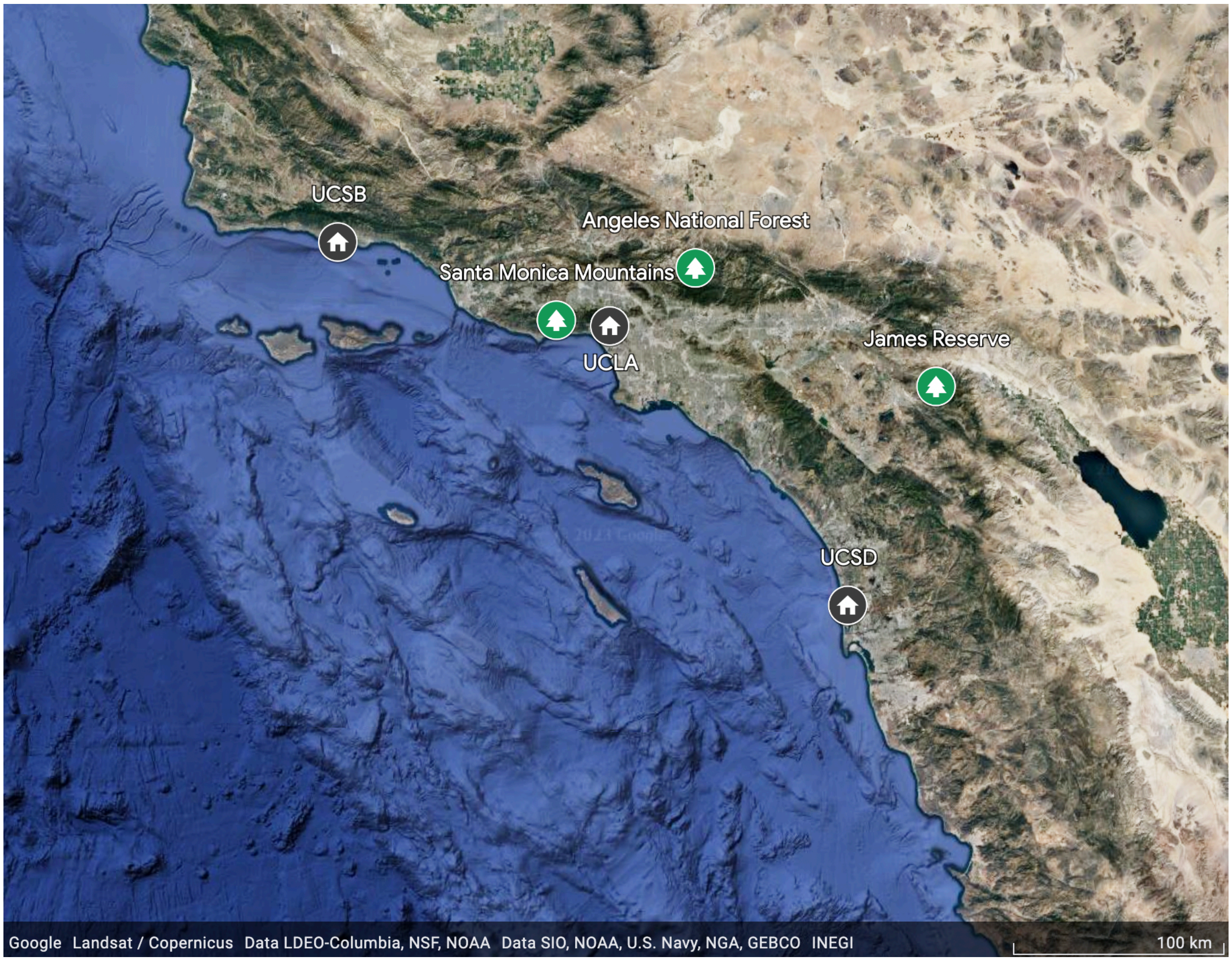

Figure 4-1. Junco banding sites across Southern California. These sites included three urban UC campuses in three cities (black circles with dwelling icons) and three non-urban mountain populations (green circles with tree icons) grouped together into one non-urban group**.**

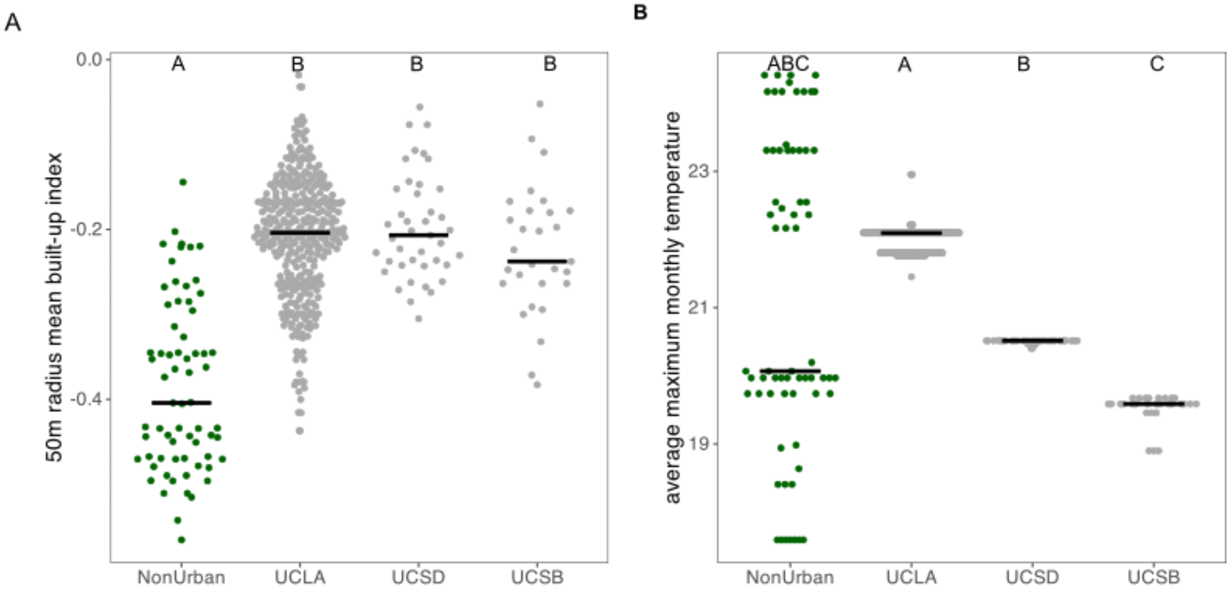

Figure 4-2. Banding locations at UC campuses in urban areas exhibited different abiotic conditions from each other and from non-urban locations. (A) All UC campuses had a higher built-up index across banding locations than the non-urban locations. (B) All UC campuses exhibited different average maximum monthly temperatures from each other, but none significantly differed from non-urban locations. Letters demonstrate statistically significant differences from (A) a Tukey HSD test and (B) a Wilcoxon signed rank test. Dots refer to data from individual bird's capture locations. Black lines refer to the median value per group. Please see Table 4-1 for sample sizes.

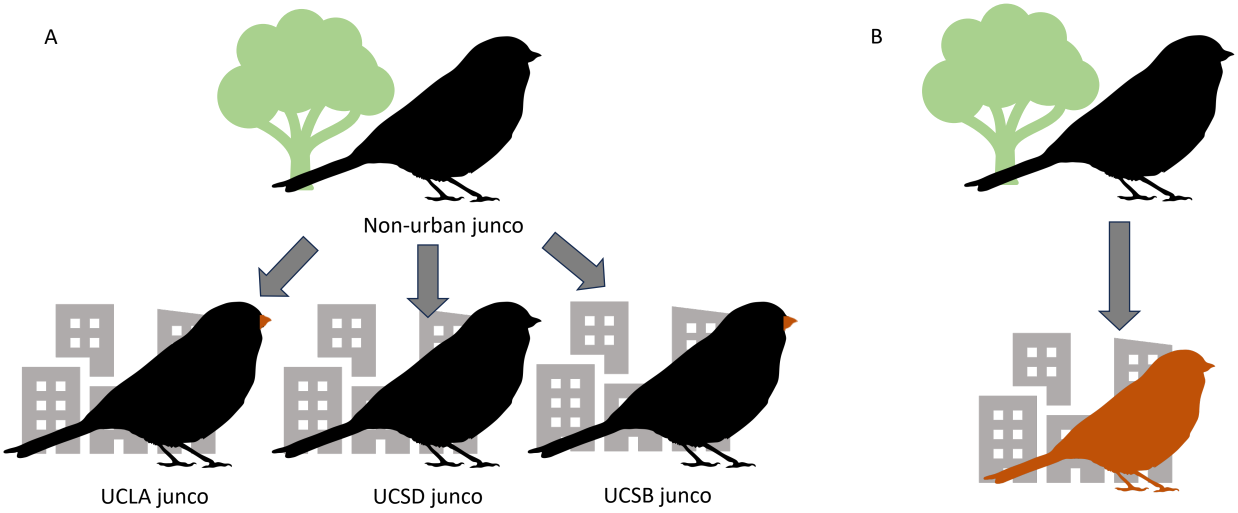

Figure 4-3. Urban and non-urban birds showed patterns of shape variation where (A) the population was associated with morphological differences between and non-urban birds and (B) the degree of built cover, regardless of urban population, was associated with morphological differences. (A) Bill size and shape, while partially explained by average maximum temperatures, differed between cities incongruently with climate-driven hypotheses. (B) Built cover index, rather than population, was associated with smaller wing size, a proxy for body size, in juncos across all populations. Negative associations between morphology and urbanization are orange. Details on statistical analyses are presented in Tables S4-2 and S4-3. Silhouetted image is of a closely related bird, *Spizelloides arborea*, and uploaded by Ferran Sayol to PhyloPic.org.

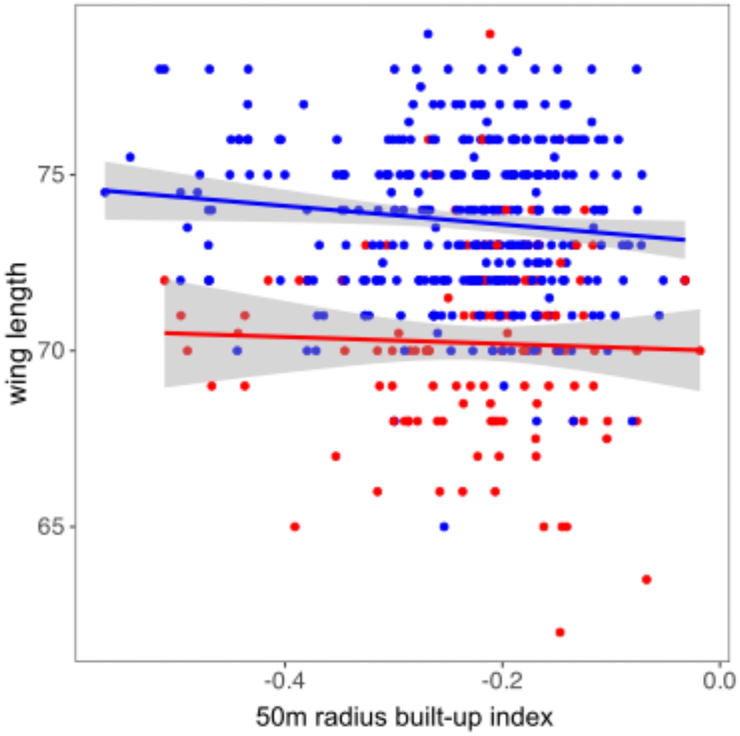

Figure 4-4. Juncos with shorter wings occupied areas with more built cover across all populations. Blue represents males and red represents females. Linear trendlines are added per group with a shaded 95% confidence interval. Model results considering fixed and random effects are presented in Table S4-2.

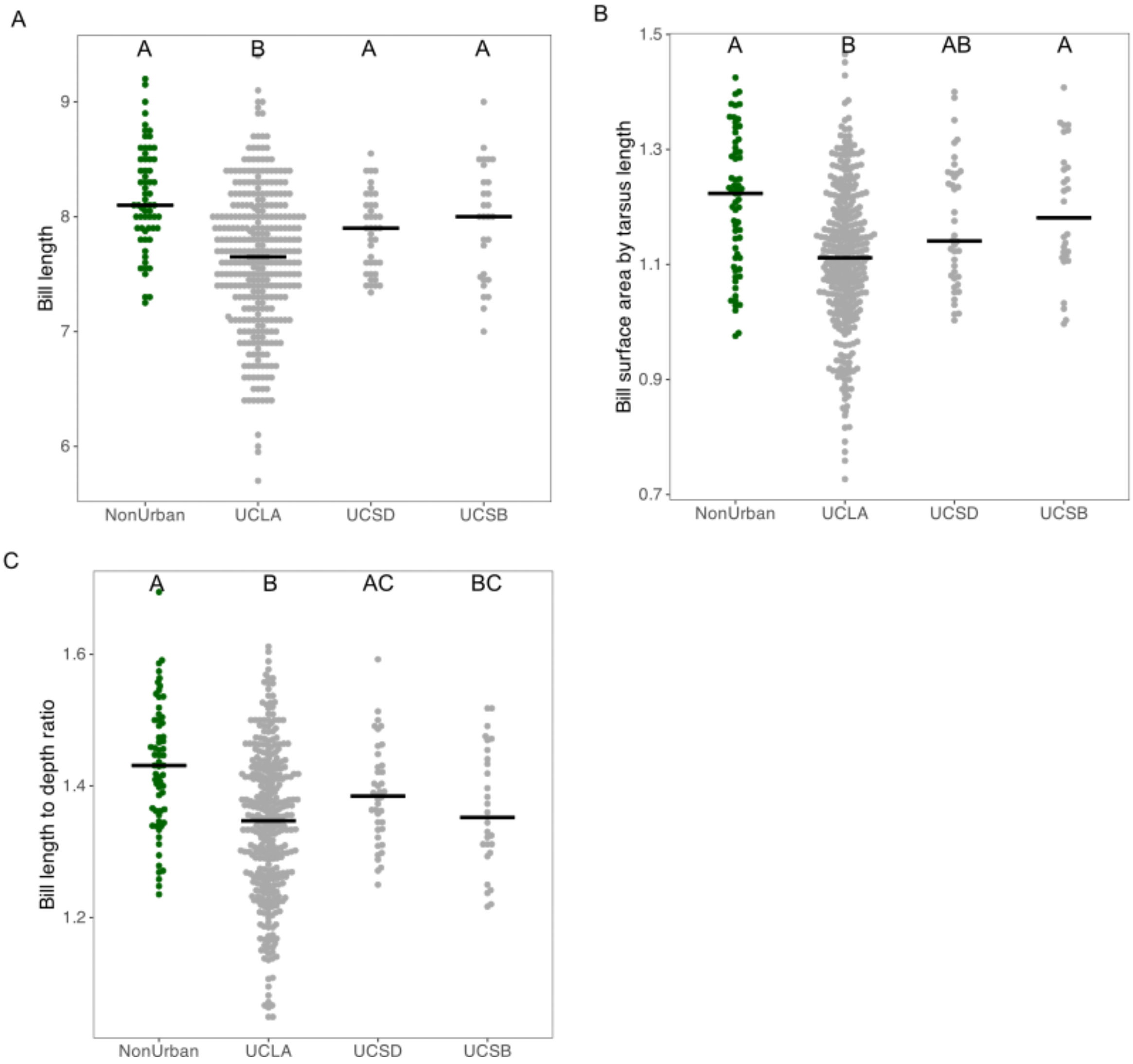

Figure 4-5. Juncos varied in bill size and shape across urban populations. (A) bill length (mm) in UCLA juncos differed from non-urban juncos while other urban junco populations did not. (B) Bill surface area by tarsus length (mm) showed similar patterns as bill length—despite a higher average maximum temperature at UCLA in comparison to other urban sites, UCLA juncos exhibited lower relative bill surface areas than UCSB. (C) bill shape differed between sites such that UCLA and UCSB exhibited relatively shorter, wider bills than non-urban juncos, while UCSD juncos did not strongly differ from non-urban juncos. Letters refer to significant groups significantly different based on pairwise contrasts (Table S4-3) and black bars represent the group's median value.

Chapter Four: Supplemental Materials

Inter- and intra-individual repeatability of measurements

Seven banders have measured juncos between 2018 and 2023. Two researchers—ESD and WAM—trained all other researchers and collectively banded across all time points. All banders have measured birds in all sites. ESD and WAM measured a song sparrow frozen specimen 4 times each (Table S4-1). New banders did not work independently until they were consistent in measurements with one of the two primary banders for at least 3 birds in the hand, including between ESD and WAM. Nonetheless, model results revealed slight differences between individual measurements and thus these were accounted for in the models with a random effect.

Table S4-1: Inter- and intra-individual variation in measurements of a song sparrow.

	Tarsus length (mm) (mean ± SD)	Culmen length (mm) (mean ± SD)	Bill depth (mm) (mean ± SD)	Bill width (mm) (mean ± SD)
ESD	20.40 (0.28)	9.19 (0.09)	6.59 (0.06)	5.88 (0.05)
WAM	20.16 (0.23)	9.10 (0.00)	6.70 (0.00)	5.78 (0.15)
ESD + WAM	20.28 (0.27)	9.14 (0.07)	6.64 (0.07)	5.83 (0.11)

Table S4-2: Model outputs for linear mixed models considering wing measurements.

Wing loading as response variable			
Fixed effects	X^2	*df*	*p-value*
Intercept	2129.7	1	**< 0.0001**
Sex	10.5	1	**0.001**
Mean built-up index in 50 m	2.28	1	0.13
Random effects	*variance*	*sd*	*p-value*
Measurer	0.0001	0.012	**< 0.0001**
Residual	0.0004	0.02	
Number of observations	412		
AIC	-2005.7		

Wing length as response variable			
Fixed effects	X^2	*df*	*p-value*
Intercept	37342.0	1	**< 0.0001**
Sex	193.3	1	**< 0.0001**
Mean built-up index in 50 m	5.26	1	**0.02**
Random effects	*variance*	*sd*	*p-value*
Measurer	0.17	0.41	0.44
Residual	5.48	2.34	
Number of observations	469		
AIC	2143.4		

Table S4-3: Model outputs for linear mixed models considering bill measurements

Bill length as a response variable

Fixed effects	X^2	*df*	*p-value*
Intercept	112.7	1	**< 0.0001**
Tmax	8.54	1	**0.003**
Sex	2.08	1	0.15
Population	77.4	3	**< 0.0001**

Random effects	*variance*	*sd*	*p-value*
Measurer	0.19	0.43	**< 0.0001**
Residual	0.23	0.48	

Number of observations	470
AIC	701.5

Pairwise contrasts: Population	*estimate*	*se*	*z-score*	*p-value*
Non-urban – UCLA	0.61	0.07	8.53	**< 0.001**
Non-urban – UCSB	0.12	0.12	1.02	0.73
Non-urban – UCSD	0.27	0.12	2.18	0.12
UCLA – UCSB	-0.49	0.12	-4.16	**< 0.001**
UCLA – UCSD	-0.34	0.11	-2.98	**0.01**
UCSB – UCSD	0.15	0.14	1.04	0.72

Bill surface area by tarsus length as a response variable

Fixed effects	X^2	*df*	*p-value*
Intercept	51.5	1	**< 0.0001**
Tmax	4.08	1	**0.043**
Population	44.6	3	**< 0.0001**

Random effects	*variance*	*sd*	*p-value*
Measurer	0.007	0.09	**< 0.0001**
Residual	0.01	0.11	

Number of observations	464
AIC	-703.9

Pairwise contrasts: Population	*estimate*	*se*	*z-score*	*p-value*
Non-urban – UCLA	0.10	0.02	6.32	**< 0.001**
Non-urban – UCSB	0.008	0.03	0.30	0.99
Non-urban – UCSD	0.37	0.03	1.37	0.50
UCLA – UCSB	-0.09	0.03	-3.50	**0.003**
UCLA – UCSD	-0.06	0.02	-2.46	0.06
UCSB – UCSD	0.03	0.03	0.93	0.78

Bill length to depth ratio as a response variable			
Fixed effects	X^2	*df*	*p-value*
Intercept	2415.0	1	**< 0.0001**
Population	50.86	3	**< 0.0001**
Random effects	*variance*	*sd*	*p-value*
Measurer	0.005	0.07	**< 0.0001**
Residual	0.009	0.10	
Number of observations	469		
AIC	-803.6		

Pairwise contrasts: Population	*estimate*	*se*	*z-score*	*p-value*
Non-urban – UCLA	0.10	0.01	6.68	**< 0.001**
Non-urban – UCSB	0.07	0.02	3.33	**0.005**
Non-urban – UCSD	0.02	0.02	0.96	0.76
UCLA – UCSB	-0.02	0.02	-0.96	0.76
UCLA – UCSD	-0.06	0.02	-3.18	**0.008**
UCSB – UCSD	-0.05	0.03	-1.78	0.27

Chapter Five: Urban birds become less fearful following COVID-19 re-openings[2]

Abstract

Following the COVID-19 pandemic, many people around the world stayed home, drastically altering human activity in cities. This exceptional moment provided researchers the opportunity to test how urban animals respond to human disturbance, in some cases testing fundamental questions on the mechanistic impact of urban behaviours on animal behaviour. However, at the end of this "anthropause," human activity returned to cities. How might each of these strong shifts affect wildlife in the short and long term? We focused on fear response, a trait essential to tolerating urban life. We measured flight initiation distance—at both individual and population-levels—for an urban bird before, during, and after the anthropause to examine if birds experienced longer-term changes after a year of lowered human presence. Dark-eyed juncos did not change fear levels during the anthropause, but they became drastically less fearful afterwards. These surprising and counter-intuitive findings, made possible by following the behaviour of individuals over time, has led to a novel understanding that fear response can be driven by plasticity, yet not habituation-like processes. The pandemic-caused changes in human activity have shown that there is great complexity in how humans modify a behavioural trait fundamental to urban tolerance in animals.

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[2] This chapter is in revision for publication with the following author order: Eleanor S. Diamant, Ian MacGregor-Fors, Daniel T. Blumstein, and Pamela J. Yeh

Background

In 2020, many countries in the world went into "lockdown" in response to the COVID-19 pandemic. With human mobility suddenly halted, these lockdowns drastically changed the dynamics of our cities and caused what has been coined as the "anthropause" [1]. While devastating for human communities, the absence of humans from the landscape provided a unique opportunity to study how animals respond to human activity, from the level of individual behavior to population dynamics to community composition. Likely because of the direct and indirect effects of human activity, such as vehicular traffic, collisions, light pollution, and noise pollution, some wildlife—specifically urban wildlife—adjusted their behaviours and patterns across the globe [1–6]. For example, during the initial pandemic lockdowns, urban white-crowned sparrows (*Zonotrichia leucophrys*) rapidly responded to the reduction in traffic noise by notably changing their songs to more high performing songs that are otherwise interrupted by urban noise [2]. Lockdowns have sporadically ended and re-occurred in different parts of the world, though human activity has broadly bounced back to pre-pandemic levels. As a result, urban animals are now faced with increased human activity and stressors following a long absence. By assessing their individual and population-level behaviours before, during, and after the anthropause, we can begin to understand how animals respond to dynamic human processes and stressors. We can also determine if and how this exceptional event continues to impact wildlife even after humans have returned to the landscape.

Determining how animals cope with urban stressors is essential to predicting wildlife response in the face of strong anthropogenic change [7,8]. Urbanization is a leading cause of habitat loss and biodiversity loss, though some animals manage to survive, adapt, and ultimately thrive in cities

[9–12]. Though the underlying causes and associations for urban success vary across species and populations, tolerating humans is essential to urban life [13–15]. Indeed, at the population and species levels, we see that urban animals typically have a reduced fear of humans [16]. The mechanisms underlying this behavioural shift are challenging to parse out: in some organisms, this is due to habitat selection wherein individuals that are less fearful choose urban habitats with increased human activity and stress [17]. However, habituation-like processes that may underly within-generational behavioural plasticity—an individual's propensity to shift their behaviour in response to differences in their environments—can also explain this observed phenomenon [18]. Here, individuals exposed to human activity might decrease their fear response with increased exposure. Once in the city, the urban environment might select for individuals that are less fearful. Plasticity itself might be under selection if certain individuals express less fear upon exposure than others and may evolve if this has reproductive consequences [19,20].

COVID-19 lockdowns and reopenings provided the opportunity for us to study the complex nature of how fear is affected by human activity, allowing us to test how plastic the response is in a successful urban bird: the dark-eyed junco (*Junco hyemalis*). This songbird, native to North America, began breeding in urban habitats in the past 20-40 years in Southern Californian cities [21–23], likely independently. By assessing fear response across lockdown conditions, we can then determine if and how non-evolutionary mechanisms, such as habituation-like processes that might lead to tolerance and habitat selection whereby tolerant individuals settle around humans while less tolerant ones avoid humans, as well as evolutionary processes like selection on plasticity itself, impact fear. Further, the relatively sudden reintroduction of humans to the landscape provided us with the opportunity to assess if dark-eyed junco behaviour returned to a pre-pandemic “normal” or if lockdowns shifted how this urban bird behaves and copes with human presence long-term.

We tested individual and population-level fear response in urban dark-eyed juncos before, during, and after COVID-19 closures to understand the immediate and longer-term effects of COVID-19 on other animals and to test fundamental questions in urban behavioural adaptation. If lower urban fear response is due to habituation, we expected fear response to lower during the COVID-19 closures and increase following reopening. On the other hand, if lower urban fear response is due to habitat selection and is less plastic, we expected fear response to remain unchanged with respect to the closures. If both habitat selection and habituation play a role, as might be seen if birds that have relatively lower fear response and a plastic habituative response are selected for, we expected fear response to increase during the closures, though not to the level of non-urban birds, and to decrease after reopening at the individual and population level. None of these hypotheses were supported by our findings. Instead, we found that, at the population level and individual level, urban birds did not change their behaviour during the COVID-19 closures but became significantly less fearful of humans following reopenings in comparison to pre-pandemic baselines.

Methods

Study Sites

To confirm that urban dark-eyed juncos have lower fearfulness across cities in comparison with non-urban dark-eyed juncos, we conducted field work at urban and non-urban sites in Southern

California between 2017 and 2022. We assessed individually colour-ringed birds across three metropolitan areas: Santa Barbara County (“Santa Barbara”), Los Angeles County (“Los Angeles”), and San Diego County (“San Diego”). At each of these metropolitan areas, we sampled birds at the local University of California (University of California Santa Barbara (UCSB), Los Angeles (UCLA), and San Diego (UCSD), respectively). In Los Angeles, specifically, we also sampled birds across the city in Occidental College and parks of various sizes across the urban core. Dark-eyed juncos likely began breeding in San Diego in the early to mid 1980s, in Los Angeles in the early to mid 2000s, and in Santa Barbara in the early 2010s. These sites were compared to non-urban, mountainous sites that are indicative of their historic breeding range [23,24]: the UC Stunt Ranch Reserve in the Santa Monica Mountains, the UC James San Jacinto Mountain Reserve, and the Angeles Forest in the San Gabriel Mountains.

Because we have a longitudinal study site in urban Los Angeles, dark-eyed juncos at UCLA were individually distinguishable by unique coloured leg rings. In March 2020, UCLA closed classes and research, except for essential researchers. UCLA remained remote until Fall 2021, when classes resumed on campus. We conducted human pedestrian surveys at UCLA to confirm that human activity was lower during campus closures than when classes are in session and in person. We previously surveyed 12 points across campus twice per week, once in the morning and once in the afternoon during the anthropause (May-July 2021) and the campus was “back to normal” (March-July 2022). Each survey lasted two minutes and all individuals, vehicles, and dogs crossing the observer’s eyeline were counted. Campus closures caused human activity to be approximately 7x lower than “normal” in 2021 [25], and therefore even lower during 2020 at the height of Los Angeles lockdowns and UCLA campus restrictions.

Individual Colour Ringing

We captured and ringed local dark-eyed juncos at each site at the start of territorial singing – around mid-January in urban sites and April in non-urban sites – to July 2017. Birds were captured between 6:30 and 11:00. They were lured into mist-nets using playback of junco song recorded at UCLA in 2018 or from the MacCauley Library (Cornell University). Each junco was fitted with 3 colour rings and 1 aluminium USGS ring in a unique combination. Birds were aged by moult limits as "second year," "after second year," or "after hatch year" (when age could not be determined) and sexed by cloacal protuberance or brood patch. When birds were not in breeding condition, they were sexed by plumage, which was later confirmed by behaviour (singing or exhibiting nesting behaviour). All birds were released after processing.

Flight Initiation Distance Assays

We assessed the fear levels of individually identifiable birds using a flight initiation distance assay repeatedly during the 2018-2022 breeding seasons (January-June/July). We determined flight initiation distances (FIDs) for each bird following established methods [26,27]. All FID tests were conducted by ESD. A marker was dropped at first site of the focal bird. The researcher walked at a steady pace of ~0.5m/sec towards the bird. A second marker was dropped at the point the researcher was when the bird flew or hopped away, and then a third where the bird was when it flew or hopped. We recorded the starting distance (the distance between the first marker and the third), FID (the distance between the second and third). Effort was made to universally assay juncos in instances with low human activity (<15 humans walking in the vicinity while assays were conducted, except for 18 data points in areas rarely empty during the academic year) and with no other humans or juncos between the investigator and the focal bird. Trial number per individual was determined for each fear response assay and varied between 1 to 10 trials per individual. We also included distance to cover—defined as habitat a bird could hide within or behind, i.e. vegetated cover, trees, or artificial cover such as benches)—that was reflective of very local habitat structure and potential risk assessment. While visibility can affect fear response, we accounted for potential variation in fog retrospectively by including a time by month covariate as, anecdotally, fog cover in Southern California typically is earlier in the year and earlier in the day. To the best of our knowledge, hourly numeric cloud cover data do not exist for Southern California. A small subset of trials were missing data on distance to cover, which were imputed with the median value across all trials.

We conducted FID tests in 2018 and 2019 in non-urban sites. At sites other than UCLA, we conducted FIDs before COVID-19 lockdowns in 2018, 2019, and up to March 2020, as the campuses closed following "safer-at-home" measures. At UCLA, we conducted FIDs on birds in 2018, 2019, 2020 ($n_{\text{pre-pandemic}}=71$; $n_{\text{2020 anthropause}}=135$), 2021 ($n=131$), and 2022 ($n=67$). Most FIDs were conducted during the pre-breeding and breeding seasons—between January and July—as birds were more conspicuous, easier to find, and not in wintering flocks, which has been found to affect FIDs in other songbirds [28]. We measured FIDs of juncos before COVID-19 restrictions started on 14 March 2020 (i.e., "pre-pandemic"). In-person classes were cancelled on that date, and the campus was mostly closed thereafter (i.e., "during anthropause"). During this time, we attempted to re-measure FIDs for individuals every 2 weeks, though this was not possible universally due to the spontaneous nature of FID testing. We re-assessed individuals following COVID-19 lockdowns from January to June 2022 following UCLA returning to in-person instruction (i.e., “post anthropause”). We categorized these time periods as “pre-pandemic” (2018-March 14, 2020), “2020 anthropause” (March 25, 2020-July 2020), “2021 anthropause,” and “post-anthropause” (2022).

Because we were also tracking reproductive behaviour prior to COVID-19 lockdowns, we know with certainty that at UCLA all chicks were hatched following the cancelation of in-person classes, and thus a significant decrease in human activity. As such, all birds ringed at UCLA in 2021 and 2022 that were in their second-year plumage were likely to have been hatched and fledged during the COVID-19 lockdown, with minimal human activity relative to older birds on campus. We also followed reproductive activities in 2021 and ringed chicks in the nest, some of which returned to campus and were assayed as second-year birds.

Measuring Human Activity

To gauge human activity shifts in the city as a whole, we accessed Google’s Community Mobility Reports [29]. This dataset compiled data from smartphones to determine where users spent time across different place categories compares aggregated data to a pre-pandemic baseline for a given community (in this case, Los Angeles) per day. We used the categories: mean change in activity (averaged across categories) and time spent in residential places as variables in our analysis. Because this dataset only began in February 2020, we calculated the mean and standard deviation for pre-pandemic levels (15 February to 14 March 2020) and randomly generated a normal distribution with the calculated mean and standard deviation. We imputed Los Angeles Community Mobility data for each date before 15 February 2020 randomly from this normal distribution.

Statistical Analysis

To determine which variables were important to include in GLMMs, we first ran a regression tree analysis using UCLA data. Here, our dependent variable was flight initiation distance (m). Independent variables we included were: sex, month, time, UCLA's anthropause status+year (2018-2020 "pre-pandemic," "2020 anthropause," "2021 anthropause," 2022 "post-anthropause"), Google's mean change in activity in Los Angeles, Google's time spent in residential places in Los Angeles, distance from cover, and starting distance. Based on this analysis, UCLA's anthropause status+year, mean change in community mobility, distance from cover, time (before or after ~9am) and starting distance were variables that were found to diagnose differences in flight initiation distance. Mean change in activity and time spent in residential places were associated with each other, because mean change in activity is calculated using time spent in residential places, in combination with other variables. The regression tree analysis found that mean change in activity was a more important variable than time spent in residential areas. Thus, we included mean change in activity in our GLMM.

To test the assumption that urban dark-eyed juncos across Southern Californian cities exhibited parallel shifts in FID in comparison to non-urban dark-eyed juncos before the pandemic, we fitted a generalized linear mixed model (GLMM) using a gamma distribution and an inverse link function. We included city and starting distance as fixed effects and bird ID as a random effect. For this analysis, we only included dark-eyed juncos in Los Angeles (*n*=119), San Diego (*n*=33), and Santa Barbara (*n*=13) that were assayed before the COVID-19 pandemic. We aggregated data (*n*=25) across non-urban populations to compare to. We then determined within model contrasts to test if urban populations were different from each other and from non-urban juncos.

We then fitted a GLMM with a gamma distribution and an inverse link, fitting the distribution of our data. We included UCLA's anthropause status+year, a given individuals' trial number, mean change in community mobility use with the Los Angeles Google Community Mobility Reports data, and starting distance as fixed effects and bird identity as a random effect. We visualized individual shifts by subsetting juncos who were assessed before and during the anthropause and/or during and after the anthropause to determine if trends at the population level were repeated at the individual level (Figure 2). Due to lower sample sizes for samples within individuals across time periods, we combined 2020 and 2021 anthropause categories into one category. To determine if patterns in these data were driven by habituation-like processes, we fitted the same GLMM, but only considered 3 anthropause categories (i.e., "before," "during," and "after" the anthropause) instead of differentiating between 2020 and 2021. All other variables remained the same.

To assess early-life effects, we compared four cohorts of second-year juncos' FID at UCLA. Second-year birds assayed in 2019 (*n*=15) hatched and were assayed in a high-human-activity environment. Second-year birds assayed in 2020 (*n*=34) hatched with high human activity and were assayed in an anthropause environment. Second-year birds assayed in 2021 (*n*=11) hatched and were assayed in an anthropause environment. Second-year birds assayed in 2022 (*n*=10) hatched in an anthropause environment yet were assayed in a high-human-activity environment. We fitted a GLMM with a gamma distribution and an inverse link function. The fixed effects were “year second-year bird was assayed” (pre-pandemic, 2020 anthropause, 2021 anthropause, 2022 post-anthropause) and starting distance. Bird ID was included as a random effect. We calculated within-model contrasts to determine if there were significant differences in FID between groups. We tested the assumptions of all models by testing for normality and linearity of residuals, as well checking for multicollinearity between independent variables. Assumptions were met for all models.

All tests were done in R v. 4.2.2 [30]. Regression trees were built and tested using R packages RSAMPLE [31] and RPART [32]. GLMMs were built and analysed using LME4 [33]. Within-model contrasts were calculated using R packages GMODELS [34] and MULTCOMP [35].

Results

Across tested Southern California populations, urban juncos have a consistent decrease of fear response in comparison to non-urban conspecifics (Supplementary Information; Figure S1).

At the population-level, fear response remained relatively unchanged during campus closure in comparison to pre-pandemic levels (*N*=404). Upon campus reopening in the 2021-22 academic year, fear response in the 2022 breeding season was significantly reduced compared to measurements from prior breeding seasons (Figure 1). We found that campus closure status (X^2=16.37, df=3, p=0.001) strongly and significantly affected fear, but relative change in human activity (X^2=1.86, df=1, p=0.17), trial number (X^2=3.57, df=1, p=0.06), distance from cover (X^2=0.03, df=1, p=0.85), and starting distance (X^2=0.02, df=1, p=0.96) did not. Additionally, there was significant individual variation in fear (sd=0.23, p<0.0001) and time by month (sd=0.01|0.01, p=0.007; Figure S2). Collectively, individual juncos varied in their fear response, but the reopening and increase of human activity following restrictions significantly lowered fear across the population (p<0.001 in 2020 anthropause and 2021 anthropause fearfulness compared with post-anthropause fearfulness, and p=0.047 in pre-pandemic fearfulness compared with post-anthropause fearfulness; Figure 1; Figure S2; Table S1).

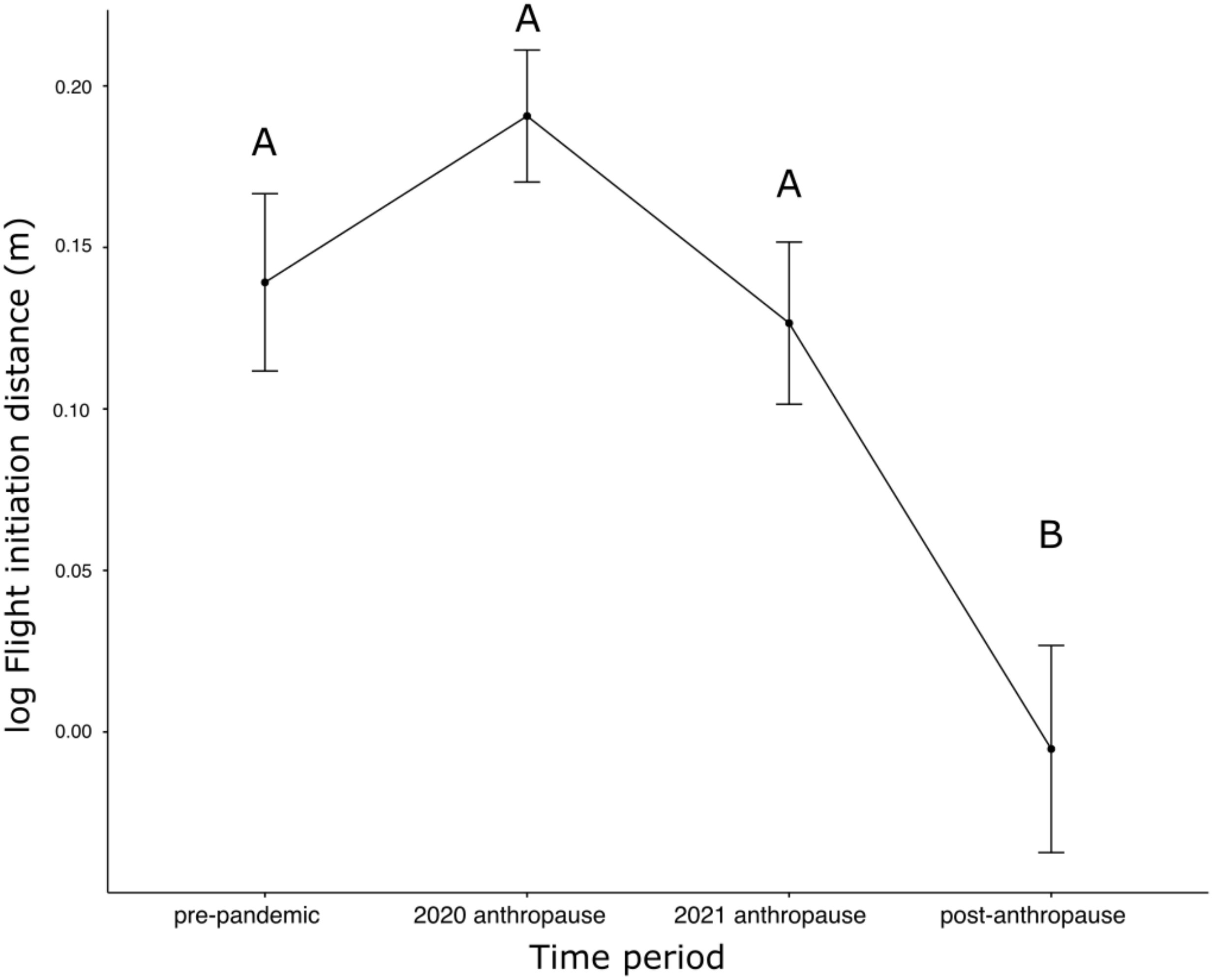

Fig. 1. Population-level fearfulness remained unchanged during lockdowns but decreased following reopenings. Population-level flight initiation distance (m) before (*n*=71), during (n_{2020}=135; n_{2021}=131), and after (*n*=67) the anthropause. The dark-eyed junco population at University of California Los Angeles (UCLA) did not shift their FID across the anthropause (GLMM contrast: p>0.05). FID significantly dropped in the 2022 post-anthropause environment in comparison to both years in the anthropause and the pre-pandemic baseline (GLMM contrasts: p<0.05 for pre-pandemic, 2020 anthropause, and 2021 anthropause compared to 2022 post-

anthropause). Flight initiation distance (FID) data are $\log_{10}$ transformed for visual aid, but not in the formal statistical analysis. Data points represent mean $\log_{10}$ FID ± standard error for each time period assessed. Groups with the same letter are not statistically significantly different from each other. Groups with different letters are statistically significantly different.

The patterns seen at the population level were repeated at the individual level (Figure 2; Table S2). A subset of individuals was tested repeatedly before, during, and after pandemic closures. While individuals varied in their fear response once lockdowns occurred, individuals nearly universally became less fearful following campus reopening (*n*=10 of 11). These results are consistent with the hypothesis that behavioural plasticity in fear response explained the pattern at the population-level. While we expected individual birds to become more tolerant to humans during the closures and return to a pre-pandemic fear response following reopening, we found that urban dark-eyed juncos had a surprisingly plastic response to increased human activity, but not to decreased human activity.

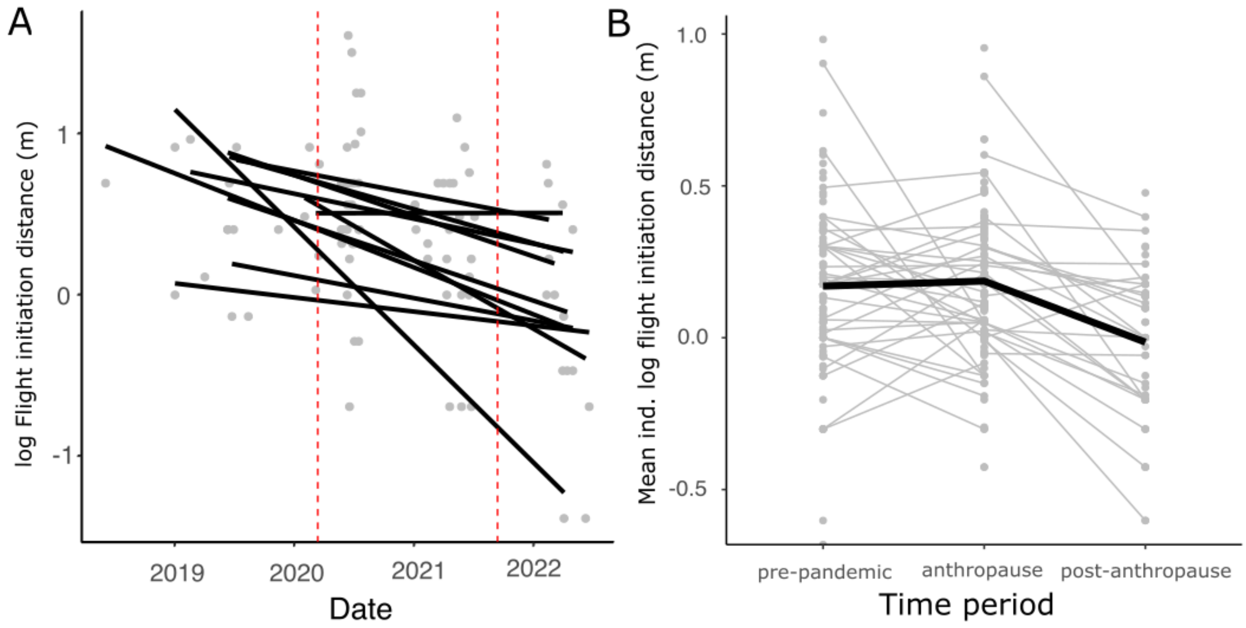

Fig. 2. Individuals became less fearful after reopenings in comparison to before the pandemic closures. Individual shifts in flight initiation distance (FID) before, during, and after anthropause. A. FID measurements only for individuals that were tested repeatably for at least one time point in each period: before, during, and after the campus closures (*n*=11). Trends reflect a similar decrease in FID from before the pandemic to the end of the anthropause. Lines are fitted linearly to demonstrate the change from before to after the anthropause. Each line represents one individual. Dashed red vertical lines denote the beginning and end of the anthropause, respectively. A GLMM, only including birds tested across all time periods and accounting for potential habituation (by including trial number) and treating the anthropause as a single category were similar. Here, differences in pre-pandemic fearfulness compared with post-anthropause fearfulness were significant (p=0.09) and differences in anthropause compared to post-anthropause fearfulness were significant (p=0.02) B. Mean FID values per individual (gray) in pre-pandemic, anthropause, and following reopening—"post-anthropause"—time periods. The thick black line represents shifts across all individuals sampled repeatedly between the pre-pandemic and anthropause (*n*=33) or the anthropause and post-anthropause time periods (*n*=24). These demonstrate pairwise shifts in mean fear response for each individual to account for individuals that might not have been tested in one of the time periods. A GLMM accounting for potential habituation (by including trial number), closest cover, time by month, and considering the anthropause as a single category, revealed significant pairwise differences between pre-pandemic and post-anthropause environments (p=0.02) and between the anthropause and post-anthropause environments (p<0.001). Though FIDs were not $\log_{10}$ transformed in our analyses (given their gamma distribution), they are $\log_{10}$ transformed here for visual ease.

Discussion

We found that fear response did not shift at the height of the COVID-19 closures, in comparison to before the pandemic, but that the re-introduction of humans led to a decrease in fear both at the population-level and at the individual-level. Thus, this urban bird did not become more like its wildland counterparts without human presence but rather, when faced with more human activity, became even less fearful than pre-pandemic levels, which is already less fearful than wildland birds. That dark-eyed juncos did not increase fearfulness with decreased human activity, even amongst birds who hatched during the COVID-19 closures (Supplementary Information; Figure S3), suggests that lower urban fear response is not dynamically driven by habituation-like processes—if these processes have a role in explaining tolerance at all. Indeed, habituation-like processes were controlled for at the individual level in all our analyses and do not explain the population-wide pattern we found. Rather, fear response expression is likely more complex and suggests that studying their ontogeny will be particularly illuminating. It could be that urban colonists from non-urban origins might have a lower baseline fear response in comparison to the non-urban population at large, and potentially decrease their fear response with human activity. We note, that in contrast to virtually all other studies of anthropause effects in birds (e.g., [1,3,4,36–38]) that used unmarked birds and were unable to focus on individuals, these insights emerged only from a detailed, longitudinal study of individuals.

A population increase coupled with a re-introduced landscape of fear might have led to higher competition for resources in 2022 and thus trade-offs favouring increased foraging despite higher perceived predator (human) risk. Changing patterns of human mobility shifted birds' use of space broadly across lockdowns [5], suggesting that human presence affects the habitability of urban spaces. Entering into COVID-19 lockdowns and reopenings, contrasting shifts in human activity—one increasing human activity and one decreasing human activity—led to contrasting fitness consequences reflecting this shift in the landscape of fear: great tits (*Parus major*) in an area with lower human activity had higher reproductive output than that with higher human activity [39]. However, there was no evidence for increased fitness by means of increased nestling condition and nest success when comparing 2021 and pre-pandemic 2019 breeding seasons in this population [25]. Additionally, there were no changes in aggressive interactions in the population following reopenings in 2022 and pre-pandemic 2019 breeding seasons [25], suggesting that fear response is not a by-product of shifting behavioural strategies due to a different socioecological context or due to indirect effects on predator density during the anthropause. Urban song rapidly shifted during San Francisco's lockdowns in a related species (white-crowned sparrows) potentially because there was a clear communicative signal being interrupted by urban stressors [2]. The relationships that exist between human activity and other urban behaviours appear more nuanced.

Fear responses could vary because ecological conditions changed, altering the trade-offs in escape behaviour following reopenings. Recent drought conditions in Southern California might have made urban birds more reliant on anthropogenic food to buffer declines in natural food resources—as was the case in an urban monkey [40]—leading to a higher tolerance of human presence in 2022. However, urban areas act as a buffer to arid conditions because of irrigation, supporting larger populations and diversity of arthropods [41]. Additionally, UCLA is an irrigated and green campus in an affluent area, which in turn is associated with increased irrigation and higher plant and bird diversity relative to non-urban arid conditions [42]. Thus, drought conditions in 2022 might not have caused strong detrimental effects, if any, to local urban resource distribution. Emergency regulations in California limiting turf irrigation only began in June 2022, making this a particularly unlikely explanation for lower fear response in the time period we were sampling, but something that could be accounted for in future studies given recent water-use restrictions.

Alternately, we could have been measuring the level juncos were distracted by stimuli. Indeed, escape behaviour can vary based on the number of stimuli as prey must divide their attention. With high human density and disturbances, prey can become distracted and either fail to respond as rapidly to an approaching threat or flee more rapidly [43,44]. Here, fear *response* in juncos would reflect the focal bird being more or less distracted, rather than more or less fearful. If distraction by the sudden increase in humans was responsible for the reduction in FID, we might expect that distracted animals were unable to detect an approaching human and therefore tolerated closer approach. Nonetheless, if fear response were solely modulated by distraction, we would have expected a higher fear response during the anthropause than after reopening; this pattern was not seen. Thus, juncos behaved surprisingly differently following the re-introduction of high human activity, though it could be due to them filtering stimuli differently than before the pandemic.

We propose two novel hypotheses explaining how fear response develops and is modulated in urban-adapted birds: urban bird fear response is either a ratchet or a spring. Birds that hatched during the anthropause mirrored the population as a whole: they became less fearful with increasing human activity following reopenings rather than expressing fearfulness at the same level as second year birds did before the pandemic, despite differences in their early life environment. Similarly, the population as a whole became less fearful than their pre-pandemic fear levels following reopenings (Supplementary Information). Thus, the prolonged absence of human activity followed by a rapid increase, rather than recurrent exposures to human activity, could be driving the expression of this fear response.

If a fear response acts as a spring that returns to pre-existing baseline with continuous exposure, we would expect that dark-eyed juncos will eventually re-sensitize to human activity and return to a pre-pandemic intermediate baseline. Alternatively, fear responses could change like a ratchet where each burst of rapid increases in human activity an urban bird is exposed to could lead to lower fear response. Rather than dynamically returning to a pre-pandemic baseline, a long absence coupled with a rapid burst of human activity drives an increase in human tolerance. Testing these hypotheses requires on-going study.

Conclusions

Collectively, our results suggest that changes in fear responses might not be as predictable as we might expect, and likely depends on *which* individuals and *how* their behaviours develop and shift in combination with strong and rapidly shifting collective human behaviours. Only through studies on individual animals tracked over time can we understand the mechanisms underlying population response, which cannot be confirmed from contradictory broadscale patterns found in metanalyses [36,38]. While the anthropause created much human hardship, it offered a unique opportunity to identify an important new avenue of ontogenetic research that can create insights which will help us better conserve biodiversity in a rapidly changing, human-dominated world.

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Preprint Statement

This paper has been uploaded as a preprint [45].

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Chapter Five: Supplementary Material

Fear response across Southern Californian populations

Urban populations across Southern California exhibited lower, yet similar FID to each other in a pre-pandemic baseline, in comparison to non-urban dark eyed juncos aggregated across non-urban populations (Figure S1). Dark-eyed junco population was a significant effect in the model (X^2=41.8, df=3, $p<0.0001$) and starting distance was not (X^2=2.59, df=1, p=0.108). All urban populations had non-significant differences in FID ($p>0.1$), but each was significantly lower than non-urban juncos ($p<0.01$), suggesting that time since urban colonization and population does not affect an urban junco population from shifting fear response lower.

Fear response across age cohorts

FID significantly varied by anthropause stage+year when assessing second-year bird fear response (X^2=8.10 df=3, p=0.03; starting distance: X^2=3.67; df=1, p=0.06; trial number: X^2=1.84; df=1, p=0.17). 2022 second-year juncos—those that hatched during the anthropause yet had exposure to human activity in adulthood—had a substantially lower FID in comparison to all other groups. These results were marginally significant in pairwise contrasts (pre-pandemic – post-anthropause: p=0.02; 2020 anthropause – 2022 post-anthropause: p=0.04; 2021 anthropause – 2022 post-anthropasue: p=0.09; Figure S3). However, all other groups did not significantly differ from each other ($p>0.90$). Low sample sizes likely affected power to detect differences between groups using an α=0.05.

Supplementary Tables

Table S1. Generalized linear mixed model (GLMM) results for population-level differences in flight initiation distance (FID) across years and anthropause status (before, during, after).

Fixed effects	X^2	*df*	*p-value*
Mean change in Los Angeles mobility across measured mobility categories	1.86	1	0.17
Trial number	3.57	1	0.06
Anthropause+Year	16.37	3	**0.001**
Starting distance	0.02	1	0.96
Distance from cover	0.03	1	0.85

Random effects	*variance*	*sd*	*p-value*
Bird ID	0.05	0.23	**< 0.0001**
Time|Month	0.01|0.01	0.09|0.1	**0.007**
Residual	0.23	0.48	

Number of observations	402
Log-likelihood	-430.5

Pairwise contrasts: Anthropause+Year	*estimate*	*se*	*z-score*	*p-value*
pre-pandemic – 2020 anthropause	-0.04	0.10	-0.43	0.97
pre-pandemic –2021 anthropause	-0.07	0.10	-0.69	0.9
pre-pandemic –post-anthropause	-0.31	0.12	-2.55	**0.047**
2020 anthropause – 2021 anthropause	-0.03	0.05	-0.56	0.94
2020 anthropause – post-anthropause	-0.27	0.07	-3.67	**< 0.001**
2021 anthropause – post-anthropause	-0.24	0.06	-3.85	**< 0.001**

GLMM is fitted to a gamma distribution with an inverse link. P-values for random effects were calculated by comparing models with and without the given random effect using ANOVA. Pairwise contrasts for anthropause stage categories for the model are included. Significant p-values are in bold. Significant p-values for pairwise contrasts reflect significant differences in FID within the model between the groups contrasted.

Table S2. Generalized linear mixed model (GLMM) results for individual-level differences in flight initiation distance (FID) across anthropause status (before, during, after) with 3 categories for COVID-19 closure status.

Fixed effects	X^2	*df*	*p-value*
Mean change in Los Angeles mobility across measured mobility categories	2.48	1	0.12
Trial number	6.38	1	**0.01**
Anthropause status	15.97	2	**0.0003**
Starting distance	0.01	1	0.92
Distance from cover	0.02	1	0.89

Random effects	*variance*	*sd*	*p-value*
Bird ID	0.05	0.22	**< 0.0001**
Time|Month	0.01|0.01	0.1|0.1	**0.003**
Residual	0.23	0.48	

Number of observations	402
Log-likelihood	-430.6

Pairwise contrasts: Anthropause	*estimate*	*se*	*z-score*	*p-value*
pre-pandemic – anthropause	-0.06	0.09	-0.65	0.63
pre-pandemic –post-anthropause	-0.31	0.12	-2.58	**0.024**
anthropause – post-anthropause	-0.25	0.06	-3.99	**< 0.001**

GLMM is fitted to a gamma distribution with an inverse link. P-values for random effects were calculated by comparing models with and without the given random effect using ANOVA. Pairwise contrasts for anthropause stage categories for the model are included. Significant p-values are in bold. Significant p-values for pairwise contrasts reflect significant differences in FID within the model between the groups contrasted.

Supplementary Figures

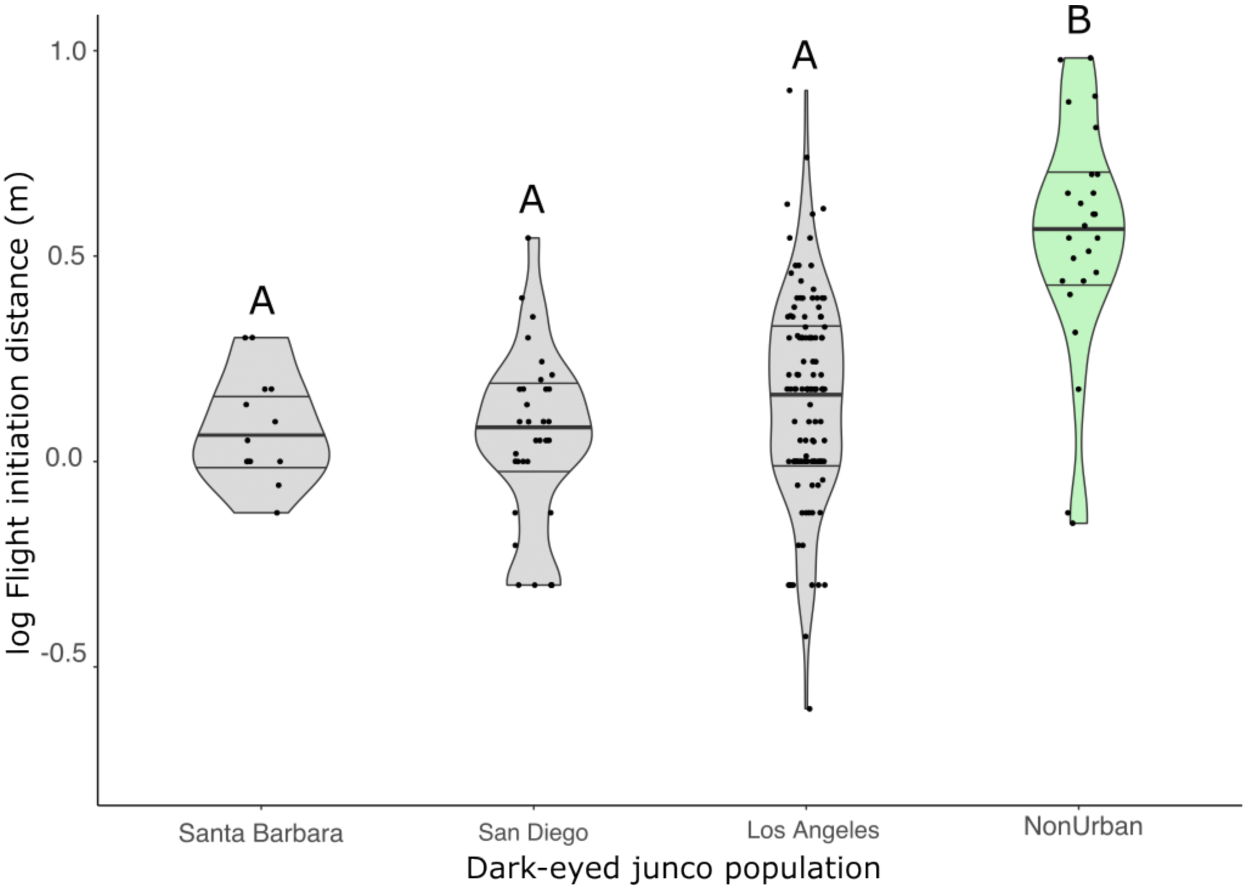

Fig. S1. Flight initiation distance (m) was lower across urban dark-eyed junco populations in comparison with non-urban populations in Southern California. Flight initiation distance in Los Angeles (*n*=119), San Diego (*n*=33), and Santa Barbara (*n*=13) were all significantly lower than that of non-urban dark-eyed juncos (*n*=25) ($p<0.01$), yet not significantly lower from each other ($p>0.05$). Flight initiation distance data are log-transformed for visual aid, but not in the analysis. Grey represents urban populations and green represents non-urban populations. Shaded violin plots represent the data distribution. Lines within the violin plots represent 25%, 50% (thicker center line), and 75% quantiles. Data points per category are jittered. Groups with the same letter are not statistically significantly different from each other. Groups with different letters are statistically significantly different.

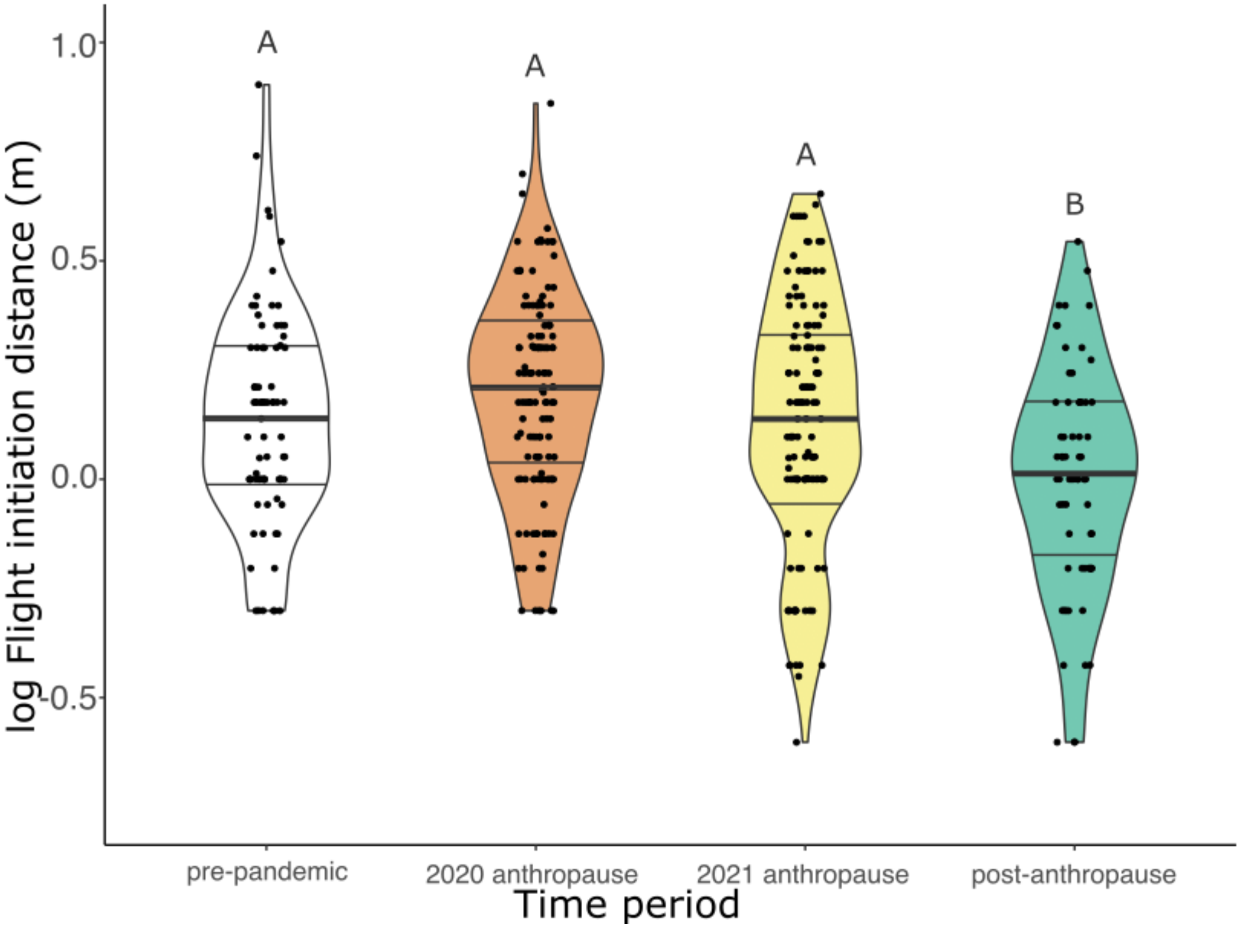

Fig. S2. Population-level fearfulness remained unchanged during lockdowns but decreased following reopenings, though exhibits strong variation across time. Population-level flight initiation distance (m) before ($n_{2018+2019+2020 \text{ pre-pandemic}}$ =71), during ($n_{2020 \text{ anthropause}}$=135; $n_{2021 \text{ anthropause}}$=131), and after ($n_{2022}$=67) the anthropause. The dark-eyed junco population at University of California Los Angeles (UCLA) did not shift their FID across campus closures (GLMM contrasts: $p>0.05$. FID significantly dropped in the 2022 post-anthropause environment in comparison to both years in the anthropause and the pre-pandemic baseline (GLMM contrasts: $p<0.05$). Flight initiation distance (FID) data are log-transformed for visual aid, but not in the analysis. Shaded violin plots represent the data distribution, with white represented a pre-pandemic baseline, orange-red representing closures in UCLA and Los Angeles as whole, yellow representing the lifting of some but not all restrictions, and green representing full reopenings and the end of the anthropause. Lines within the violin plots represent 25%, 50% (thicker center line), and 75% quantiles. Data points per category are jittered. Groups with the same letter are not statistically significantly different from each other. Groups with different letters are statistically significantly different.

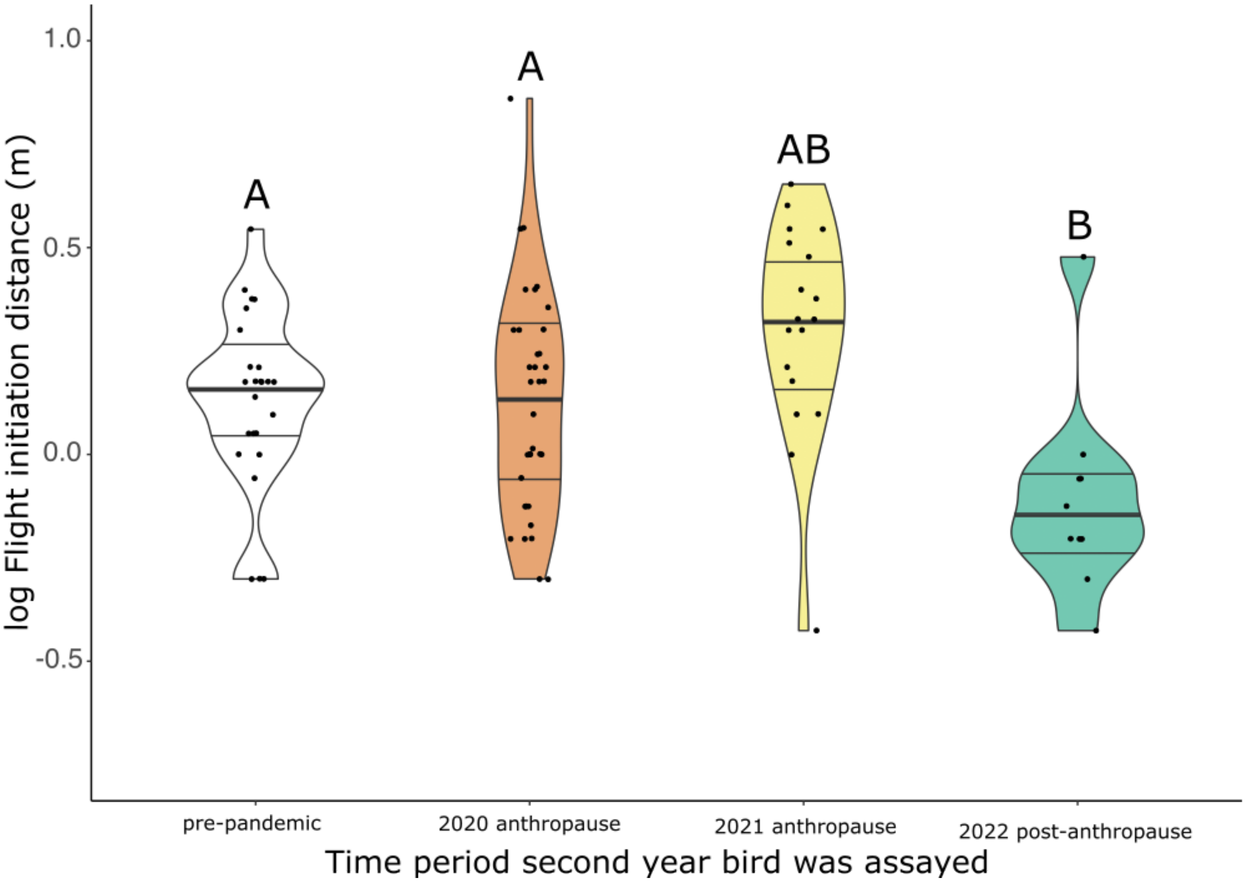

Fig. S3. Early life and adulthood exposure to human activity does not affect adult fear response. Each column corresponds to only second-year birds assayed in different years in a natural full factorial experiment. The left column represents second-year birds assayed in 2019 (*n*=15) hatched and were tested in a pre-pandemic, high human activity environment. Second-year birds assayed in 2020 (*n*=34) hatched in an environment with high human activity yet were assayed as adults during the anthropause. Next, second-year birds assayed in 2021 (*n*=11) hatched and were tested during the anthropause, never having been exposed to high human activity. Finally, the right column represents second-year birds assayed in 2022 (*n*=10): hatched during the anthropause and naively exposed to high human activity in adulthood. 2022 second-year birds have a marginally significantly shorter FID than the pre-pandemic group (2019-2022: p=0.04) with no clear distinction between other groups (2020-2022: p=0.11; 2021-2022: p=0.57; 2019-2020: p=0.97; 2019-2020: p=0.93; 2020-2021: p=0.78). Data are restricted to dark-eyed juncos at UCLA. Shaded violin plots represent the data distribution. Flight initiation distance data are log-transformed for visual aid, but not in the analysis. Shaded violin plots represent the data distribution, with white represented a pre-pandemic baseline, orange-red representing closures in UCLA and Los Angeles as whole, yellow representing the lifting of some but not all restrictions, and green representing full reopenings and the end of the anthropause. Lines within the violin plots represent 25%, 50% (thicker centre line), and 75% quantiles. Data points per category are jittered. Groups with the same letter are not statistically significantly different from each other. Groups with different letters are statistically significantly different.

Chapter Six: Phenotypic plasticity in the anthropause: Does reduced human activity impact novel nesting behaviour in an urban bird?[3]

Abstract

The COVID-19 pandemic temporarily transformed urban ecosystems by restricting public human activity to only the most essential societal functions, even as other landscape-level factors such as the built environment remained unchanged. In so doing, it provided a unique opportunity to experimentally answer questions about the role of human disturbance in driving behavioural adaptation in urban wildlife. We compared nesting data collected on an urban dark-eyed junco (*Junco hyemalis*) population nesting on the University of California, Los Angeles (UCLA) campus during the 2021 nesting season, when the campus restrictions were in effect, to a similar dataset collected in 2019, before the pandemic, in order to examine (1) whether urban dark-eyed juncos on the UCLA campus altered their use of novel off-ground and artificial nesting sites in response to reduced human activity, and (2) if reduced human activity impacted nesting success. We found that after a >80% reduction in human activity, junco nesting success during the COVID-19 pandemic modestly increased compared to pre-pandemic levels. However, nest-site selection remained unchanged. Our findings suggest that the landscape of the built environment or urban predators, rather than disturbance by human activity, drives novel nest-site selection in urban birds.

Keywords: COVID-19, dark-eyed junco, human disturbance, urban adaptation, urbanization

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[3] This chapter is in revision and provisionally accepted for publication Animal Behaviour with the following author order: Samuel A. Bressler*, Eleanor S. Diamant*, Christina Cen, and Pamela J. Yeh.
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Introduction

The COVID-19 pandemic, and the social distancing, isolation, and quarantine measures enacted in response to it, resulted in the extraordinary, multi-year phenomenon of cities emptied of people, with environmental effects ranging from reduced human and vehicular traffic (Marinello et al., 2021) to attenuated smog (Ravina et al., 2022) and sound pollution (Aletta et al., 2020; Guenaga et al., 2021; Lecocq et al., 2020). The unique environmental conditions arising in this period, subsequently dubbed the “anthropause” (Rutz et al., 2020), radically shifted wildlife behaviour and ecology in many cities across the world (Manenti et al., 2020; Miraglia & Di Brita, 2022; Montgomery et al., 2021). Furthermore, the anthropause created the conditions of a natural experiment for testing the direct impact of human activity on urban wildlife. In the short-period of time since the onset of the COVID-19 pandemic, urban ecologists have linked reduced human activity in urban areas to a range of effects on animals, including declines in vehicle-induced mortality in hedgehogs (Łopucki et al., 2021), increased use of urban areas by birds (Schrimpf et al., 2021), a shift to lower song frequencies in urban white-crowned sparrows (Derryberry et al., 2020), and a shift in spatial foraging patterns by urban mountain lions (Benson et al., 2021). While most national and regional governments have largely lifted pandemic-era restrictions on human activity and movement, ecological and behavioural data collected during this period continues to inform our understanding of the relationship between humans and wildlife in the urban environment.

Prior research across many species and cities has established and quantified the impacts of human activity on urban animal behaviour. For example, urban wildlife typically exhibit reduced fear response (Møller, 2008; Samia et al., 2015; Stansell et al., 2022), reduced stress levels compared to their wildland counterparts (Atwell et al., 2012; French et al., 2008), and vocalize at different times of day to avoid competition with anthropogenic noise (Arroyo-Solís et al., 2013; Cartwright et al., 2014; Vargas-Salinas et al., 2014). Nevertheless, studies of human-animal interactions in cities are limited by human omnipresence itself: we cannot shut down a city to study how animals might respond to our absence. As a result, the extent to which organisms respond to the physical presence of humans, versus our infrastructure, landscapes, and commensals, is difficult to tease apart. The pandemic-driven decoupling of human activity from other urban features has made it possible to experimentally test whether behaviourally plastic traits in urban wildlife vary in response to human activity or are tied to the built environment (Bates et al., 2020; Derryberry et al., 2020; Seress et al., 2021). Urban birds, due to their abundance and conspicuousness within the city, as well as their dominance within the prior urban ecology literature, are an ideal model for testing these hypotheses.

Nesting behaviour in birds is an important natural history trait that is subject to filtration, plasticity, and natural selection in urban areas (Croci et al., 2008; Reynolds et al., 2019). Cities often have stressors that select against certain nesting guilds, such as ground nesters (Cooper et al., 2020; Croci et al., 2008; Evans et al., 2011), while favouring those that can make use of artificial cavities (Jokimäki et al., 2016; Tomasevic & Marzluff, 2017). In addition, many species that do adapt to city life develop flexibility in reproductive traits. For example, many urban bird populations use a wider variety of nest sites than their wildland conspecifics (Yeh et al., 2007), develop modified breeding phenology (Capilla-Lasheras et al., 2022; Fudickar et al., 2017), and incorporate anthropogenic materials into their nests (Antczak et al., 2010; Potvin et al., 2021; Suárez-Rodríguez et al., 2013). At least some evidence indicates that birds may alter nesting behaviour in urban areas to cope with increased levels of human activity. For example, nest height in a Singaporean population of Eurasian magpies (*Pica pica*) was positively correlated with pedestrian activity (Wang et al., 2008), while species with less plastic nesting strategies, such as American kestrels (*Falco sparverius*), reduced incubation and nest provisioning in response to human presence (Strasser & Heath, 2013).

The COVID-19 pandemic created the conditions for comparisons of urban nesting behaviour before and after the onset of reduced human activity. In doing so, it opened up new lines of inquiry into the impact of human activity on nesting behaviour, such as the relative importance of human disturbance on nest-site placement, as opposed to other potential drivers of urban nesting behaviour, such as predation by human commensals (Schmidt et al., 2006) or the inherent desirability of novel nesting sites in urban areas (Bressler et al., 2020; Finley & Finley, 1924).

The dark-eyed junco (*Junco hyemalis*) is a small sparrow that breeds in woodlands and forests throughout much of North America (Nolan et al., 2020). In Southern California, this species was historically restricted as a breeder to montane wildland habitats for nesting, but over the past several decades, it has successfully colonized numerous cities in Southern California (Bressler et al., 2020; Yeh & Price, 2004). Urban juncos have diverged from their wildland counterparts behaviourally, including through reduced aggression (Newman et al., 2006), a longer breeding season (Price et al., 2008), and the use of novel nesting sites (Yeh et al., 2007). Novel nesting behaviours adopted by urban juncos include an increased use of off-ground nesting, largely driven by abundant use of crevices and ledges on artificial structures for nesting. This behaviour is correlated to increased nesting success in cities (Bressler et al., 2020; Yeh et al., 2007). Individual juncos also display a high level of nesting plasticity, as breeding pairs have been observed to shift between the use of traditional and novel nesting sites; furthermore, informed re-nesting has been demonstrated in at least one population: pairs are more likely to select a nest site with similar characteristics to a prior nest if the prior nesting attempt was successful, rather than a failure (Bressler et al., 2020). Thus, if nest placement is sensitive to human disturbance, then shifts in nest locations should be readily observable with changes in human activity.

In this paper, we examine the impact of an unexpected reduction in levels of human activity due to the COVID-19 pandemic on nesting behaviour in juncos on the campus of the University of California, Los Angeles (UCLA). We compared the proportion of nests placed off the ground and in novel nesting locations during the 2021 breeding season—when human activity was severely reduced as a result of limitations on campus access during the COVID-19 pandemic—with data previously collected during the 2019 breeding season before the pandemic. We predicted that if novel nest placement were a reaction to disturbance by human activity, fewer nests would be placed off-ground in 2021 than in 2019. By contrast, if nest placement were driven by landscape-level, non-human factors such as availability of nest sites, then there would be no difference in off-ground nesting between 2019 and 2021. Understanding the drivers of nest placement in cities will better inform wildlife and habitat management not just in cities but wherever humans and wildlife coexist.

Methods

Study Site and Field Methods

The UCLA campus is located in western Los Angeles, California. It lies in the foothills of the Santa Monica Mountains and has a Mediterranean climate characterized by hot, dry summers and cool, wet winters. The campus is heavily urbanized, with many large buildings interspersed with stretches of lawns, planted trees (particularly non-native pines (*Pinus* sp.), sycamores

(*Platanus* sp.), and various palm species (family Arecaceae). The campus is surrounded by the economically affluent neighbourhoods of Westwood, Beverly Hills, and Bel Air, and lies within a matrix of urban commercial and suburban residential development with a high level of relatively high vegetation and tree canopy cover. The nearest undeveloped open space is 2 km away in the Santa Monica Mountains.

As a result of the coronavirus pandemic, between March 2020 and September 2021, on-campus classes were moved online, a large majority of students left the on-campus residence halls, and research/laboratory activity was heavily curtailed on campus. On-campus amenities such as cafés and stores were closed, and any person entering campus was required to complete an online COVID-19 symptom questionnaire. These actions led to a dramatic decline in human activity on campus, as formerly bustling plazas, commons, and walkways emptied of people. While restrictions gradually loosened over the course of the pandemic, human activity would not approach pre-pandemic levels until the resumption of in-person classes in September 2021. Human pedestrian surveys were conducted across campus from May to July in 2021 and from April to June in 2022 to compare human activity during campus closure and after campus activities returned to normal conditions (Walters et al., 2023). Twice weekly, two-minute surveys were conducted at 13 sites distributed approximately evenly across campus. All pedestrians crossing a line of sight were recorded over the course. There was a six-fold increase in human activity—as a broad measure of the presence of people across campus—following campus re-opening in comparison to campus closures (Walters et al., 2023), suggesting a dramatic drop in human activity between pre-pandemic and pandemic conditions.

As part of a longer-term study, juncos have been mist-netted across much of the campus since 2017. Targeted netting using junco playback is employed to facilitate trapping, and individuals are fitted with one aluminium leg band and three coloured plastic leg bands for individual recognition before being released. A majority of nests identified in both years belonged to at least one banded adult.

Nest-hunting was conducted by teams of researchers led by one investigator in 2019 and a second investigator in 2021. The first investigator trained the second investigator in 2019. Methods employed in nest hunting remained identical between the 2 years. Nesting began each year in early March and was largely complete by early August; during this period, most pairs raised between two and four broods. Nests were located by observing and monitoring adult juncos during the nesting season for behavioural cues, and upon discovery, the height of the nest above ground was estimated, and whether the nest was placed on an artificial substrate (such as directly on a building ledge or within ivy covering the exterior of a building) or natural substrate (such as in ornamental vegetation). In the interest of conciseness, these two types of nest locations are hereafter referred to as “artificial” and “natural” nests respectively. Nests were found during the nest building stage by observing female juncos repeatedly depositing nesting material such as dried grass or pine needles in a concealed location. These nests were checked every second day until the first egg was laid, so that the first egg date could be ascertained. Nests were found during the nesting stage by monitoring female juncos animatedly foraging before retreating to a potential nesting site. Subsequently, the general area of the nest would be methodically searched until the nest was found, with special care being taken not to trample the nest during the process. Nests found or observed with eggs were checked weekly until hatching. Nests found or observed with young (<7 days old) nestlings were aged based on morphological and behavioural characteristics, and the nestlings were banded once they reached 7 to 8 days of age. Nests found with nestlings older than 8 days old were not banded to avoid pre-fledging the nest. Subsequently, all nests were monitored from a safe distance every 2 to 3 days until nestlings fledged and were observed being fed by adults outside the nest. Nests were considered successful if they produced at least one fledgling. Prior research of this population found that approximately 85% of nest failure was due to some form of predation (Bressler et al., 2020). While the cause of nest predation remained unknown for most nests, a variety of potential nest predators are common on the study site, including American crows (*Corvus*), feral and domestic cats (*Felis cattus*), eastern fox squirrels (*Sciurus niger*), and rats (*Rattus* sp.) At all stages of nest hunting and nest monitoring, care was taken to avoid causing nest predation by scanning the area for potential predators before approaching the nest, minimizing time spent at the nest during monitoring and banding sessions, and taking care to avoid unnecessary disturbance to surrounding vegetation.

Certain nests were not banded due to inaccessibility, particularly those placed on trees or buildings not accessible by a standard stepladder. These nests were instead monitored once every week until vocal nestlings could be heard (approximately 5 days after hatching), then every 2 to 3 days until fledging.

The location description and characteristics of each nest were logged using the Citizen Science app NestWatch. We estimated height in meters of each nest, and we also recorded the substrate in which each nest was built.

Statistical Analysis

All statistical analysis was conducted in R v. 3.6.1 (R Core Team, 2021). The primary variables examined in this study were: nest year (coded as a two-factor categorical variable, either 2019 or 2021), use of artificial substrate and use of off-ground sites (both of which were coded as binary variables), and age of the nest at discovery (calculated by subtracting the measured or estimated first egg date from the date the nest was first discovered). Discovery age was included as a variable in the models as nests placed higher up or in hard-to-reach locations, such as on trees or the sides of buildings, were more difficult to find than those at ground level or in low-growing vegetation, and often could only be confirmed at the nestling stage, when adult birds would be observed making frequent trips to the nest carrying food. As nests found at more advanced stages could be expected to show greater success (as fewer days between discovery and fledging would mean a smaller window for predation), including discovery age as an explanatory variable would remove this potential source of bias. This method was used instead of Mayfield's method of calculating daily survivorship rates (Mayfield, 1975; Shaffer, 2004) due to our monitoring protocol involving purposefully infrequent site visits, particularly during the incubation stage, to minimize disturbance. Parental identity was initially included as a random variable in all models but was subsequently removed as the number of nests observed was not sufficient to allow for model convergence. Longitudinal studies of individual pairs were not conducted due to lack of data, as there were only three banded female juncos for whom at least one nest was found in both 2019 and 2021.

A pair of two-proportion z-tests was used to determine the impact of reduced human activity in 2021 on off-ground nest placement and artificial substrate use. Additionally, we used a Bayesian analysis to determine posterior distributions of parameter values for a pair of logistic regression models. In each model, success was used as the response variable and discovery age was used as an explanatory variable. We were unable to determine the first egg date and discovery age for some nests; to avoid discarding samples, we imputed values for these nests using the multiple imputation package *mice* (van Buuren & Groothuis-Oudshoorn, 2011). An interaction variable between year and either artificial substrate or off-ground nest placement was incorporated into the first and second models, respectively. The off-ground and artificial substrate variables were not included in the same model because artificial substrate was entirely nested within the off-ground nest site and the phi coefficient of similarity (package *psych*) (Revelle, 2022) between the two variables showed a high degree of similarity (PHI = 0.67). We used a Bayesian approach to fit a logistic regression model as residuals were not normally distributed in a generalized linear mixed modelling approach and thus p-values could not be interpreted for significance. In our Bayesian logistic regression model, the posterior distribution of each variable's coefficient was calculated using 3 MCMC chains and 20,000 iterations per chain, with a thinning rate of 10. We used weakly informative priors, with a distribution of 0 - 0.1 for each coefficient, and a burn in of 5,000 iterations. Traces of the MCMC chains as well as posterior distribution density curves were assessed to ensure that chains did not diverge and that posterior distributions were informative (Figures S1 through S4). We interpreted independent variables as statistically affecting nest success when the 95% confidence intervals of their parameters did not overlap with 0. We removed interaction effects as their confidence intervals overlapped with zero (Figure S5), the posterior distribution of the term was normally distributed for all chains, and because of the challenges of interpreting interaction terms in non-linear models (Ai & Norton, 2003; Berry et al., 2010). We then re-ran the models without interaction effects. We checked the two models' fits (Conn et al., 2018) by determining that residuals were normally distributed (Figure S6), by conducting posterior predictive checks (Figure S7) and calculating Bayesian p-values. While Bayesian p-values are known to be conservative (Conn et al., 2018), we found strong goodness of fit (0.50 for both models). These methods suggested that both of our models were well fit for both the model with artificial substrate as a response variable and with off-ground nesting site as a response variable. These analyses were conducted using JAGS (Plummer, 2003) in R (R Core Team, 2021) using packages *R2Jags* (Su & Yajima, 2021), *MCMCvis* (Youngflesh, 2018), *jagsUI* (Kellner & Meredith, 2021) and *bayesmix* (Grün, 2004).

Ethical Note

All animals were cared for and tested according to institutional guideline and approval (IACUC #ARC-2018-007). Procedures were conducted under United States Geographical Survey Banding Permit 23809 and California Department of Fish and Wildlife Specific Collection Permits S-183270002-18337-001, S-183040004-18313-001, and S-193110002-20012-001. Mist nets were always monitored visually such that birds were immediately extracted upon capture. They were placed in a cotton bag and processed rapidly. During each stage, birds were monitored for signs of stress (panting, drooping eyelids) and released immediately in a safe location, such as under a bush, if they showed signs of stress. They were then observed from a distance to ensure they flew away safely. As part of an ongoing long-term study on urban juncos in Los Angeles, we aimed to individually band the UCLA junco population so that each could be individually identified and both the male and female in each pair would be uniquely known. Bands did not impede normal songbird behaviour.

Results

A total of 164 and 117 junco nests were found and monitored during the 2019 and 2021 nesting seasons, respectively. Of these, 38.5% of nests were placed off the ground in 2021, versus 34.8% in 2019. This difference was not significant ($X_1^2 = 0.26$; p = 0.61; Figure 1B).

Similarly, 22.2% of nests were placed on artificial substrates in 2021 versus 18.9% in 2019; this difference was also not significant ($X_1^2 = 0.28$; p = 0.59; Figure 1A).

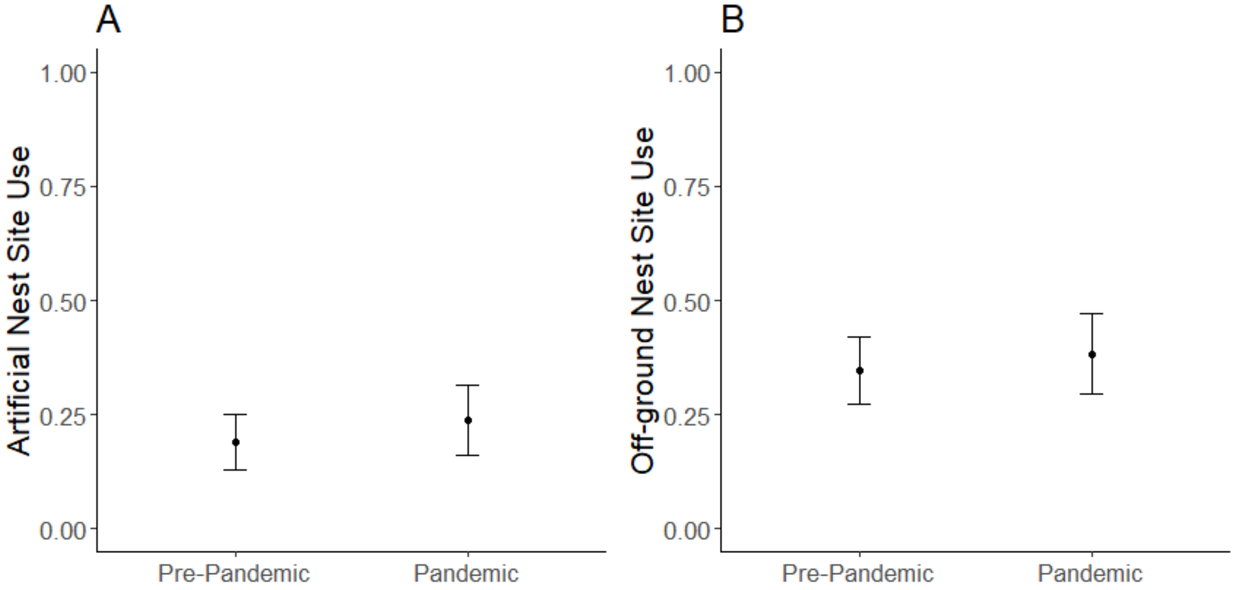

Figure 1. Effect of reduced human activity associated with the pandemic restrictions of 2021 on the frequency of (A) artificial and (B) off-ground nest site use. Error bars represent 95% confidence intervals. Neither artificial nor off-ground site use was significantly affected by reduced human activity in 2021 (p = 0.59 and p = 0.61 respectively).

Artificial nest placement had a positive effect on nest success compared to nests placed on non-artificial substrates (mean coefficient of artificial nest placement [95% CI] = 1.83 [0.74, 3.11]). Year had a positive effect on nest success (74.4% in 2019 versus 82.1% in 2021; mean coefficient of year [95% CI] = 0.69 [0.03, 1.37]; Figures 2A and S8). This model had a good fit, with a Bayesian p = 0.50.

Off-ground nesting also had a positive effect on success (mean coefficient [95% CI] = 1.16 [0.43, 1.95]). Year had a positive effect on nesting success in the model that included off-ground nesting but not artificial nesting (mean coefficient [95% CI] = 0.71 [0.05, 1.42]; Figures 2B and S8). This model had a good fit, with a Bayesian p = 0.50. We report the details of diagnostic plots in the supplement for both analyses (Figures S1-S4 and Figures S6-S7).

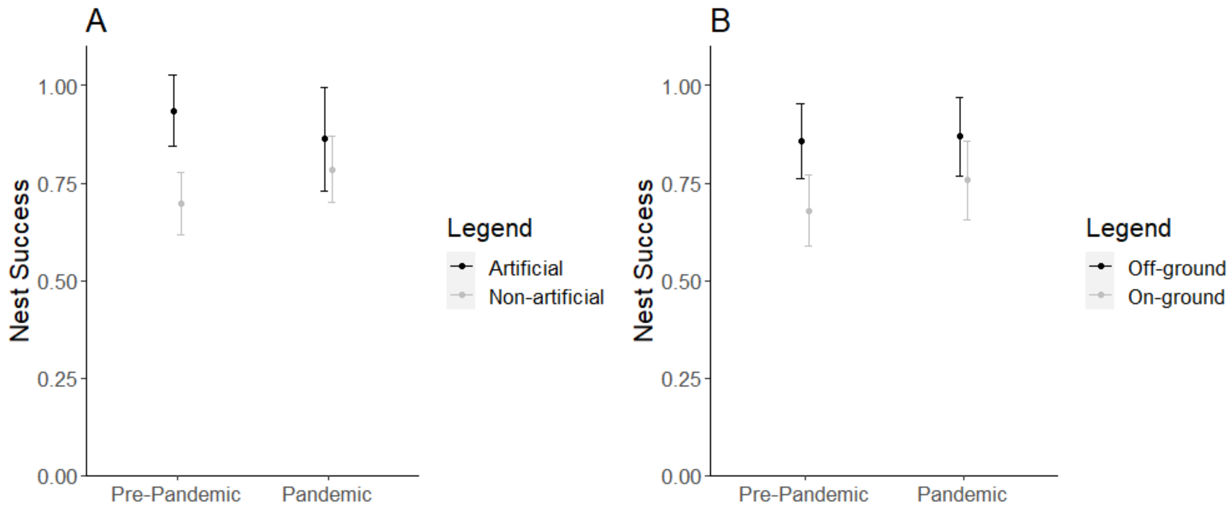

Figure 2. Effect of reduced human activity associated with the pandemic restrictions of 2021 on the relative nesting success of (A) artificial vs. non-artificial nests and (B) off-ground vs. on-ground nest sites. Error bars represent 95% confidence intervals. Reduced human activity predicted nest success in both models, though the confidence interval is close to zero (95% CI) = 0.69 (0.03, 1.37) in the former and (95% CI) = 0.71 (0.05, 1.42) in the latter model.

Discussion

We found that reduced human activity during the anthropause did not significantly change the frequency of novel nesting behaviours in response to reduced human activity in dark-eyed juncos. Despite the greatly reduced human footprint in the study site during 2021, the use of artificial and off-ground nesting sites did not change. We also found a small but significant increase in nest success during the pandemic. Our findings suggest that plasticity in nest-site selection of this established population of urban juncos is likely not driven by human activity and disturbance.

Other studies have found little to no impact of the anthropause on many urban bird behaviours or life history metrics, particularly those associated with reproduction. For instance, pandemic-induced restrictions on human recreation in urban parks did not impact nestling body size in great tits (*Parus major*) (Seress et al., 2021), various reproductive life history traits such as first egg date and clutch size in blue tits (*Parus cyanistes*) and great tits (Corsini et al., 2022), or fear response in dark-eyed juncos (Diamant et al., 2023). These results contrast with other studies finding impacts of reduced human activity on behaviours associated with communication, such as changes in song characteristics in urban white-crowned sparrows (*Zonotrichia leucophrys*) (Derryberry et al., 2020), a shift to earlier singing times in a Catalonia bird community (Gordo et al., 2021), and reduced territorial aggression in dark-eyed juncos (Walters et al., 2022).

What can be made of these contradictory effects of reduced human activity on urban avian behaviour? One possible explanation is that urban bird behaviour is affected more strongly by human disturbance in the realm of communication compared to predation or other threats. That is, human activity may contribute less to the landscape of fear that governs individual anti-predator behaviours and decision-making (Laundre et al., 2010), but birds nevertheless do respond behaviourally to the *masking* effect of human activity on essential communication such as establishing territories, attracting mates, and responding to interlopers. This has not been universally observed, however. A few pandemic-era studies contradict this pattern: for nestling great tits, there were no differences in condition in urban parks that saw reduced human activity during the pandemic compared to pre-pandemic conditions, but nestling condition decreased in parks that experienced abnormally high levels of human recreation during the same time period (Seress et al., 2021).

It is possible that our finding of no change in nest site selection might be due to the unusually rapid collapse in human activity, and that if anthropause levels of human activity continued for more than 2 years, then the juncos might gradually have shifted their nesting behaviour. However, we consider this scenario unlikely, as high levels of nesting plasticity have been found within individual pairs: rather than most pairs exhibiting either nesting exclusively on or exclusively off the ground, many pairs switch repeatedly between the two strategies, at least partially in response to prior nest success (Bressler et al., 2020). Thus, we would expect real or perceived changes in site suitability to be rapidly incorporated into nesting decisions. Juncos in this population have been shown to use information gleaned from their environment in choosing nesting sites (Bressler et al., 2020), but human presence does not appear to contribute to the decision-making process.

Our findings suggest that novel nest site selection is driven by the urban landscape structure itself, rather than direct human activity. Instead of predation pressure "pushing" juncos to novel nesting site selection, juncos might instead be "pulled" to these atypical nesting sites due to the relative abundance of these sites in urban environments. Such a pre-existing affinity for artificial nest sites has been hypothesized as the reason why chimney swifts (*Chaetura pelagica*) began using chimneys and similar structures as nesting and roosting sites as soon as several decades after the arrival of Europeans in North America (Graves, 2004). Cities contain abundant novel nesting sites, such as window ledges and outdoor lamps that are of sufficient size and shape to host junco nests.

Alternatively, novel nesting behaviours may be a response to predation pressure by cats, rats, fox squirrels, or other human commensal mesopredators. Predation is one of the primary threats to birds in urban areas, particularly ground-nesting species such as juncos. Indeed, artificial ground nests have had a higher predation rate closer to town centres in Finland (Jokimäki & Huhta, 2000). Predation pressure is a known driver of nesting plasticity in a range of songbirds, including orange-crowned warblers (Hays et al., 2022; Peluc et al., 2008), Siberian jays (Eggers et al., 2006), and dusky warblers (Forstmeier & Weiss, 2004). Furthermore, mesopredator abundance, spatial distribution, and behaviours were also impacted by the pandemic; for example, urban Norway rats (*Rattus norvegicus*) in Sydney, Australia, declined in abundance after the initial stages of lockdown (Bedoya-Pérez et al., 2020), while rat populations in New York, USA, and Tokyo, Japan, shifted spatially to forage closer to clusters of urban restaurants. However, such shifts were not found in Warsaw, Poland (Parsons et al., 2021).

There may have been little fitness benefit for juncos to switch to ground nesting in response to reduced human activity if predation pressure remained unchanged; indeed, predation pressure might even increase if mesopredators lost access to human scraps and refuse and subsequently switched to natural food sources such as eggs and nestlings. Our analysis of reduced human activity on nesting success did not yield clear results regarding mesopredators. Our models predicted a small but significant increase in nesting success between 2019 and 2021, but this may not be due to a lack of human activity. The 8% difference in nest success between 2019 and 2021 was roughly in-line with year-to-year differences in an intensive four-year junco nesting study conducted by Yeh et al. (2007), which reported annual nesting success rates within a 20% range (36-56%) over the course of the study. Thus the differences in nest success we found may reflect natural stochasticity in nesting success due to factors other than human disturbance. The interaction between nesting behaviour and year did not predict nest success in either model, suggesting that reduced human activity did not differentially impact nest success in artificial versus natural nest sites.

Individuals, populations, and species lacking behavioural responses to human activity and disturbance may be superior competitors in urban environments. Human-tolerant populations such as the Los Angeles juncos may navigate the urban landscape differently from either their non-urban ancestors, or conspecific or heterospecific populations residing in nearby wildlands. For these individuals, human presence may be simply an incidental component of their environment, contributing minimally to behaviour and decision-making. Such individuals would minimize energy-costly and maladaptive fear responses such as alarm calling and mobbing, thus increasing reproductive success compared to less tolerant species. If tolerance of humans occurs concurrently with invasion of urban habitats, this might lead to a priority effect in urban community assembly, whereby early-colonizing species have more time to habituate to human activities and thereby outcompete more recent, less-adapted colonizers more likely to prioritize human avoidance (Alberti et al., 2020). Alternatively, tolerance of humans may serve as an ecological filter, whereby only species that are pre-adapted or possess sufficient behavioural variation are able to colonize urban areas (Cooper et al., 2020; Croci et al., 2008). Urban juncos seem to be one such example of this: in addition to nesting behaviour, fear response was not impacted by reduced human activity (Diamant et al., 2023); this tolerance of human disturbance might be mediated hormonally, such as through reduced corticosterone levels in urban birds (Atwell et al., 2012). In the case of nesting, this adaptive plasticity where nest site location is driven by the landscape might facilitate adaptive evolution (Ghalambor et al., 2007).

While early headlines in popular newspapers emphasized the novel ways birds and other animals adapted to human absence from urban spaces (Silva-Rodríguez et al., 2021), one of the enduring legacies of the COVID-19 anthropause in the field of urban ecology may be how urban wildlife maintained much of their typical behaviour and ecology in the face of a nearly unprecedented transformation of their ecosystem. The indirect impacts of human presence on urban wildlife, from community assemblage to adaptation, are a defining characteristic of the urban ecosystem itself, so it is perhaps ironic that some behaviours in certain urban-adapted species are not strongly impacted by physical human presence. Further research on avian behaviour during and after the anthropause will more clearly identify the nature, extent, and limits of avian responses to human disturbance.

Data availability: data is available for peer review through figshare.com: https://figshare.com/s/1e55b465dd00f99c1ae7. Data will be made publicly available upon acceptance.

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Chapter Six: Supplementary Materials

Supplementary Material for:

Phenotypic plasticity in the anthropause: Does reduced human activity impact novel nesting behaviour in an urban bird?

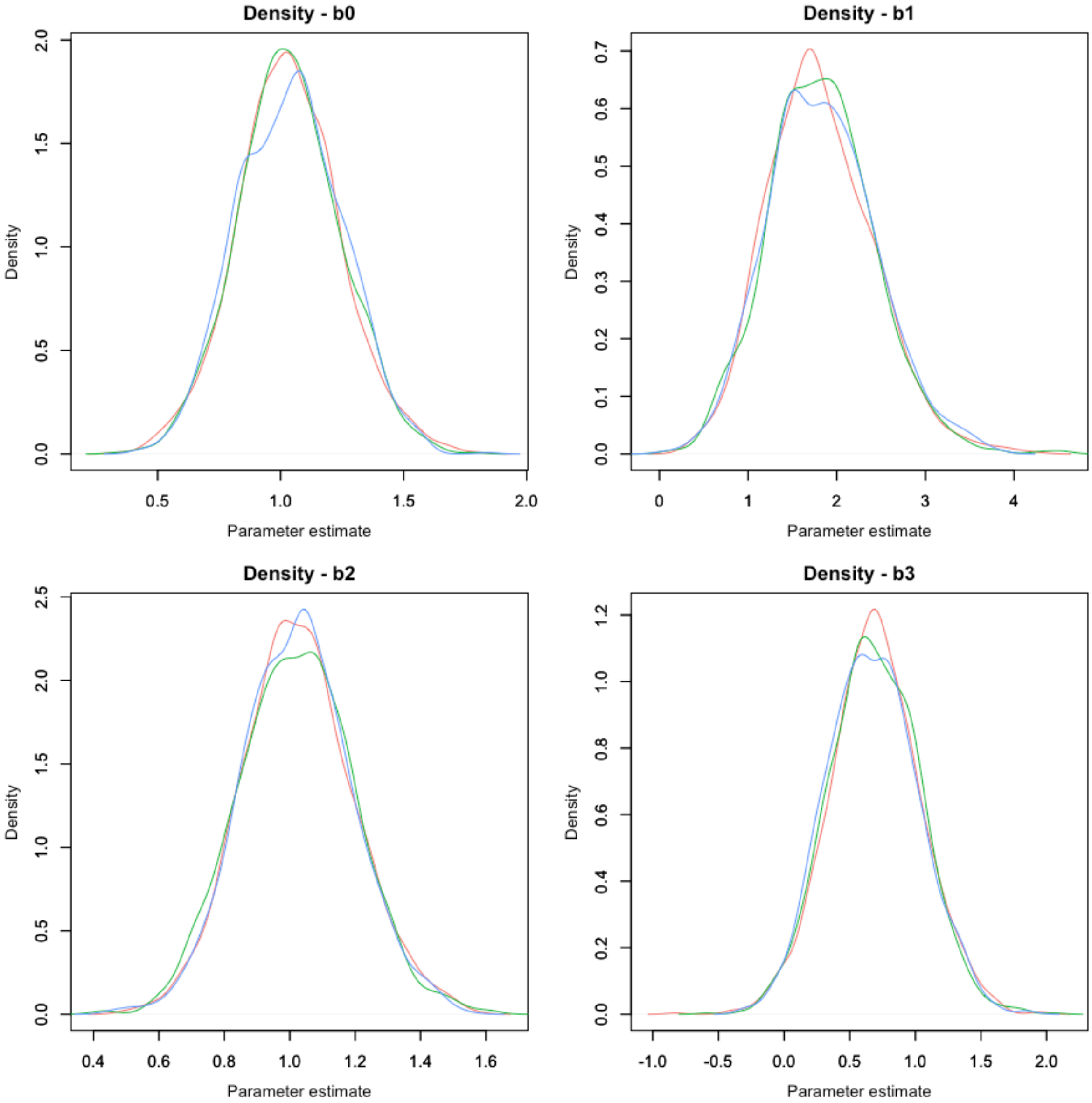

Fig. S1. Density plots of the posterior distributions of parameter estimates per MCMC chain for modeling a logistic regression with artificial (vs. non-artificial) nest site as a predictor. Here, b0 refers to the intercept, b1 to the coefficient of artificial (vs. non-artificial) nest site, b2 to discovery age, and b3 to year. Each color (red, blue, and green) refers to one MCMC chain. Priors were distributed between 0 and 0.1 for all parameters.

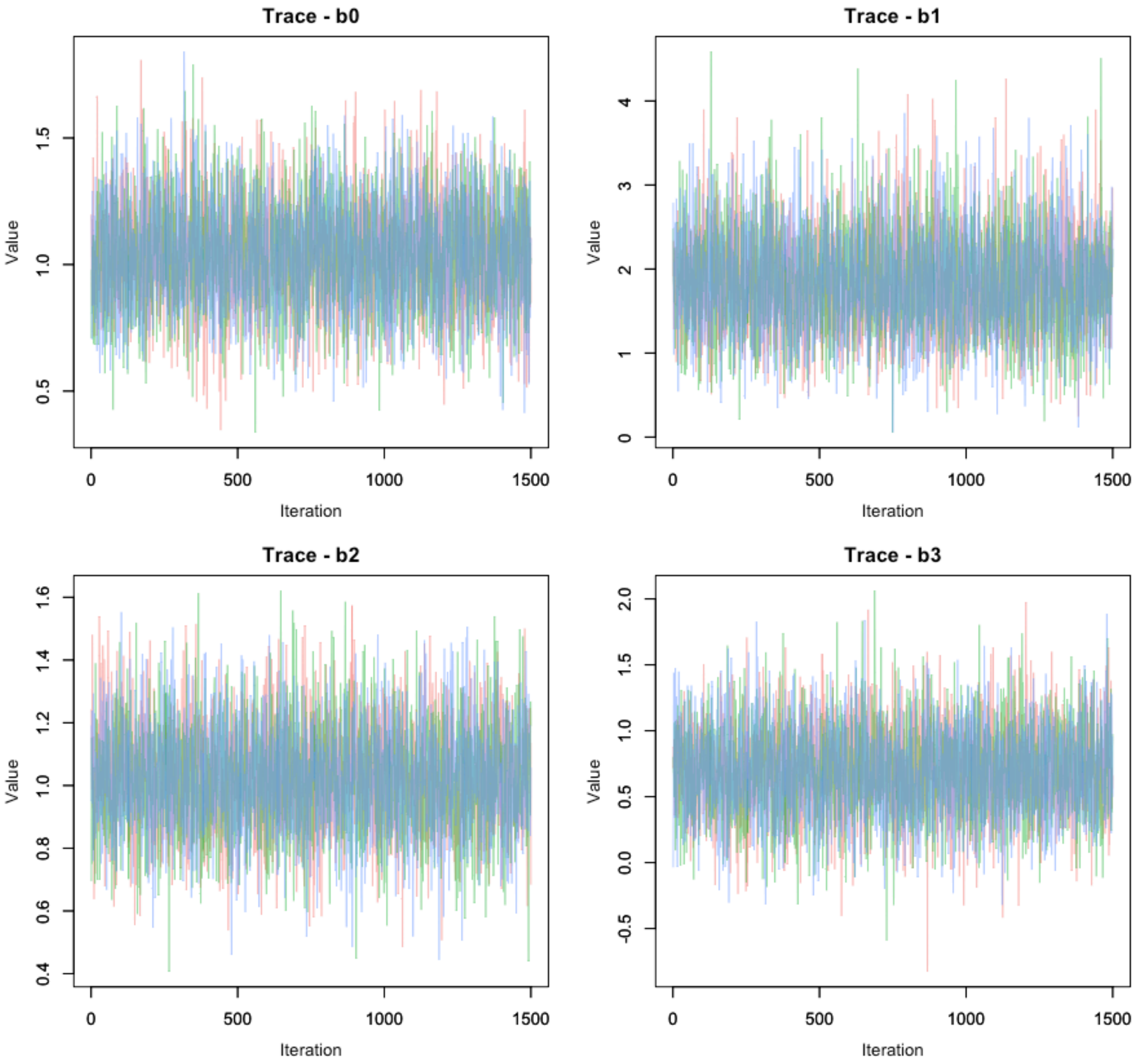

Fig. S2. Trace plots of the parameter estimates per MCMC chain over all iterations for modeling a logistic regression with artificial (vs. non-artificial) nest site as a predictor. Here, b0 refers to the intercept, b1 to the coefficient of artificial (vs. non-artificial) nest site, b2 to discovery age, and b3 to year. Each color (red, blue, and green) refers to one MCMC chain. Priors were distributed between 0 and 0.1 for all parameters.

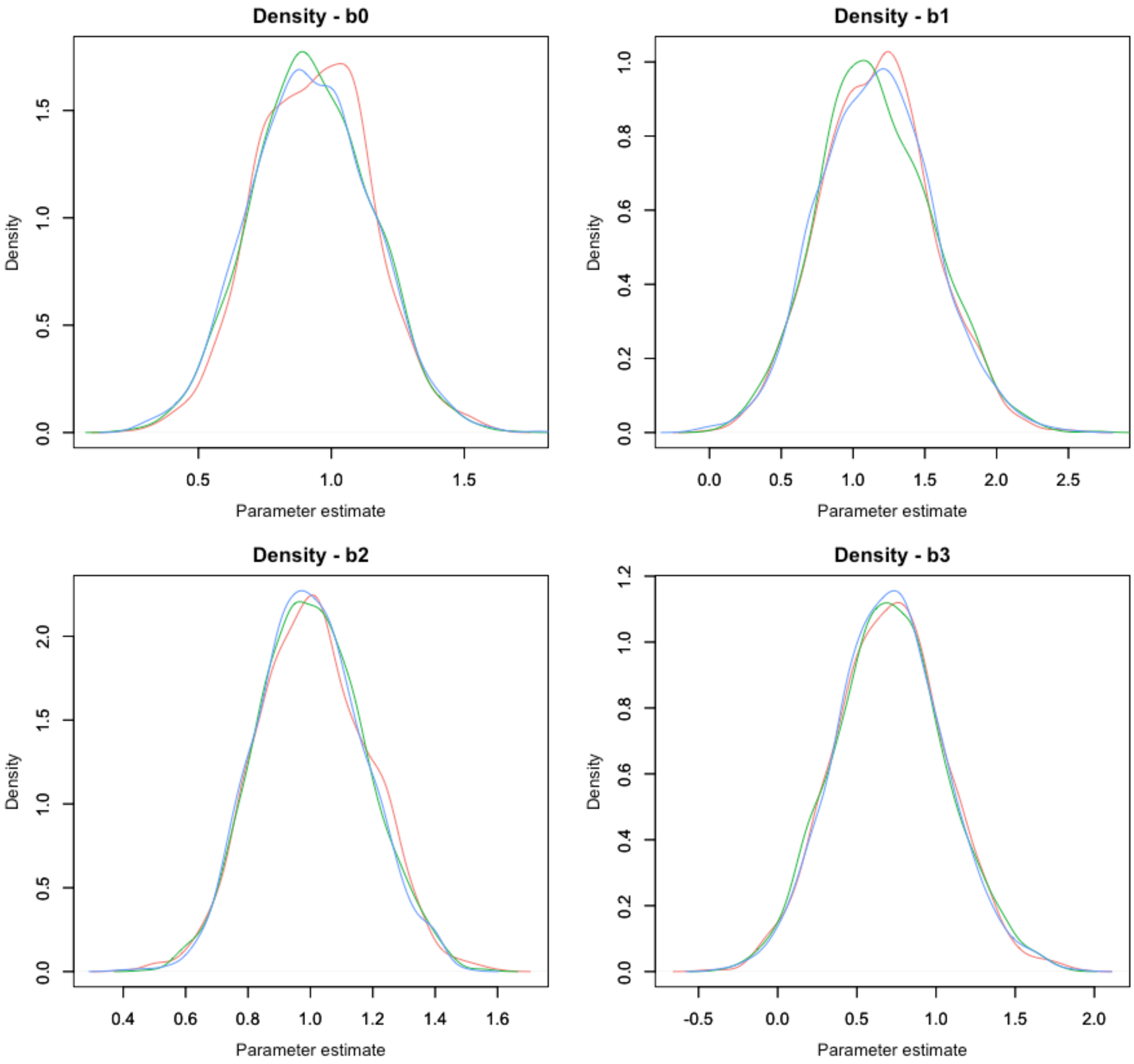

Fig. S3. Density plots of the posterior distributions of parameter estimates per MCMC chain for modeling a logistic regression with off-ground (vs. on-ground) nest site as a predictor. Here, b0 refers to the intercept, b1 to the coefficient of off-ground (vs. on-ground) nest site, b2 to discovery age, and b3 to year. Each color (red, blue, and green) refers to one MCMC chain. Priors were distributed between 0 and 0.1 for all parameters.

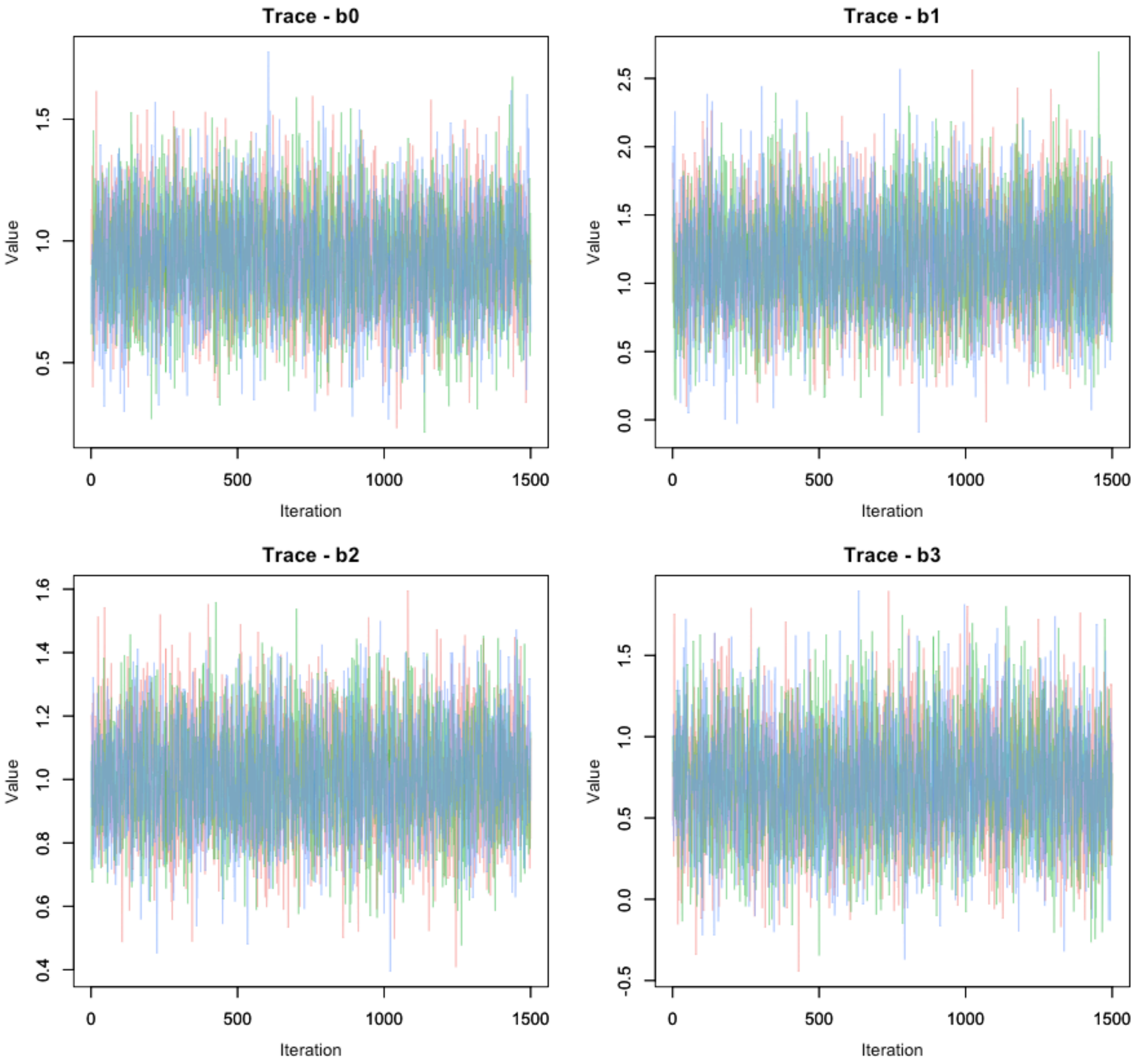

Fig. S4. Trace plots of the parameter estimates per MCMC chain over all iterations for modeling a logistic regression with off-ground (vs. on-ground) nest site as a predictor. Here, b0 refers to the intercept, b1 to the coefficient of off-ground (vs. on-ground) nest site, b2 to discovery age, and b3 to year. Each color (red, blue, and green) refers to one MCMC chain. Priors were distributed between 0 and 0.1 for all parameters.

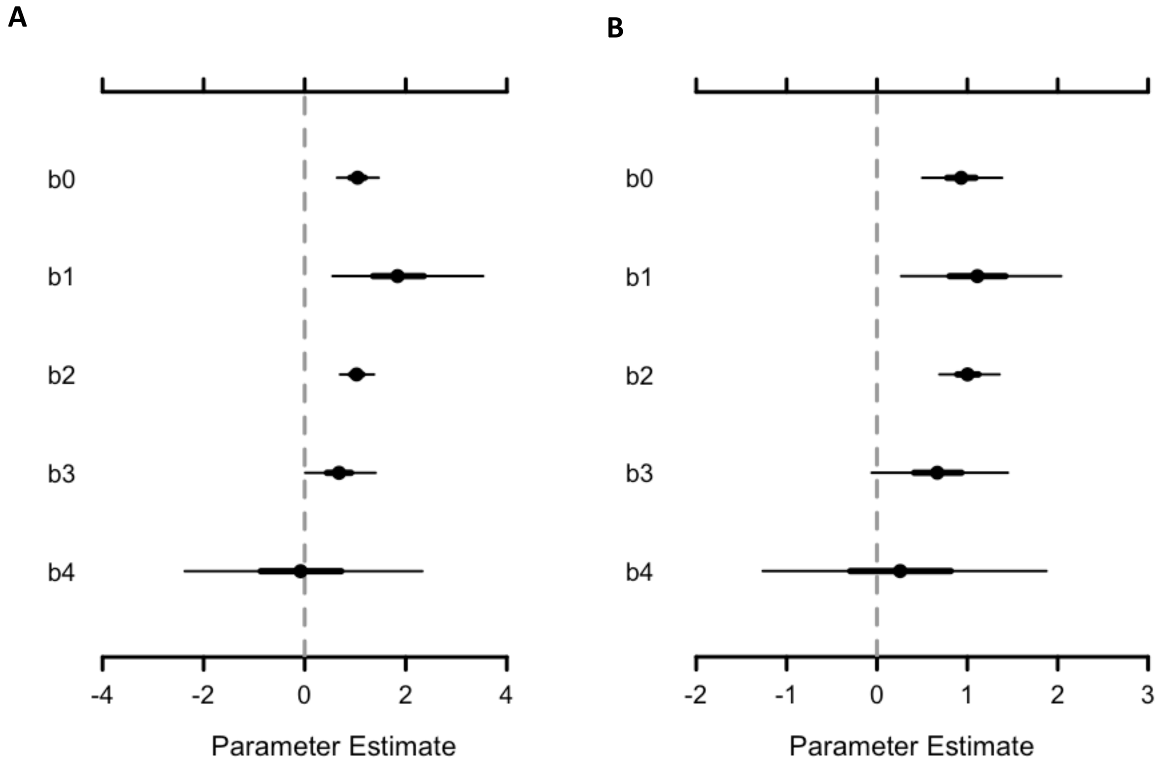

Fig S5. Parameter estimates of the posterior distributions of parameter estimates for modeling a logistic regression including an interaction with (A) artificial (vs. non-artificial) nest site as a predictor and (B) off-ground (vs. on-ground) as a predictor. Here, b0 refers to the intercept, b1 to the coefficient of (A) artificial (vs. non-artificial) nest site or (B) off-ground (vs. on ground) as a predictor, b2 to discovery age, b3 to year, and b4 to the interaction of nest location and year. Circles refer to the medians, bold lines refer to 50% confidence intervals. Un-bolded lines refer to 95% confidence intervals.

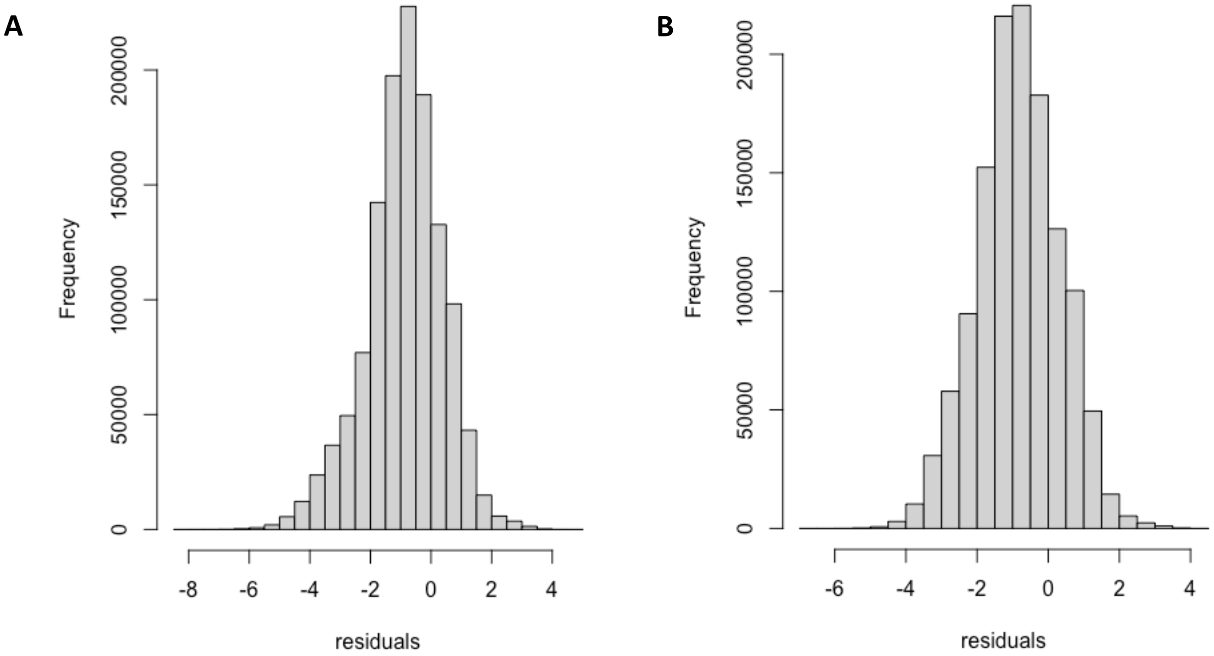

Fig S6. Logistic regression model residuals with (A) artificial (vs. non-artificial) nest site as a predictor and (B) off-ground (vs. on-ground) as a predictor. Both models have normally distributed residuals, suggesting goodness of fit.

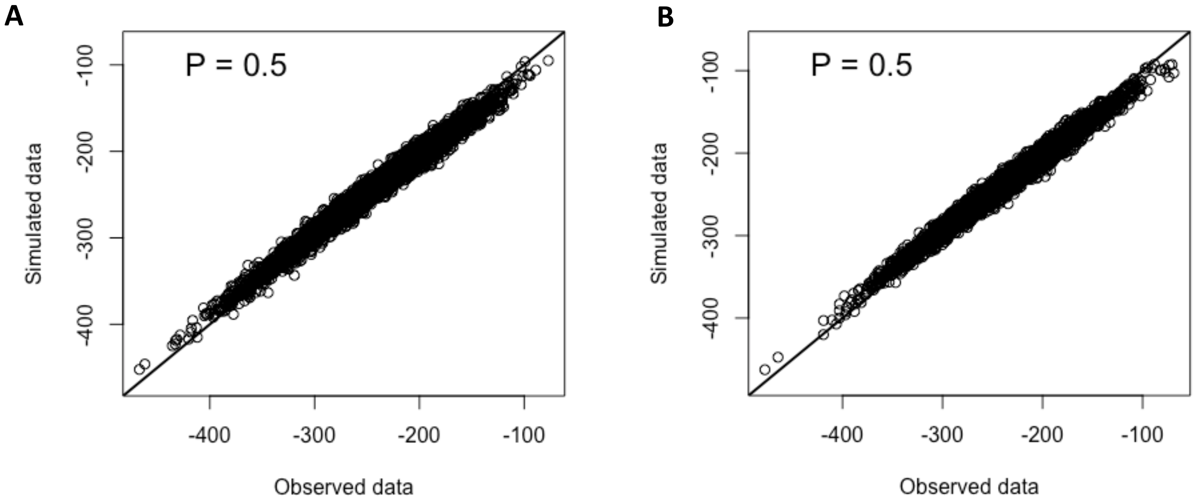

Fig S7. Posterior predictive checks for predicted logistic regression models with (A) artificial (vs. non-artificial) nest site as a predictor and (B) off-ground (vs. on-ground) as a predictor variable. Observed data of success were compared with simulated success data based on the posterior distributions of variable parameters. Each model had a Bayesian p-value of 0.50, suggesting a strong goodness of fit, as models with p-values closer to 0.50 are better fit.

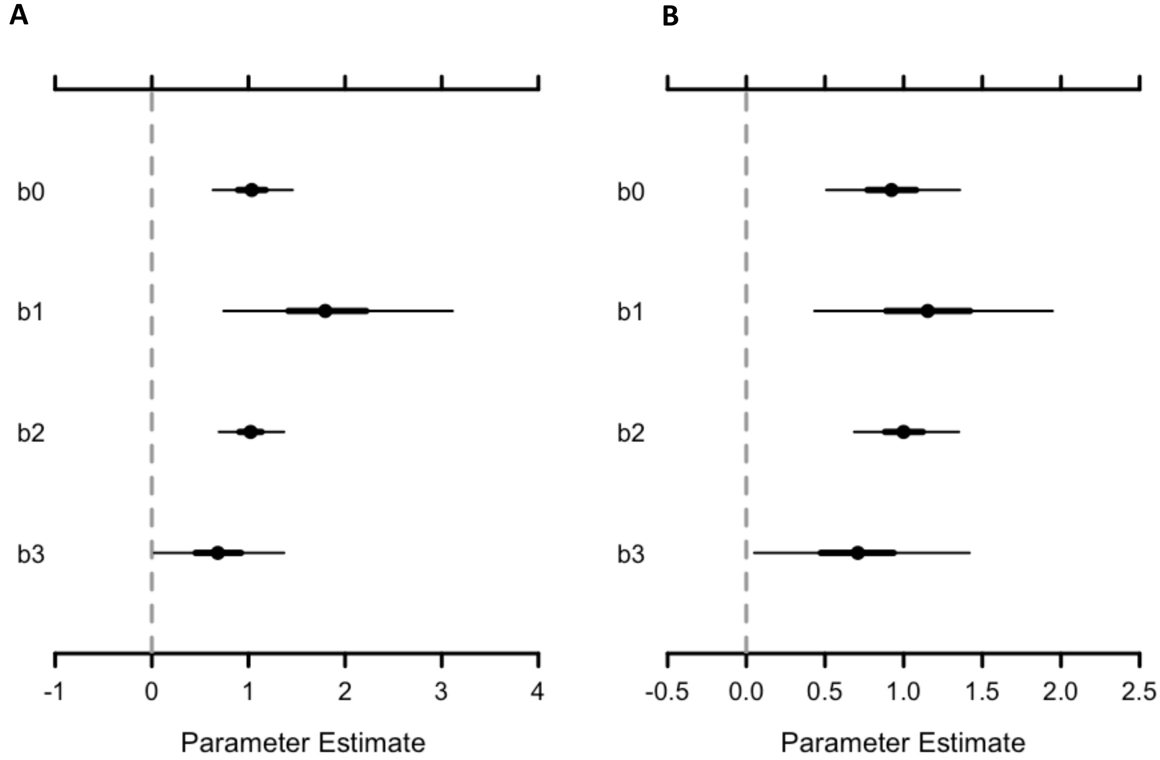

Fig S8. Parameter estimates of the posterior distributions of parameter estimates for modeling a logistic regression with (A) artificial (vs. non-artificial) nest site as a predictor and (B) off-ground (vs. on-ground) as a predictor. Here, b0 refers to the intercept, b1 to the coefficient of (A) artificial (vs. non-artificial) nest site or (B) off-ground (vs. on ground) as a predictor, b2 to discovery age, and b3 to year. Circles refer to the medians, bold lines refer to 50% confidence intervals. Un-bolded lines refer to 95% confidence intervals.