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RESEARCH ARTICLE

BugBook: Determining multiple stressor interactions in mass-reared insects based on principles of ecotoxicology

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Abstract

Insect mass-rearing is a rapidly expanding industry for the production of protein for food and feed. These highly productive artificial rearing environments can expose insects to a range of biotic and abiotic stressors, including insect pathogens, which may result in population crashes. Interactions between insect pathogens with multiple stressors can exacerbate the effects of individual pathogens on host insects. However, reliable predictions on the combined effects of individual stressors based on mechanisms of action are lacking within the field of insect pathology. We review how ecotoxicological modelling of multiple stressors can be applied to mass-reared insect systems and discuss the importance of standardization across research fields investigating multiple stressors. Important considerations in multiple stressor terminology, experimental design, endpoints and analysis of results are discussed to improve understanding of multiple stressors and their impact on insects for food and feed. This is essential for ensuring optimal rearing conditions for mass-reared insect populations.

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additivity – antagonism – entomopathogens – mixtures – synergism

1 Introduction

Mass-rearing of insects for food and feed is an industry that has seen exponential growth over the past few years. The Food and Agricultural Organization advocating for insects being a sustainable future food source has increased interest in the mass-rearing of insects as animal feed and human food (van Huis and Oonincx, 2017). Insects provide comparable levels of protein, fat, amino acids, vitamins, and minerals to more conventional food and feed sources, whilst their production requires less water and land use with reduced emission of greenhouse gases than that associated with rearing conventional livestock (Oonincx *et al.*, 2010; van Huis and Oonincx, 2017). In addition, insects can be reared on recycled organic waste, converting low quality biomass by-products from the agro-food industry to a nutritionally rich protein source for animal feed through bioconversion processes (Sangiorgio *et al.*, 2021).

Mass-reared insects are exposed to a multitude of different abiotic and biotic factors, both directly and indirectly, that may affect their performance and health, and therefore their quality as a food or food product. The presence of pathogens in insects that are mass-reared is a common occurrence; for example, honey bees *Apis mellifera* have a known number of viral pathogens that occur globally (Tantillo *et al.*, 2015), and diseases in mass-reared silkworms *Bombyx mori* have been recorded for more than 6000 years (Rosalind, 2012), and to this day still cause serious losses in the silk industry (Jiang and Xia, 2014). Moreover, devastating losses of commercial populations of the European House Cricket *Acheta domesticus* due to the Acheta domestica densovirus (AdDV) have been frequently highlighted across Europe and North America (Liu *et al.*, 2011; Szelei *et al.*, 2011). In closed rearing systems, such as those expanding rapidly in Europe, insects are reared in high density populations under tightly controlled optimal rearing and production conditions. Despite a high level of environmental control, the high density and proximity of insects provides an ideal environment for the spread of insect pathogens, potentially causing devastating disease outbreaks (Eilenberg *et al.*, 2015). Insect pathogens may cause overt mortality or reduce insect fitness through sub-lethal effects, such as reduced fecundity and longer development time (Cabodevilla *et al.*, 2011; Sait *et al.*, 1994). In addition, insects reared at high densities may be exposed to elevated temperatures due to metabolic heat production, to increased gas concentrations such as carbon dioxide produced during respiration or other abiotic factors such as changes in relative humidity (Herren *et al.*, 2023). Any physical, chemical or biological entity that is able to move an organism out of its normal operating range may be considered a 'stressor' (Segner *et al.*, 2014).

The study of multiple stressors (both biotic and abiotic) has been a focus of much research in toxicology/eco-toxicology and has now expanded to different disciplines, receiving attention in both mainstream ecology and biology (Orr *et al.*, 2020; Siviter *et al.*, 2021). Understanding how multiple stressors perturbate natural systems and impacts on ecological processes is one of the most pressing issues being addressed by ecologists. Consequently, terminology and concepts adopted by different areas of research have evolved, but often with little cross-disciplinary conjuncture. Whilst the fundamental approaches to understanding the impacts of multiple stressors underpin all disciplines, the crossover of ideas, models and conceptual theories has been more limited (Orr *et al.*, 2020; Roell *et al.*, 2017; Xu *et al.*, 2011). Moreover, applying the same approach for the provision of beneficial symbionts in mass-reared environments (Savio *et al.*, 2022) could provide standardised results for measuring their effects on insect health in conditions characterized by the presence of mixed stressors.

To enhance understanding of how multi-stressor theory applies to pathogens, beneficial microorganisms, and stressor interactions in mass-reared insects, we held a workshop bringing together ideas from Ecotoxicology and Invertebrate Pathology. The workshop, titled "Mixtures and Combined Stressors; Use of Multi-Stressor Theory for Invertebrate Function-Based Endpoints" took place on 5–8 April 2022 as part of the INSECT DOCTORS programme (a Marie Skłodowska Curie ITN supporting PhD research in infectious disease challenges in commercial insect production). A total of 22 participants, including ESRs (early-stage researchers) and experts from both fields, collaborated to discuss unifying approaches to understanding how pathogens, beneficial microorganisms, and other stressors impact insects reared for food and feed.

The primary output from the workshop was reaching a common understanding between the participants from the different disciplines on how established ecotoxicological mixture terminology and approaches could be adapted and adopted to improve understanding of the impacts of multiple stressors on insect mass-rearing systems. In this review, we outline the main considerations, with particular emphasis on important stressor combinations found in mass-reared insect systems. We concentrate specifically on microbial pathogens of insects and the diseases that they can cause in insects. Our examples and case studies come primarily from the bacteria, fungi, viruses, microsporidia and other protists. For general reviews of insect pathogens and diseases in insects reared for food and feed, we recommend reviews by Eilenberg *et al.* (2015) and Maciel-Vergara *et al.* (2021). For specific review of viruses of insects, we refer the reader to Maciel-Vergara and Ros (2017), Bertola and Mutinelli (2021), and for protists to Bessette and Williams (2022). From these examples, we outline recommendations on how to adapt and adopt ecotoxicological mixture theory in insect pathology to produce standardised multiple stressor research.

2 Stressors in mass-reared insects

More than 1600 insect species are identified as being consumed as human food (Van Itterbeeck and Pelozuelo, 2022), with the European Food Safety Authority (EFSA) recognising twelve insect species that have the biggest potential for use in food and feed production in Europe (EFSA, 2015). In Europe, several insect species are sold as live or dead whole insects or are processed into powders (flour) to be used as ingredients in energy bars, burgers or animal feed. Banded crickets (*Gryllodes sigillatus*), house crickets (*Acheta domesticus*), lesser mealworms (*Alphitobius. diaperinus*) and yellow mealworms (*Tenebrio molitor*) are the most commonly reared insects for human consumption in the European Union. Black soldier flies (*Hermetia illucens*), yellow mealworms and lesser mealworms are often reared for animal feed products, with 80% of this industry being dominated by black soldier flies. Additionally, yellow mealworms (*T. molitor*), lesser mealworms (*A. diaperinus*), black soldier flies (*H. illucens*), wax moths (*Galleria melonella*), grasshoppers (*Locusta migratoria*), silk moths (*B. mori*) and field crickets (*Gryllus assimilis*) are reared in Europe for pet food and zoo animals (Lourenço *et al.*, 2022).

To optimise rearing conditions of these insects whilst ensuring the health of the insect population, an increased understanding of how multiple stressors interact is needed to predict the impacts of stressor interactions on insects in a mass-reared environment. Different abiotic and biotic stressors may interact with each other outside the insect host (e.g. high humidity allows fungal conidia to germinate) or they may interact indirectly via the insect host (e.g. a temperature change alters the innate immune responses, which affects the susceptibility of insects to pathogens) (Herren *et al.*, 2023; Figure 1). Synergistic interactions may occur between environmental conditions and pathogens. In wax moth (*G. mellonella*) larvae, for example, the susceptibility (measured as mortality) to the fungal pathogen *Cordyceps militaris* increases when exposed to low temperature (15 °C) in comparison to a control temperature (25 °C) (Kryukov *et al.*, 2020). However, other factors such as nutritional status or density are highly relevant in the mass-rearing of insects and these factors potentially also interact synergistically with pathogens, leading to a lowered production. For example, Mormon crickets (*Anabrus simplex*) fed with a low protein diet showed higher mortality when exposed to the fungal pathogen *Beauveria bassiana* compared to crickets fed with a high protein diet. This increased mortality coincided with a reduction of several innate immune response parameters (Srygley and Jaronski, 2018). Moreover, different pathogen species and genetic strains can interact with each other resulting in a larger effect than predicted from the effects of the individual infections (Kean *et al.*, 2017; Tokarev *et al.*, 2011).

Conversely, an infection by one pathogen species can protect the insect host against a secondary infection by a different pathogen species through a process known as immune priming. This has been demonstrated, for instance, in *T. molitor* when exposed to various bacterial and fungal pathogens (Dhinaut *et al.*, 2018; Medina-Gómez *et al.*, 2018). Antagonistic interactions between different stressors may be beneficial in insect rearing, as effects on the insects will be smaller overall than expected when the stressors are combined. Mechanical and heat stress, for example, has been shown to increase the survival rates of *G. mellonella* larvae exposed to fungal pathogens (Browne *et al.*, 2014; Kryukov *et al.*, 2018; Mowlds *et al.*, 2008). However, beneficial effects on survival of heat stressed *T. molitor* larvae exposed to *Metarhizium brunneum* have been shown to be only of short duration, waning five days after exposure to the heat stress (Herren *et al.*, 2024). Moreover, the effects of the environment may be dependent on the

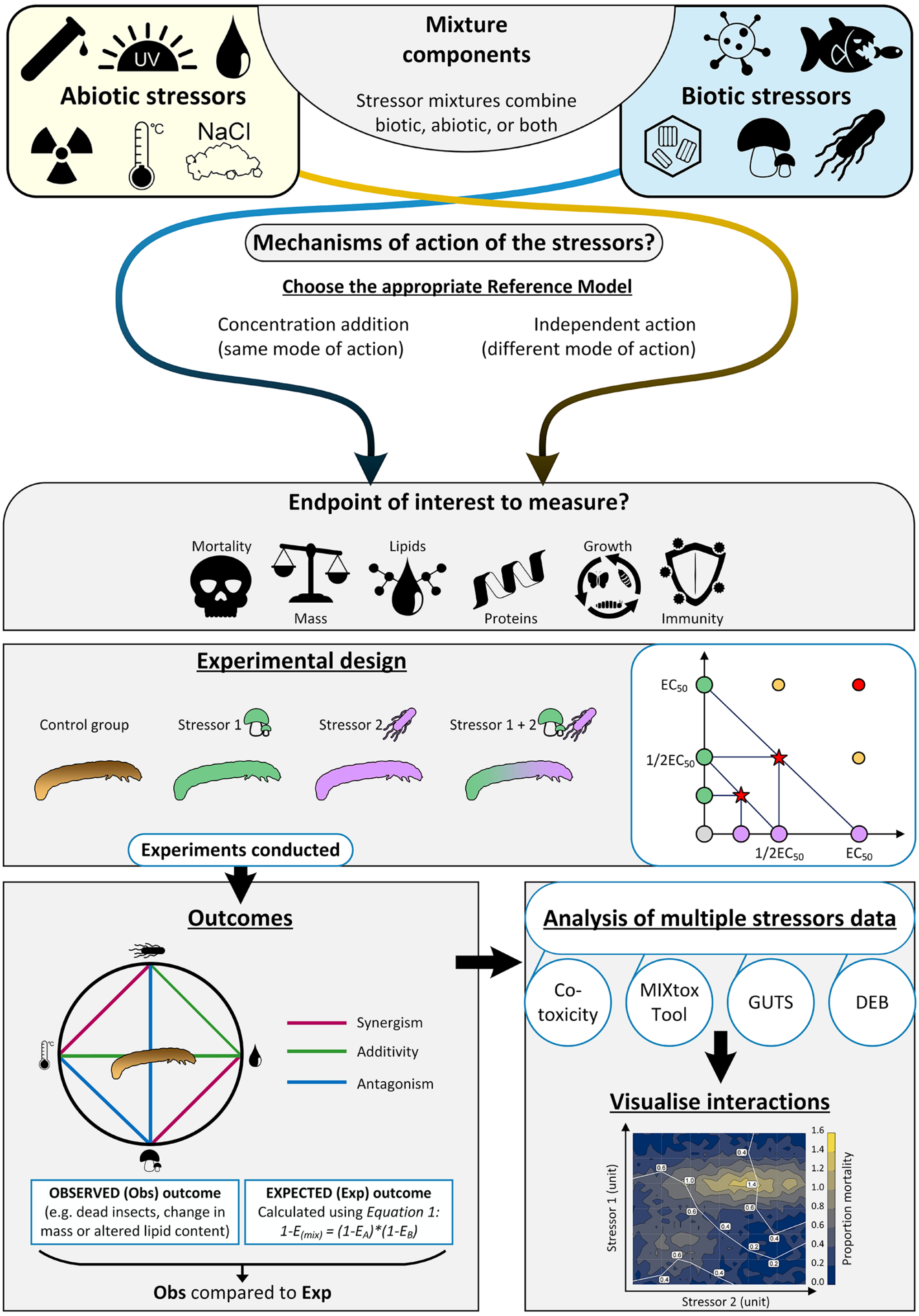

FIGURE 1 Design and use of mixture theory and experimental methods to assess the impact of combined stressors on insects mass-reared for food and feed. Mixtures may include abiotic or biotic stressors that interact externally or within the insect host. An appropriate reference model should be selected, depending on the mechanism of action of each mixture component (independent action model when stressors do not interact; concentration addition model when interactions occur). Effects are quantified using lethal and sublethal endpoints of interest. The simplest experimental design includes a control, single stressor groups, and a combined-stressor group, with at least five dose combinations to produce a reliable reference model. Experimental results should be compared to expected outcomes to identify deviations from theoretical additive effects. Antagonism occurs when observed effects are less than predicted, whilst synergism occurs when they are greater than predicted. Ecotoxicological models and visualisation tools (e.g. contour plots) can be used to analyse complex stressor interactions, enabling comprehensive predictions of multiple stressor effects.

pathogen species; for example, Texas field crickets (*Gryllus texensis*) exposed to heat stress showed increased resistance to the bacterium species *Serratia marcescens* but decreased resistance to another bacterial species, namely *Bacillus cereus* (Adamo and Lovett, 2011).

3 Advancing the understanding of multi-stressor theory for application to mass-reared insects

The recent increase in mass insect rearing for food and feed has identified a need to better understand the interacting factors influencing quality, yield and disease spread. Understanding the interactions between pathogens and other stressors has garnered significant attention in invertebrate pathology. However, there remains a notable gap in expertise when it comes to analysing and quantifying such interactions. In comparison, the field of ecotoxicology has embedded for decades an understanding of interactions between environmental contaminants, such as chemical pollutants, which are determined and quantified using mathematical models. These models also consider interactions between environmental contaminants and abiotic factors, for example, temperature and gas concentrations (e.g. oxygen, CO_2), which also interfere with the uptake, elimination and internal distribution over different tissues and how fast effects occur as a result of the interaction of the substance(s) at target site(s) (Pereira *et al.*, 2019; Saari *et al.*, 2020). By doing so, risk assessments into the likelihood of harm to organisms in the environment can be conducted. These approaches can be adapted to determine the interactions between multiple stressors in insect rearing, with an aim to quantify their effects on important industry-relevant endpoints.

Understanding multiple stressors using ecotoxicological approaches

Mathematical models to determine joint effects of multiple stressors have long been established in the field of pharmacology, being transposed to ecotoxicological approaches, within which the combined effect of multiple environmental contaminants can be further predicted for risk assessment purposes. Similarly in a biomedical and pharmacological setting, additivity and interaction between treatments for cancer and diseases are established using the same basic principles (Pegram *et al.*, 2004; Tallarida, 2002, 2007). Recently, the study of interacting stressors has been investigated by terrestrial ecologists, for example to assess the impact of multiple environmental contaminants on ecosystem services or the potential exploitation of synergistic interactions between pesticides for pest control (Siviter *et al.*, 2021b) and in interacting stressors influencing the amount and quality of product in insect rearing systems (Kangassalo *et al.*, 2018; Krams *et al.*, 2015; Peters and Barbosa, 2003). However, the language used between and within these different disciplines is not consistent and methods of testing multiple stressors in terrestrial ecology are less refined than those used in ecotoxicological studies (Orr *et al.*, 2020).

Terminology and concepts used in multiple stressor studies

In multiple stressor studies, the concept of additivity is fundamental as interactions between stressors are identified as deviations from the expected additive effect. Additivity occurs when the combined effect of two or more stressors matches the expected outcome, assuming no interaction between them. (Jonker *et al.*, 2005). However, additivity is not determined simply by summing the individual effects of each stressor. For example, if we consider the joint effect of two stressors which individually cause >50% effect e.g. stressor A causes 60% and stressor B causes 65% effects, respectively. If the effects of these stressors are simply summed, the estimated combined effect is equal to >100%, which is not possible. Instead, the additive effect can be estimated with the use of two reference models (Figure 1), independent action (Bliss, 1939) and concentration addition (Loewe and Muischnek, 1926). Interactions between multiple stressors are identified by deviation from the theoretical additive effect (Figure 2; Cedergreen, 2014). Antagonism occurs when there is an interaction between components in a mixture, leading to the mixture being less potent or toxic than expected (Schäfer and Piggott, 2018). Alternatively, synergism and potentiation result in a greater effect than expected (Schäfer and Piggott, 2018).

Use of each reference model is dependent on the mechanism of action of the stressors being investigated (Figure 1). If we consider the simplest multiple stressor interaction, with just two stressors 'A' and 'B', their combined effect can be measured as a response variable. According to Plackett and Hewlett (1952), interactions of two chemicals occur inside the organism because: the presence of chemical A influences the ability of chemical B to reach its target site in the organism or the presence of chemical A changes the products produced when chemical B reaches its target site in the organism, and the same for chemical B on chemical A. There-

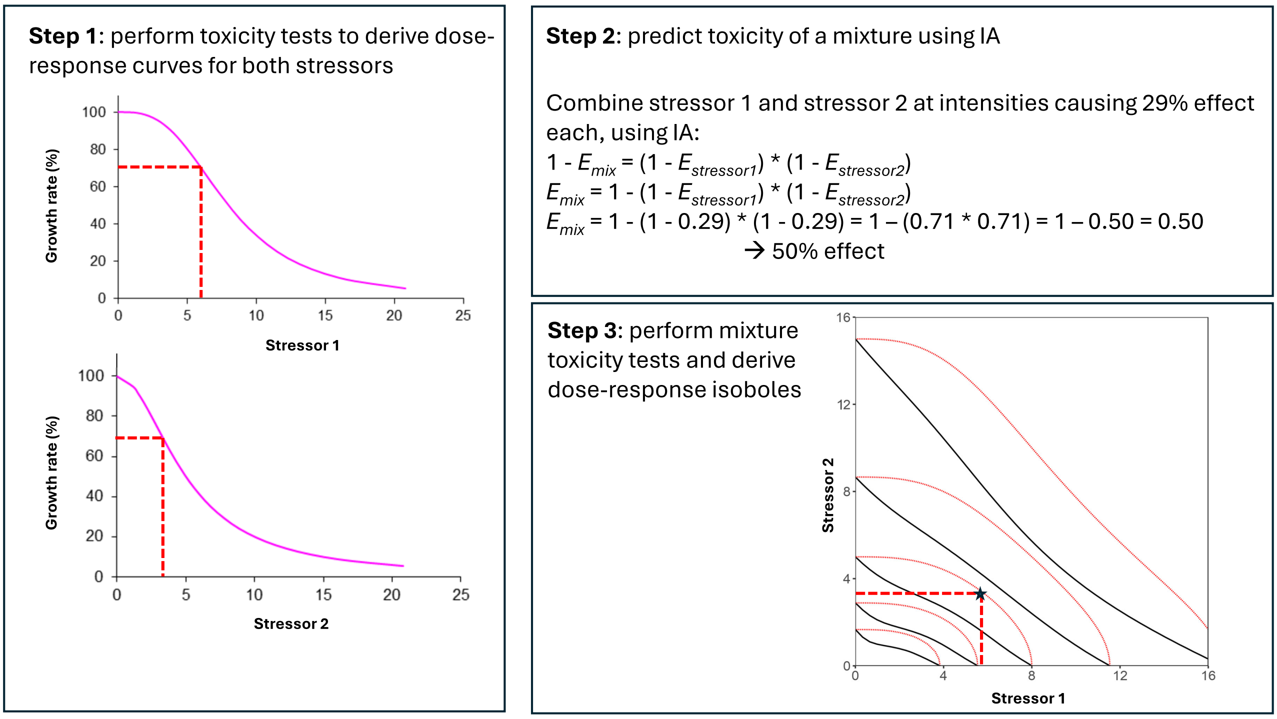

FIGURE 2 Example of an approach to assess the combined effect of two stressors, using the Independent Action (IA) conceptual model. The figures under step 1 show hypothetical dose-response curves for the effects of two stressors on insect growth rate (%). The dashed red lines indicate the 29% effect level for each stressor. Predicted toxicity of the two stressors is calculated using IA as shown in step 2. The figure under step 3 gives an example of isoboles for the combined effects of the two stressors on insect growth rate (%). Red isoboles show IA-predicted effects (from step 2) and black isoboles show synergism, for 10%, 25%, 50%, 75% and 90% effect. The dashed red lines show the expected effect when combining the two stressors at levels giving 29% effect each, the star shows the IA-predicted 50% effect.

fore, mixture theory in ecotoxicology is concerned with investigating the joint effects of chemical substances and potential interactions occurring inside the organism.

To establish whether interactions are occurring between two chemicals (or stressors), we must identify significant deviation of the measured response variable from the expected theoretical response (Figure 2). The combined impact of two or more chemicals can be predicted, though the approach chosen to predict combined effects differs depending on whether stressors have the same mechanism of action (concentration addition; (Loewe and Muischnek, 1926)), or different mode/mechanism of action (independent action; (Bliss, 1939; see examples in Figure 2). In the context of mass-reared insects, it is an appropriate mechanistic assumption that any two stressors are likely to follow different mechanisms of action, including infection by two strains of the same species of pathogen, which will not have identical mechanisms of action due to their multimodal effects on an insect. Under this assumption, a simple equation can be used to estimate the effect of two stressors on an insect (see Figure 2, step 2). This theoretical value is equal to the additive effect, i.e. the effect that stressors cause when no interactions are occurring by the product of two fractional responses (Equation 1; outcomes in Figure 1).

$$1 - E_{\text{mix}} = (1 - E_{\text{A}}) * (1 - E_{\text{B}}) \qquad (1)$$

where $1 - E_{\text{mix}}$ is the unaffected fraction of the combined effect of two stressors, $1 - E_{\text{A}}$ is the unaffected fraction of stressor A at the concentration at which it is present in the mixture, and $1 - E_{\text{B}}$ is the unaffected fraction of stressor B at its concentration present in the mixture (Figure 2).

The effects of biotic and abiotic stressors can influence the effects of chemical toxicants through interactions with the organism. Within systems involving one or more entomopathogens as well as additional biological and abiotic stressors, these interactions happen inside the organism and therefore are ruled by toxicokinetic and toxicodynamic processes. Note that if interactions between stressors happen at the exposure level (outside the organisms), pharmacokinetic models are no longer valid to determine these interactions (Van Gestel *et al.*, 2019). In environmental risk assessments, the variability in response of organisms to chemical toxicants caused by interactions with additional stressors is often accounted for by applying an uncertainty factor. However, assuming the response of mass-reared insects to biotic and abiotic stressors follows a dose response curve and interactions between stressors occur within the insect, we can determine their individual effects and, as a result, model the additive response of a combination of stressors in insect hosts.

An exception to this concentration response occurs when the population being tested has a covert infection. Covert infections are characterised by the absence of visible signs and symptoms of disease and are also known as 'silent' or 'dormant'. Instead of a directional response, whereby increasing stress causes an increase in effect, the covert infection will have little to no observable effect on the organism until a physiological or environmental stressor causes the infection to become overt leading to sudden lethal effects (Hughes *et al.*, 1993; Williams *et al.*, 2017).

Applying ecotoxicological theory in insect pathology to describe combined effects of multiple stressors

To establish the combined effect of multiple stressors it is essential to first understand the effects of these stressors individually (Figure 2). Within the field of ecotoxicology, risk assessments focus on the exposure, uptake, metabolization and excretion of individual toxins (toxicokinetics), as well as the molecular interactions of the toxin within an organism (toxicodynamics), which result in an effect (Van Gestel *et al.*, 2019). These approaches are directly applicable to stressors in mass-reared insects because much like a chemical toxicant, stressors within this system can have a directional and measurable effect on the insect (Cardoso *et al.*, 2023). The uptake of chemicals in organisms is modelled using toxicokinetic assumptions such as first order kinetics, whereby rates of exchange are proportional to concentration. Change in concentration with time (dC/dt) is related to the concentration and a rate constant (k):

$$dC/dt = kC \tag{2}$$

This equation may be directly applicable to some stressors of mass-reared insects, such as chemical contaminants of food and water supplies or cleaning products and allows for accounting for different routes of exposure (Meyer *et al.*, 2021). Unlike chemicals, pathogens can reproduce within the insect host, affecting the bioaccumulation of infective propagules without further uptake from the environment. As such, it can be difficult to predict over time the level of infection in infected insects compared to predicted chemical bioaccumulation which can be estimated from its toxicokinetics in the organism. This is because factors influencing the organism which are normally accounted for in toxicological studies, are also affecting the pathogen in addition to the immune response triggered by pathogen invasion of the host, which acts as an additional 'elimination' process.

Generally, exposure to pathogens in mass-reared insects is either reported as an application concentration (e.g. Mastore *et al.*, 2019), an estimated dose based on rate of consumption (e.g. Upfold *et al.*, 2023) or contact with the insect cuticle (e.g. Herren *et al.*, 2023). Alternatively, quantification of pathogens in infected individuals is possible throughout the infection process by DNA or RNA extraction (e.g. Hansen and De Fine Licht, 2017). Once uptake of a chemical has occurred, the internal processing and molecular mechanisms, which result in adverse effects, are described using toxicokinetics and toxicodynamics, respectively. Adverse effects from chemical exposure are a result of a biomolecular interaction between the chemical and the organism which are categorised by specific mechanisms of action (van Gestel *et al.*, 2019). Determining the toxicodynamics of a pathogen stressor within an insect becomes more complex for pathogens that likely operate through multiple modes of action. According to EFSA (More *et al.*, 2019) to conduct mixture analysis in ecotoxicology, it is not necessary to know the specific modes or mechanisms of action of individual chemicals. This is because interactions of multiple stressors can still be determined using mixture toxicity models if a dose-related response is observed.

A sigmoidal curve is traditionally used to describe the effect caused by the binding of chemicals to biological receptors (the Hill equation; Goutelle *et al.*, 2008). In ecology, this response function may also be applicable in modelling environmental or biological factors, on biological processes. For example, the unimodal distribution produced by the Sharpe–Schoolfield model (Schoolfield *et al.*, 1981), which describes the rate of biological processes influenced by temperature. For temperature responses where there is an optimal effect and the response is bell-shaped, the estimate of combined effects may need to be modelled separately (Lima *et al.*, 2011). Even in complex multimodal infections of insects by pathogens (entomopathogens), each interaction outcome is determined by a rate limiting step, the slowest reaction in a series of chemical reactions which occur during an infection (Cherry and Perrimon, 2004), such as the process of invasion by a pathogen as well as the immune response triggered by the insect host (e.g. phenoloxidase pathway) (Nakhleh *et al.*, 2017). Mechanistic assumptions simplify these infection processes to improve understanding of factors or stressors that may affect reactions in both the insect and the infecting pathogen. As such, several biological processes can be modelled based on the Michaelis-Menten equation, which describes the saturation curve of an enzyme reaction based on substrate concentration and reaction rate (Cornish-Bowden, 2015; Michaelis and Menten, 1913). Therefore, even without knowing the multitude of molecular interactions occurring within the host in response to a pathogen or other stressors, the effects of these stressors can be quantified by measuring endpoints. Information about the mechanisms of stressors can be inferred from all levels of observable effects, ranging from molecular to ecological (Pirotta *et al.*, 2022). In the case of mass-reared insects, the extent of the effect depends on the insect itself, the exact endpoint being measured and other factors interacting with the insect and the stressors.

A challenge observed across disciplines is that the effect of multiple stressors cannot be reliably estimated because the observed effects of individual stressors are influenced by other stressors acting upon the system (Orr *et al.*, 2020; Pirotta *et al.*, 2022; Schäfer and Piggott, 2018). Variation in stressor investigations can be minimised through test standardisation, by clearly and sufficiently describing methods for culturing and handling test organisms, test procedures, exposure conditions and data analysis (Table 1). Significant effort must be made to cope with the biological variation of test insects and entomopathogens within and between experiments, ensuring sufficient replication, careful test designs and a good choice of endpoints to enable proper estimates of stressor effects. Unless a variable is being investigated, it should be controlled or monitored and reported with results. However, standardisation of covert infections can be complicated, whether aiming to rear a 'clean' population for control groups or conducting an experiment on test populations with differing levels of infection. Whilst it may not be possible to ensure that test insects are 'clean' from covert infections, the presence and level of infection within the population may be determined by molecular studies (Carballo *et al.*, 2017).

When investigating interactions between multiple stressors in mass-reared insects, it is important to standardize key factors before conducting experiments, including environmental conditions (e.g. temperature, humidity, gas concentrations, and light), diet (e.g. composition, water content, quantity, feeding regime, and absence of agricultural chemicals), and insect-related variables (e.g. genetic strain, covert infections, life stage, and size). Protocols to standardise these experiments will differ depending on the insect species being mass-reared. For example, a protocol for standardisation of

TABLE 1 Key experimental endpoints and standardised recording guidelines for assessing multiple stressors in mass-reared insects

Endpoint	Relevance	Recording criteria
Survival and mortality	Survival rates provide a fundamental measure of insect resilience under different conditions.	· Clearly defined criteria for determining the endpoint of survival and mortality (e.g. adult emergence, time to death). · Consistent and well-documented stressor exposure periods.
Total Biomass	Total biomass is indicative of the combined effects of stressors on the growth and reproduction of insects, offering insights into the overall productivity and fitness of the population.	· Standardised measurement techniques (e.g. consistent methods for insect collection, sample preparation, and weighing). · Use of same life stage for biomass measurement. · Reporting biomass data on a per-individual or per-unit basis.
Dried Biomass	Dried biomass offers a standardised metric for comparing the impact of stressors on insect body composition, aiding in the assessment of nutritional efficiency and resource utilisation.	· Standardised drying protocols to ensure consistent water content removal. · Reporting dried biomass as a percentage of total biomass. · Consistent use of life stages for dried biomass measurements.

TABLE 1 (Continued)

Endpoint	Relevance	Recording criteria
Weight gain	Tracking individual weight gain helps assess the nutritional status and feeding behaviour of insects offering insights into the efficiency of resource utilisation and potential nutritional deficiencies.	· Standardised measurement of individual insect weights. · Recording weights at specific time points or developmental stages. · Consistent feeding regimes and nutritional sources. · Controlling for factors influencing weight gain, such as sex and age.
Protein and/or fat content	Changes in protein and fat content offer insights into the metabolic responses of insects to stress and can act as quality indicators.	· Reporting content as a percentage of total biomass or on a per-individual basis. · Consistent sampling methods for protein/fat analysis across experiments.
Feed conversion rate	Critical parameter for understanding the energetic cost of stressors on insect populations, indicating how efficiently resources are utilised for growth and development.	· Standardised measurement of food consumption including diet composition and feeding frequency. · Clear definition of conversion rate calculation (e.g. biomass gained per unit of food consumed). · Control for potential variations in metabolic rates.
Innate immune response parameters	Provide information about the insect's ability to combat stress-induced challenges. For example, gene expression, antimicrobial peptide (AMP) concentration, phenoloxidase (PO) activity, and haemocyte concentration are key indicators of the insect's immune system activation, offering insights into the impact of stressors on immune function and overall health.	· Consistent methods for collecting and processing samples (e.g. haemolymph, tissues). · Standardised laboratory techniques for gene expression analysis and biomolecule quantification. · Temporal changes of response after stressor exposure.

Specific multiple stressor experimental endpoints (a measurable response or outcome used to assess the effects of the combined stressors on the study organism or system) of particular importance for mass-reared insects were identified by participants in the INSECT DOCTORS programme workshop "Mixtures and Combined Stressors; Use of Multi-Stressor Theory for Invertebrate Function-Based Endpoints". For each endpoint, we identify the specific data that should be recorded to enhance standardisation and facilitate meaningful comparisons across studies. Following these recording guidelines will help researchers ensure consistency between experiments, enabling more robust and reliable conclusions about the effects of multiple stressors on mass-reared insects.

feeding experiments has been developed by Deruytter *et al.* (2024) for black soldier fly larvae.

Methods for designing multiple stressor experiments

Determination of an interaction is possible by statistical comparisons of the observed mixture effect and the expected additive effect, predicted by Equation 1. The simplest experimental design to determine effects of two stressors contains four treatment groups; a control group, a group exposed to stressor one, a group exposed to stressor two and a group exposed to both stressors (e.g. Yaroslavtseva *et al.*, 2017; Figures 1–2). An issue with this experimental design is that determination of interactions is limited to one exposure dose. But, as the susceptibility of individuals differs in a population, the additional effect observed when an organism is exposed to a second stressor is not constant across all doses of the first stressor, even if additivity is observed (Orr *et al.*, 2020; Pirotta *et al.*, 2022; Schäfer and Piggott, 2018). Additionally, interactions between stressors can vary depending on the relative ratio of stressors in the exposure treatment (Jonker *et al.*, 2005).

To analyse the effects caused by two stressors, data can be visualised across a surface in which the additive effect differs according to dose of both stressors (see van Gestel *et al.*, 2019). In order to produce this surface response model, full dose response curves must be conducted for each stressor to produce a null (additive) reference model as well as combined stressor exposures to detect deviation from the null reference model (Figure 2). Additionally, individual, and multiple stressor tests must be conducted at the same time to reduce variability in the organisms involved (van Gestel *et al.*, 2019).

The minimum number of dose combinations between two stressors that could produce a reliable additive reference model has previously been reported to be at least five treatment levels in a factorial design (Schäfer and Piggott, 2018). Unfortunately, most multiple stressor studies in ecology do not conduct enough exposure level treatments to determine stressor interactions which do not follow a linear relationship across a range of doses (Orr *et al.* 2020). In contrast, it is not always appropriate to conduct full factorial experimental designs in multiple stressor studies. For example, Wu *et al.* (2017) studied the fungus gnat (*Bradysia odoriphaga*) following exposure to the nematode species *Steinernema feltiae* in combination with thiamethoxam for pest control purposes, investigating interactions which may affect total mortality. A total of 20 treatments were applied in this mixture experiment, including seven treatments of the control agents applied individually, twelve mixture treatments of each possible concentration combination and a control treatment. However, based on their calculations, three of the mixture treatments were calculated to cause an expected mortality higher than 100%. In treatments where all exposed organisms are expected to die, interactions other than strong antagonism between stressors cannot be determined and this leads to wasted experimental effort.

Basing mixture treatments on Lethal Concentrations (LC) (or Effective Concentrations: EC) values of each component allows researchers to predict the expected outcome of a mixture before conducting the experiment and avoid treatments which do not allow interactions to be determined, thereby rationalising the experimental design. Therefore, it is important to understand the effects of individual stressors and consider their expected joint effects when designing multiple stressor exposure studies (Figure 1). So, depending on the endpoint, full factorial designs can be chosen when survival is the endpoint, while fixed ratio designs, not reaching extreme doses/combinations, are used for non-lethal endpoints.

Key parameters and endpoints in assessing multiple stressor effects

Generally, survival, growth and reproduction are the most measured parameters in studies determining the effects of single or multiple stressors in mass-reared insects (El Deen *et al.*, 2021; Herren *et al.*, 2023; Jordan and Tomberlin, 2021; Marshall and Sinclair, 2010). Survivorship or mortality can be determined at the end of the exposure period by simply counting individual insects or as a proportion compared to the initial number in the treatment group. If multiple exposure treatments are conducted, the increasing mortality observed with increasing severity of an individual stressor can be plotted as a dose response curve from which LC_{50} values can be extracted. The LC_{50} is the concentration of the stressor required to result in 50% mortality of the exposed population (Figure 2).

However, other non-lethal endpoints could be of greater value to stakeholders of mass-reared insects and should be considered when designing multiple stressor experiments (Table 1, Figure 1). For example, conditions that support higher survivorship of a harvest may be less valuable compared to those which affect the quality of the final product for insects reared for consumption by humans or animals. For example, the lipid and protein content of *T. molitor* mealworms has been shown to be significantly affected by diet composition (El Deen *et al.*, 2021; van Broekhoven *et al.*, 2015) and variation in rearing temperatures (Bjørge *et al.*, 2018). Products generated during the bioconversion process, such as exuviae and frass, which can be used as organic fertilisers, or the insects themselves used as animal feed after being reared on organic waste, should be monitored for contamination (van der Fels-Klerx *et al.*, 2018). Metals have a high tendency to adsorb to organic matter, which can then bioaccumulate in insects consuming the organic material. Uptake of silver nanoparticles from insect diets has been shown in *T. molitor*, as well as being discharged in exuviae when moulting (Khodaparast *et al.*, 2021). Whilst individual *T. molitor* were shown to tolerate the exposure to these silver nanoparticles, insects or insect products contaminated with metals are of little market value. Polycyclic aromatic hydrocarbons (PAHs) can be also of high concern regarding their ability to accumulate in fat, with a low elimination rate. The mealworm *T. molitor* was exposed to B(a)P (benzo(a)pyrene) (B(a)P) revealing the ability to bioaccumulate this PAH in a dose dependent way and needing long term elimination periods to become safe for food and feed (Cardoso *et al.*, 2024).

Novel techniques have become available with the rise of molecular biology and can provide additional information on the effects of stressors, such as quantification of pathogen load following infection of the host and changes in molecular mechanisms of detoxification and pathogen-immune responses in insects, which can allude to modes or mechanisms of action of individual stressors (Jia *et al.*, 2016). These endpoints could be essential for developing new methods to improve insect health. For example, the measurement of the expression of immune-related genes in insects under abiotic or biotic stress would be helpful to assess the health status of an insect population.

These non-lethal endpoints can also be quantified with increasing severity of each stressor and plotted as a dose response curve. Effect concentrations, such as the EC_{50} (median effect concentration where the performance of test organisms is reduced by 50% compared to the untreated controls), can then be extracted from the plot (Figure 1). To detect these sub-lethal effects, lower exposure doses or concentrations of stressors are investigated over a longer period of time compared to acute exposure studies which often only determine survivorship over short exposure periods at high exposure concentrations. Another ecotoxicological endpoint that may be crucial is the No Observed Effect Concentration (NOEC). In sustainable rearing processes, the NOEC must not be exceeded. The NOEC is defined as the highest exposure concentration tested at which there still is no significant reduction in the performance of exposed organisms compared to the untreated controls. A problem of the NOEC approach is that there still may be a considerable reduction of an endpoint at this concentration. It therefore is advisable to always mention the effect level observed at the statistically established NOEC. Chronic exposure studies could be particularly inciteful when considering the effects of multiple stressors in the rearing of longer-lived insects such as crickets or mealworms. There are several considerations for the organism when observing sub-lethal effects in chronic exposure studies (van Gestel *et al.*, 2019). In addition to these, when exposing insect populations to pathogens, infection ecology of the pathogen must also be considered. Depending on the length of the exposure, secondary transmission of a pathogen may occur from infected to healthy individuals and for some pathogens, vertical transmission directly to offspring is possible.

Statistical and ecotoxicological modelling approaches to analyse multiple stressor effects

Commonly, insect pathologists may determine whether differences between predicted and observed mixture effects are statistically significant with the use of a Chi Square test (e.g. Brousseau *et al.*, 1998). Whilst this type of analysis is acceptable, it is lacking in descriptive power compared to other approaches of multiple stressor analysis. For example, all data points from experiments which investigate multiple exposure doses of each stressor could be analysed concurrently to establish relationships between the dose combinations studied, assuming important aspects relating to standardisation and experimental design previously discussed have been adhered to.

Alternatively, co-toxicity coefficients are also commonly used in mixed stressor interactions in invertebrate pathology, though the equations, thresholds and citations used vary across the field. Originally, Sun and Johnson (1960) published this analysis using toxicity indexes to incorporate the dose response curve of two mixture components into the calculation of expected mixture mortality. Sun and Johnson (1960) stated that three dose response curves should be conducted for effective use of this analysis; two dose response curves for the individual mixture components and one dose response of both components applied together. Three equations are used for this analysis;

$$Toxicity\ index\ (TI)\ of\ agent = \left(\frac{LC_{50}\ of\ standard\ agent}{LC_{50}\ of\ supplied\ agent}\right) \times 100 \qquad (3)$$

$$\begin{aligned} &Theoretical\ toxicity\ index\ of\ the\ mixed\ formulation \\ &= (toxicity\ index\ of\ agent\ 1 \times \%\ of\ agent\ 1\ in\ the\ mixed\ formulation) \\ &+ (toxicity\ index\ of\ agent\ 2 \times \%\ of\ agent\ 2\ in\ the\ mixed\ formulation) \qquad (4) \end{aligned}$$

$$co\text{-}toxicity\ coefficient = \left(\frac{actual\ toxicity\ index\ of\ the\ mixed\ formulation}{theoretical\ toxicity\ index\ of\ the\ mixed\ formulation}\right) \times 100 \qquad (5)$$

A co-toxicity coefficient equal to 100 indicates that the observed mortality is identical to the expected mortality. If the calculated co-efficient is more than 100, synergism has occurred. In contrast, if the coefficient is less than 100, antagonism has occurred.

However, application of this method is limited because the definitions of standard and supplied agents are uncertain and not clearly defined by the authors. In addition, threshold values for interaction are varied and arbitrary and do not provide a statistical determination of an interaction. Despite this, co-toxicity coefficients are regularly used to determine whether differences between observed and expected mixture effects are classified as antagonistic or synergistic (Magholli *et al.*, 2013; Norris and Bloomquist, 2021; Wei, 2020). There is much variation in the equations used and significance levels specified for co-toxicity coefficients in different studies, and this identifies the need to standardise approaches of analyses in this research area.

Multiple stressor effects can be reliably modelled using ecotoxicological approaches, by making assumptions based on the mechanisms of action of each stressor on the target organism (Dearlove, 2022). In ecotoxicology, producing a dose-response surface for the combined effect of two stressors relies on data to predict the effects of individual and combined stressor effects and is therefore unbiased for chemical combinations. The surface response model allows combined effects to be described and predicted based on interactions across the doses of each stressor. Therefore, these models should not be used to describe or predict combined effects outside the range of doses tested (Jonker *et al.*, 2005; Pirotta *et al.*, 2022). Surfaces of response to multiple stressor doses have been fitted for environmental stressors and varied responses in different levels of biological organisation in mussel assemblages (Brooks *et al.*, 2023), behavioural responses to ecological disturbances (Dunlop *et al.*, 2021) and abiotic and biotic stressors of freshwater plankton communities (Hampton *et al.*, 2013), or the combined exposure of abiotic and chemical stressors in earthworms and plants (Lima *et al.*, 2011). Despite the diversity in their applications, these models share the same foundational principles of mixture theory as those described in this manuscript, incorporating varying degrees of assumptions shaped by chemical, biological, and ecological insights. Pirotta *et al.* (2022) reviewed the approaches used to model multiple stressor interactions and demonstrated they lie across a spectrum, whereby the greater the number of assumptions made, the higher the predictive power and analytical precision, but the greater the potential for bias if the underlying assumptions are incorrect.

The combined effect of two stressors on an organism at a given time can be predicted (and described) in a dose-response surface produced by analyses such as the MIXTOX tool (Figure 1) developed by Jonker *et al.* (2005). This tool can help analysing mixture responses against the classic models of CA (concentration addition) and IA (independent action) and check for potential synergistic/antagonistic (S/A), dose-level (DL) or dose-ratio (DR) dependent deviations from this model, quantifying specific doses where interactions may switch from synergism to antagonism or vice versa (Jonker *et al.*, 2005). The MIXTOX tool has recently been used to describe complex interactions between a fungal (*Metarhizium brunneum*) and a bacterial (*Bacillus thuringiensis*) pathogen in *T. molitor* under varying carbon dioxide (CO_2) concentrations (Herren *et al.*, 2025) as well as interactions between entomopathogenic fungi in combination with an insecticide for control of greenhouse whitefly (Dearlove *et al.* 2024). Comparatively, mechanistic effect models (toxicokinetic – toxicodynamic models) are used to determine the effects of multiple stressors over time. When monitoring survivorship, the general unified threshold model for survival (GUTS; (Jager *et al.*, 2011)) is the leading framework in ecotoxicology (Jager, 2020). Application of these models could be insightful for interactions such as those described by Candian and Tedeschi (2023), investigating the impact of diet on susceptibility to entomopathogenic fungi exposure for *T. molitor*. Type and timing of diet administration has been shown to have an effect on the immune response of *T. molitor* and *H. illucens* and their survivorship (Candian *et al.*, 2023; Candian and Tedeschi, 2023).

Further, several ecotoxicological models have been developed based on dynamic energy budget (DEB) theory and a summary of the range of these models can be found in Jager *et al.* (2014). These models, collectively known as DEBtox, also establish the effects of long-term exposures to multiple stressors but deal with a range of responses such as growth, reproduction, body composition, etc. (Jager, 2020). Continued monitoring of insect nutritional profiles, including protein and lipid content, could be used as indicators of quality of mass-reared insects (Anankware *et al.*, 2021). Bioenergetic models similar to these can allow the integration of multiple stressor effects even if they act across different response pathways (Pirotta *et al.*, 2022). For example, sea water temperature can alter the metabolism of the Mediterranean mussel *Mytilus galloprovincialis*, increasing their susceptibility to parasites which further impacts their energy dynamics (Anestis *et al.*, 2010). In the common house mosquito *Culex pipiens* biotype molestus, which is vector of several arboviruses of human health importance such as the West Nile virus, the combined exposure to two stressors were studied (Tran *et al.*, 2018). Overall lower offspring survival and a delayed offspring metamorphosis were seen when mosquitos were exposed to a warmer temperature (4 °C) and to the pesticide chlorpyrifos. Similarly, polyphenols included in mosquito meals can promote the activation of AMPK (AMP-dependent protein kinases) in the yellow fever mosquito *Aedes aegypti*. Following this activation, AMPK is able to positively regulate the level of midgut autophagy, leading to a decrease in bacterial proliferation. The decrease in bacterial communities in the midgut of polyphenol-fed mosquitoes was the result of the activation of the immune response of the mosquito itself. An additional result is the increase in vector lifespan (Nunes *et al.*, 2016).

Selection of the model to determine multiple stressor outcomes depends on data availability and the validity of assumptions required to apply mathematical equations to the study system. From a production perspective, determining mechanistic interactions between stressors is secondary to establishing practical measures to reduce risk to insect populations (Herren *et al.*, 2023). Understanding the effects of multiple stressors could be used to define management goals whereby one stressor in a mixed exposure could be manipulated to maintain risk to insect populations below acceptable thresholds.

4 Summary and conclusion

Given the rapid uptake of mass-reared insect production, several authors have indicated the importance of understanding the effects of multiple stressors within these systems, in order to effectively manage populations to prevent population declines. In recent years, there have been several publications calling for a cohesive and collaborative approach to multiple stressor investigation across a range of disciplines. In particular, it is imperative that researchers and stakeholders within different disciplines use shared terminology and methodology, despite being concerned with different stressors.

We demonstrate that the field of insect pathology could benefit from existing methods of ecotoxicological approaches for analysing multiple stressor effects, applying chemical mixture theory to non-chemical stressors specifically considered for mass-reared insect systems. Assumptions based on the mechanistic effects of stressors can be applied to improve our understanding of stressor interactions, through suitable experimental design, informative experimental endpoints and holistic methods of analysis. Within this system, we consider important stressors and their interactions in relation to management objectives. We also outline basic investigations required to understand the system and find solutions to risks posed towards mass-reared insect populations allowing us to infer best practises for continued maximum production.

In conclusion, integrating ecotoxicological methods and chemical mixture theory into the field of insect pathology, offers a robust framework for addressing biotic and abiotic multiple stressor interaction studies. By emphasising shared terminology, standardised methodology, and a holistic analytical approach, we aim to advance research on stressor interactions, deepen the understanding of their outcomes and inform best practices for mass-rearing insects sustainably for food and feed.

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