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Contaminant-induced behavioural changes in amphibians: a meta-analysis

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Running title: How contaminants influence amphibian behaviour

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Abstract

Environmental contamination contributes to the threatened status of many amphibian populations. Many contaminants alter behaviour at concentrations commonly experienced in the environment, with negative consequences for individual fitness, populations and communities. A comprehensive, quantitative evaluation of the behavioural sensitivity of amphibians is warranted to better understand the population-level and resultant ecological impacts of contaminants. We conducted a systematic review and meta-analysis evaluating behavioural changes following exposure to contaminants. Most studies were conducted in North America and Europe on larval stages, and 64% of the 116 studies focussed on the effects of insecticides. We found that a suite of contaminants influence a wide range of behaviours in amphibians, with insecticides typically invoking the strongest responses. In particular, insecticides increased rates of abnormal swimming, and reduced escape responses to simulated predator attacks. Our analysis identified five key needs for future research, in particular the need: (1) for researchers to provide more details of experimental protocols and results (2) to develop a strong research base for future quantitative reviews, (3) to broaden the suite of contaminants tested, (4) to better study and thus understand the effects of multiple stressors, and (5) to establish the ecological importance of behavioural alterations. Behavioural endpoints provide useful sub-lethal indicators of how contaminants influence amphibians, and coupled with standard ecotoxicological endpoints, can provide valuable information for population models assessing the broader ecological consequences of environmental contamination.

Keywords

frogs; toads; tadpoles; ecotoxicology; behavior; log response ratio

1. Introduction

Amphibians are one of the most imperilled taxa globally (Monastersky 2014) and are vulnerable to negative effects associated with a wide range of anthropogenic stressors (Alford and Richards 1999, Cushman 2006, Pounds et al. 2006). Amphibians may be particularly vulnerable to contaminant exposure since they often live and breed in areas designed to receive contaminated waters (e.g. stormwater wetlands receiving urban runoff; Brand and Snodgrass 2010, Sievers et al. 2018c) and in locations where chemicals are intentionally added (e.g. agricultural wetlands receiving pesticides and fertilisers; Hazell et al. 2001). Many of the contaminants common in these environments are known to affect amphibian growth, development and survival, including heavy metals (Lefcort et al. 1998), pesticides (Egea- Serrano et al. 2012), hydrocarbons (Jelaso et al. 2002) and fertilisers (Marco et al. 1999). These impacts have been well conceptualised and discussed within the literature in several comprehensive reviews (Rohr and McCoy 2010, Egea‐Serrano et al. 2012, Baker et al. 2013). However, the ways by which common environmental contaminants influence amphibian behaviour have not been systematically assessed.

Many animals respond to environmental stressors by altering their behaviour (Tuomainen and Candolin 2011, Wong and Candolin 2015). These behavioural changes – intentional or unintentional – play a pivotal role in determining how well animals can be expected to cope with a changing environment (Saaristo et al. 2018). This is because many behaviours directly influence correlates of fitness such as survival, growth and reproduction, and because behavioural alterations tend to manifest in response to more subtle changes to the environment than these fitness indicators (Zala and Penn 2004, Melvin and Wilson 2013, Montiglio and Royauté 2014). As such, behavioural responses are being increasingly used to

assess the impacts of environmental stressors including contaminants.

Increasing evidence demonstrates that contaminants influence amphibian behaviour (Shuman-Goodier and Propper 2016) and researchers have been measuring how contaminant-induced sub-lethal effects relate to population-level responses. Some of the most common altered behaviours are erratic swimming, surface swimming, and changes in feeding and activity rates, although other endpoints have also been measured (Mitchkash et al. 2014, Moore et al. 2015, Miko et al. 2017, Sievers et al. 2018b). For example, widespread contaminants such as copper increase the time wood frog (*Lithobates sylveticus*) tadpoles spend swimming at the water surface (Hayden et al. 2015), the herbicide glyphosate decreases activity and increases hiding in agile frog (*Rana dalmatina*) tadpoles (Miko et al. 2017), and the suite of contaminants in stormwater wetlands reduced the capacity of spotted marsh frog (*Limnodynastes tasmaniensis*) tadpoles to detect olfactory cues and ultimately interferes with normal anti-predator behaviour (Sievers et al. 2018d).

Given the dire status of global amphibian populations, an evaluation of the usefulness and sensitivity of behavioural responses for studying the ecological impacts of environmental contaminants in amphibians is required. This evaluation will also help to identify avenues for future research that may lead to better understanding of how contaminants exert their sub-lethal effects on amphibians, and promote the inclusion of behavioural alterations as components of risk assessment and population modelling (O'Brien 2017).

When assessing how contaminants affect behaviour, it is important that study designs accurately reflect natural conditions to correctly evaluate responses. However, the methods that are used to do so can influence what responses are observed. For example, some studies use a solvent control which matches the solvent used in the contaminant formulation, but for which the impacts of are not of interest, whilst other times use a water control (uncontaminated or the culture medium). This choice might influence the magnitude of

impacts that are observed and hence the conclusions made about the broader ramifications of contaminants (Eddleston et al. 2012). Meta-analysis offers an opportunity to address these challenges by compiling data from many existing studies and quantitatively evaluate the combined dataset for important trends. Systematic evaluations of this sort can then provide the evidence to inform conservation decisions (Sutherland et al. 2004).

Here, we conduct a systematic review and meta-analysis to: (1) evaluate the sensitivity of behavioural endpoints by assessing the magnitude and direction of responses to various contaminants; (2) determine how the characteristics of experimental design may influence the outcomes of a study, and; (3) identify research gaps and highlight key recommendations. Since most animals are affected by multiple stressors simultaneously (Ormerod et al. 2010, Jackson et al. 2016), we also record studies that consider non-chemical stressors in addition to contaminants. We hypothesise that many common behavioural measures will be affected by a range of contaminants, but are unsure whether different contaminant classes will modify behaviours similarly or differentially due to these different modes of action. Importantly, our intention here is not to explore the nature and modes of action for different classes of contaminants, nor to discuss the physiological mechanisms causing behavioural changes. Instead, we focus on the utility of using behavioural responses to study contaminant effects in amphibians and conclude with considerations for future research.

2. Methods

2.1. Literature search

We performed a literature search on 6th December 2018 using ISI Web of Science (all databases) and the following term: (amphibia* OR frog* OR toad* OR newt* OR

salamander* OR anuran* OR caecilian*) AND (behavio* OR locomotion OR attract* OR avoid* OR swim* OR perform* OR social interaction OR feeding OR forag* OR hiding OR hide OR navigat* OR speed OR endurance) AND (contamin* OR toxic* OR pollut* OR light OR noise OR sound OR metal OR pesticide OR herbicide OR fertili* OR fungicide OR chemical OR pharma* OR petro* OR hydrocarbon OR acid OR nitr* OR UV OR ultraviolet OR salinity OR conductivity OR salt* OR ozone OR disruptor OR inhibitor OR endocrine OR drug* OR *estrogen OR pharma* OR therapeutant*). Initial results were filtered to include the following research areas: zoology, ecology, behavioural sciences, environmental sciences, toxicology, physiology, biology, evolutionary biology, multidisciplinary sciences, marine freshwater science, developmental biology, acoustics, and reproductive biology, with no restriction on publication date. In addition, we examined the reference lists of selected studies, including related reviews and meta-analyses. Excluding duplicates, we were left with a pool of 4,628 potentially relevant studies. The PRISMA flow diagram (Moher et al. 2009) shows the procedure used for selection of studies for the systematic review (Figure 1, and see Appendix A for a bibliography of the included studies).

2.2. Data extraction and classification

To select studies for potential inclusion, we first scanned titles for those remotely relevant to the topic. We then read abstracts and included all papers that did or might have contained relevant data. Then, at the full paper level, a study must have published original quantitative data on amphibian behavioural responses following exposure to a contaminant to be included. Although we had a restriction that the paper had to be in English, we did not restrict the journal or date of publishing. We did not include field studies that compared exposed populations to unexposed populations (e.g. stormwater wetlands versus natural), since the contaminant(s) responsible for an observed behavioural response could not be conclusively

identified and unknown confounding factors could not be ruled out. From each of the 123 studies we included, we recorded the location (continent and country), year of study, control type, contaminant class (e.g. insecticides, herbicides, fertilisers, metals, pharmaceuticals, salts), contaminant name (e.g. cadmium, atrazine), exposure concentration, duration of the experiment, additional stressors and stressor type (e.g. predator, warming), biological information of the test species (order, genus, species and life-history stage), number of replicates, and the behavioural response (endpoint) that was quantified (see Table 1 for types of behavioural endpoints used in studies).

2.3.Effect-size calculation and statistical analysis

We extracted data from exposed and unexposed (control) groups from the text, tables or figures (using open source graphical digitiser software; Huwalt 2001) of the included studies and used this information to calculate response ratios (RRs): $\ln[RR] = \ln[E \div U]$, where $\ln[RR]$ is the natural log response ratio, E is the exposed group mean, U is the unexposed group mean (Hedges et al. 1999). For different behaviours, the direction of the RR can imply a positive or negative effect. For example, a positive RR for abnormal swimming would indicate higher rates of abnormal swimming in exposed individuals (a negative response to the contaminant), whereas a negative RR for feeding would be indicative of contaminant-induced reductions to feeding rate (another negative response).

For studies that considered the effect of different contaminants or multiple concentrations of the same contaminant, we calculated RRs for each. We also calculated RRs for each combination when two or more contaminants were investigated simultaneously. Our modelling approach (see below) ensured that studies with a high number of contaminants or concentrations were equally weighted.

A $\ln[RR]$ cannot be defined in situations where the numerator or denominator is zero,

but adding a constant to these values can lead to serious bias (Rosenberg et al. 2013). As such, we took the more conservative approach of excluding these data from further analysis. However, to include as much of the available information as possible, if the response was provided as a percentage or proportion with one zero value (e.g. proportion individuals feeding; 0% versus 60%), we converted both values to represent the opposite (i.e. proportion not feeding; 100% versus 40%), and then reversed the sign of the resultant RR. This meant that only 62 RRs (2% of the dataset) were excluded from analysis.

Not all combinations of behavioural measures and contaminant classes were represented in the final dataset, precluding the exploration of complex interactions. Instead, for each behavioural measure we constructed separate generalised linear mixed-effects models with contaminant class (e.g. metal) or name (e.g. cadmium) fitted as fixed effects, and 'replicate' (contaminant-specific concentration) nested within 'study' (reference ID) fitted as a random effect (Mengersen et al. 2013a, Sievers et al. 2018a). Replicate nested within study accounted for any correlation amongst responses for a given contaminant and accounted for common contextual effects. The study random effect accounted for any systematic differences due to study-specific methodologies or biases. This model structure allowed us to analyse multiple RRs from within a given study rather than having to aggregate data to a single mean value per study, and to ultimately take into consideration the non-independence of multiple entries extracted from the same study (Krist 2011, Davidson et al. 2017).

When variance estimates are not provided in all studies within a meta-analysis, alternative weighting approaches are often used, such as weighting based on sample size (Mengersen et al. 2013b). Many of the studies included in our meta-analysis did not report sufficient information to calculate variance (e.g. no information or presented metrics such as inter-quartile ranges). This precluded the calculation of standard weightings used in some meta-analyses for all included studies (Lajeunesse 2015). Instead of omitting a high

proportion of studies or simply relying on unweighted analyses, we calculated weights based on the sum of sample sizes, reducing the influence of estimates based on less replicated studies (Stanley and Doucouliagos 2015). Furthermore, since the models used maximum likelihood methods, studies were automatically (implicitly) weighted by the uncertainty of the estimates, since the regression analyses and the variation in the regression estimates were included as part of the model (Mengersen et al. 2013a).

We produced unbiased parameter estimates and 95% confidence intervals using restricted maximum-likelihood estimation (REML) and suppressed intercepts. For each behavioural class, we also performed sub-group analysis for studies that included both water controls and solvent controls to assess differences in these experimental designs. We used the lmerTest package (Kuznetsova et al. 2015) in R v.3.2.2 (R Development Core Team 2015) to build models and extract least-squares means and confidence intervals (Stanley and Doucouliagos 2015).

3. Results

Most studies investigating contaminant-induced behavioural changes were conducted in North America (58%) and Europe (27%), with Australasia, Asia and South America contributing a relatively small proportion of the research effort (total 15%). After excluding behavioural measures with insufficient replication to quantitatively analyse (i.e. ventilation, proportion head out of water, oviposition-site selection), we were left with 116 studies and 2,280 RRs (mean ± SD per study: 23 ± 45) based on experiments with 59 species (Appendix B). Of these studies, the majority focussed on pesticides (64%) with fewer on metals (14%), fertilisers (13%), pharmaceuticals (8%), salinity (6%), and others (<5%; Appendix C; Table

2) (note: summed percentages here and below are typically >100% as several studies focus on more than one contaminant, behaviour, developmental stage, etc.). Of the 72 studies on pesticides, 53% were on insecticides, 36% herbicides, 6% fungicides, and 5% others (e.g. molluscicides, surfactants). Metal studies focussed on copper (31%), lead (25%), cadmium (19%), aluminium (13%), mercury (13%), arsenic (6%) and zinc (6%). Eight studies looked at pharmaceuticals, six of which were on endocrine-disrupting chemicals (75%).

In terms of developmental stages, most studies used larvae (81%), with only 16% studying adults and 6% metamorphs (Appendix D). In terms of behavioural endpoints, most studies measured activity levels (57%), followed by velocity (21%), feeding behaviours (21%), reproductive behaviours (11%), hiding (11%), abnormal swimming patterns (8%), escape responses (8%), and surface activity (6%; Appendix C). In addition, 7% of the studies measured rates of amphibian predation following exposure. Experimental exposure durations ranged from less than 1 day to over 100 days, and showed considerable variability amongst contaminants, as did the number of replicates per trial and the exposure concentrations examined (Table 2; Appendix D).

Exposure to contaminants increased rates of abnormal swimming (+168%), with most of the evidence coming from studies testing insecticides (Figure 2). Overall, activity levels were reduced in exposed individuals (-36%), again largely driven by responses to insecticides and, to a lesser extent, metals (Figure 2). Reproductive behaviours were reduced overall following exposure to contaminants (-20%), but confidence intervals were wide for individual contaminant classes even though all mean RRs were negative (Figure 2). Exposure to herbicides and insecticides reduced the capacity to exhibit escape responses (grand mean: -42%), but exposure to metals and fertilisers did not produce the same effect (although mean responses were still negative; Figure 2). Feeding rates were reduced in exposed individuals (-43%), with insecticides largely driving the trend of negative response (Figure 2). We found

little evidence that exposure to contaminants affected levels of hiding, swimming speeds or surface behaviours, with confidence intervals overlapping zero (Figure 2). For forest plots of responses to each contaminant (e.g. atrazine, cadmium), see Appendix E.

Exposure to contaminants increased rates of amphibian predation – the culmination of multiple behaviours – when predators were not also exposed (+176%), but this trend reversed when predators were exposed along with the amphibians (i.e. the prey; -62%; Figure 3). We found little evidence that using a solvent control produced consistently different results to using a water control (uncontaminated or culture medium; Figure 4). Although RRs for hiding did differ statistically depending on the control type used, the size of this effect was small, and hiding was not a sensitive indicator of contamination. Out of the 112 studies, 42 considered an additional stressor, mostly the threat of predation (67%), acidity (12%) and temperature (warming; 10%).

4. **Discussion**

4.1. *Where and how the effects of contaminants on amphibian behaviour are being studied*

The majority of selected studies were conducted in North America, followed by Europe, mirroring the findings of other ecological meta-analyses (e.g. Conrad et al. 2011, Sievers et al. 2018a). Although not surprising, it is concerning since amphibian richness is highest in South America, Africa and Asia, and the proportion of threatened amphibians is greatest in the central and south American bioregion (Stuart et al. 2008). The observed geographical bias towards affluent areas also raises issues surrounding the transferability of findings. For example, it is possible that the ecological impact of contaminants will be more severe in

developing countries due to comparatively lower investment in, and less advanced technology for, pollution management (Kivaisi 2001). Conversely, these populations and species may be more adapted to polluted environments and exhibit reduced behavioural impacts (Flynn et al. 2019). Alternatively, tropical species may have traits that influence exposure pathways or alter how they react to contaminants (Clements et al. 2012). These uncertainties can only be addressed through increased research efforts in less studied regions, with comparison of the findings to existing research.

Most of research was conducted on larval amphibians (primarily tadpoles), likely due to a combination of (1) their fully aquatic lifestyle and fact that contaminants primarily enter aquatic environments, (2) larval life-stages being particularly vulnerable to predation, (3) easier ethics approval for studying tadpoles compared to adults, and (4) greater access to a larger number of comparable individuals relative to adult stages. Therefore, there is significantly less known about the impacts of contaminants on adult amphibians, including effects of exposure during the larval phase that may not manifest until adulthood, such as changes in reproductive behaviour and/or success. However, it is also possible that the known impacts on tadpoles are enough to cause alarm, and that additional studies with adults will not necessarily afford greater protection or concern.

Decisions regarding experimental design can have a large impact on the conclusions that are drawn from a study (Skelly 2002). Nevertheless, in terms of control type, we found little evidence that the magnitude or direction of behavioural responses to contaminants differed when experiments used a solvent control or water control. Our findings are consistent with those observed for pesticides by Shuman-Goodier and Propper (2016), where the relative impact of exposure on swimming speed and activity was independent of control type. Since solvents can exhibit toxic effects or increase the toxicity of particular contaminants (e.g. Eddleston et al. 2012, Melvin et al. 2018), the choice of control used

should be dictated by the study goal. For example, if the goal of the study is to quantify the impact of the active ingredient within a contaminant formulation, then using a solvent control (where the formulation uses one) is appropriate. For assessing potential ecological outcomes from contamination, we suggest that using a water control, and thus assessing the impact of the formulation against a contaminant-free environment, is more appropriate. However, when possible, experiments would ideally be conducted using both control and solvent-control; in this way, the effects of active ingredients and solvents can be partitioned to give a better idea of the causal factors of any behavioural changes. Finally, most studies do not appear to provide data on the quality of the exposure water. It is therefore not possible to observe whether the contaminant has affected the quality of the water or the animals or both, and thus whether impacts are direct or indirect (through contaminant-induced changes to water quality). We recommend that greater emphasis is placed on monitoring and reporting water quality during experiments, as this can be an important influence on the results of ecotoxicological studies (Hale et al. 2014).

Studies spanned a range of exposure periods, contaminant concentrations, and number of replicates (Table 2; Appendix D). The considerable variability likely represents site-specific conditions and consequent choices made by researchers. Mean exposure duration for each contaminant ranged between less than 1 day to over 60 days, suggesting that exposures are realistic, at least in longer-term studies. Given the high variability and purpose of this review, we do not delve into and critically assess the exact suitability of these experimental designs, but rely on researchers – who know intimately their study species and environments – to have conducted suitable and realistic experiments. Of course, these variables can influence responses, as with all experimental work, and examining environmental concentrations (as opposed to ‘laboratory concentrations’, which might be above concentrations experienced in the wild) under realistic conditions is critical if we are to

develop an accurate understanding of contaminant induced behavioural alterations. Taking this into consideration, we still suggest our meta-analytical and statistical approach still provides accurate generalisations about the influence of contaminants on amphibian behaviours.

4.2. How and which behaviours are being affected by contaminant exposure

We found that contaminants are broadly capable of influencing a wide range of behaviours in amphibians. Overall grand means (i.e. the mean behavioural response to all contaminants combined) were affected for many measures of behaviour, particularly for those that are well-studied (i.e. high replication). For these behaviours, contaminants typically had a reducing effect; that is, reducing rates of activity, breeding, feeding, normal swimming (i.e. opposite of abnormal swimming) and the ability of tadpoles to escape predation (Figure 2). In particular, our review highlights the considerable potential for insecticides to alter the behaviour of amphibians. Herbicides, fertilisers, endocrine disruptors and metals were tested as commonly as insecticides (i.e. often equal replication for response ratio calculations), but often had lesser impact.

Our findings match those from a meta-analysis on pesticides by Shuman-Goodier and Propper (2016), who found that pesticides reduced activity levels and swimming speeds in frogs and fish. Although our mean RR for speed matched that from Shuman-Goodier and Propper (2016), our confidence intervals overlapped zero, potentially following the inclusion of more recent studies or as a result of our methodology (see Key Recommendations below). These authors found that changes in swimming speed were more variable than changes in activity levels and suggested that this may be due to the breadth of behavioural endpoints measured. For detailed discussion of the nature and modes of action for each contaminant-

type and the physiological mechanisms causing changes to swimming speeds and activity levels, see Shuman-Goodier and Propper (2016).

Escape responses were hindered while rates of abnormal swimming increased following exposure to insecticides. Interestingly, Denoel et al. (2012) found that abnormal behaviours, such as swirling rapidly, only occurred in the presence of pesticides, in this case the organochlorine endosulfan. On the other hand, Sievers et al. (2018b) examined abnormal swimming in uncontaminated water immediately following exposure to imidacloprid and still observed considerable increases in rates of erratic swimming and decreases in tadpoles' capacity to escape simulated predators. These differences could be an artefact of the tested concentrations, but either way, such behavioural changes are likely to manifest into increased rates of predation in the wild.

Indeed, rates of amphibian predation increased following exposure to contaminants. However, this only occurred when predators were not themselves exposed. In studies that also exposed predators, predation rates declined substantially, often below rates when both predator and prey were unexposed (i.e. natural rates). The lack of effect following exposure to metals is driven by one individual study (Hayden et al. 2015). For this study, we calculated response ratios for both non-lethal and lethal predatory attacks. These two measures, however, exhibited opposite trends, essentially cancelling each other out (a RR of zero); the frequency of non-lethal attacks was reduced, but lethal attacks increased, following exposure. The authors frame their conclusions as predation increasing under contamination, as the rate of fatal attacks (the more important and ecologically relevant measure) was much greater following exposure (Hayden et al. 2015). Taken together, under more natural conditions (i.e. when all animals are exposed to the same conditions), contamination may in fact lead to reduced predation upon amphibians, suggesting that the (sublethal) sensitivity of predators is greater than that of prey. However, evidence to support this comes from few studies. Our

findings highlight the pressing need to assess the ecological effects of contaminants under realistic scenarios (also see Mandrillon and Saglio 2007). Finally, studies that examine rates of predation following exposure to contaminants should aim to tease apart the effects of the contaminant on locomotion versus neurological changes in response to the presence of predators.

4.3.Key recommendations

4.3.1. Including greater details of experimental results

Several papers did not provide quantitative data for behavioural responses with statistically non-significant differences between exposed and unexposed groups (i.e. when RRs would be close to zero, or when statistical power was low). Therefore, it is possible that mean response ratios would be closer to zero for some behavioural responses if all possible data were available, but this is currently unknown. This is a well-known yet still pervasive issue extending beyond ecotoxicology and ecology (Csada et al. 1996, Koricheva 2003). We strongly urge that all results (especially information needed to calculate effect sizes such as means, measures of variation, sample size) are presented in future papers regardless of whether statistically significant results are detected.

4.3.2. *Developing a strong research base for future quantitative reviews*

Since we included response ratios for all concentrations of contaminants tested (albeit with our nested random effect), the relatively large confidence intervals for specific classes of contaminants may reflect dose dependent responses. The nature of these dose-response relationships is not clear from our analysis (e.g. directional versus non-monotonic), and this is an avenue for future study. As more studies on contaminant-induced behavioural changes become available, future meta-analyses will have increasing confidence in the pooled

evidence. With sufficient replication, these future quantitative reviews can also assess dose-response relationships that may exist, documenting contaminant-specific concentration thresholds below which no behavioural impacts are observed. Ultimately, a strong research base will be invaluable for providing direction to regulators and industries that use or deal with these contaminants.

4.3.3. Broadening the suite of contaminants tested and where studies are conducted

Although studying prevalent and ubiquitous contaminants such as insecticides and herbicides in well-studied locations is important, new and emerging contaminants and data-poor areas desperately need greater focus, as impacts are largely unknown but potentially substantial. For example, endocrine disruptors (although these can include pesticides and metals) are found in waste and surface waters around the world (Aris et al. 2014) and can impair the natural hormonal pathways of aquatic animals. However, the impacts of endocrine disruptors on amphibian behaviours are not well studied (although see Tamschick et al. 2016), despite evidence that these substances are highly relevant to amphibian populations (Lambert et al. 2015, Lambert and Skelly 2016, Bókony et al. 2018). Broadening the suite of contaminants being tested and where they are being tested, will help reveal how different contaminant classes with disparate modes of action influence behaviour around the world, and will further help to unravel how amphibians respond to exposure in the wild.

4.3.4. Exploring more the effects of multiple stressors

Our focus here was on investigating the effects of individual contaminants on amphibian behaviours. However, ecosystems are stressed in many ways, and it is important to identify how these additional stressors (e.g. presence of predators, warming) contribute to changes in

behaviour in conjunction with chemical stressors. We found that 40% of studies investigated additional, non-chemical stressors. Assessing interactions amongst multiple stressors can be extremely complex, but is critical to evaluate the impact of chemical and other threats under realistic conditions (Côté et al. 2016). This includes assessing behavioural responses to multiple stressors (Hale et al. 2017). Given that different interaction types can produce very different ecological outcomes, future research should investigate responses to combinations of stressors that occur in nature (Halfwerk and Slabbekoorn 2015). Understanding when and how stressors interact will be important for managing their effects, i.e. should management focus on mitigating one or more stressors, and if the latter, which?

4.3.5. *Establishing the ecological importance of alterations to behaviour*

Behaviours used to assess the impact of contaminants should have probable relevance for fitness outcomes such as responses to predator scents or simulated attacks. Throughout the literature, links between such behavioural responses and fitness outcomes are often implied rather than directly tested, particularly in terms of predation rates (Bridges 1999, Sievers et al. 2018b). On occasion, opposite response types are interpreted in a way that suits the study narrative (typically, that the response is maladaptive). For instance, contaminant-induced reductions in overall activity – particularly when described as immobility or lethargy – have been suggested to increase predation due to a lower capacity to escape predators, whereas increased activity has been suggested to increase predation due to increased detectability (Azevedo-Ramos et al. 1992, Jung and Jagoe 1995, Lavorato et al. 2013, Sievers et al. 2018b).

Well-designed studies are necessary to link fitness outcomes to contaminant-induced behavioural responses and avoid misappropriation of responses. Future research on contaminant-induced behavioural responses in amphibians should focus on how behaviour

correlates with fitness (e.g. Hayden et al. 2015) and how reduced fitness might scale-up to influence population demographics or even community-level metrics (see Wong and Candolin 2015). Interdisciplinary work between ecotoxicologists, behavioural ecologists and modellers can help to unravel these pathways (also see Relyea and Hoverman 2006). Population modelling provides tools to undertake targeted risk assessments that align with what needs to be protected, such as populations and communities (O'Brien 2017). Meta-analyses on behavioural and fitness responses to contaminants, particularly those that provide detailed information across all life-history stages, can provide a basis for understanding the population processes that mediate the effects of contaminants (O'Brien 2017). Models that use data on individual-level responses to predict demographic responses have long existed (Chapman 2002), but we have yet to see proper application of these tools, likely due to perceived costs, complexities, or lack of suitable data (see O'Brien 2017). We suggest that greater interdisciplinary collaboration will help break down these barriers, culminating in more effective predictions about the potential impacts of contaminants and how best to mitigate them.

5. Conclusions

Many contaminants influence a wide range of amphibian behaviours. Insecticides in particular strongly altered amphibian behaviours that intuitively are capable of reducing fitness for exposed individuals, potentially due to comparatively higher concentrations being tested. During our data extraction, we noted a pervasive issue that has long permeated ecology and ecotoxicology; many responses deemed ‘non-significant’ are not presented in publications, with likely implications for future studies and meta-analyses. Overall, our review suggests that researchers need to report all results of experiments regardless of statistical significance, design experiments to accurately reflect environmental conditions,

experiment with novel contaminants in under-studied regions, and expand experiments to try to unravel the true ecological implications of behavioural effects. Given that stressors rarely occur in isolation in nature, our understanding of contaminant impacts would also greatly benefit from more multiple stressor studies. Revised data show that behavioural endpoints can provide useful indicators of contamination. The sub-lethal nature of behavioural responses also makes them a more ethical and perhaps more ecologically relevant endpoint to use in ecotoxicological research.

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8. Author contributions

MS, RH, KP, SS: Conceptualization; MS: Data curation; MS: Formal analysis; MS, RH, SS, KP: Investigation; MS, RH, KP, SS: Methodology; All authors: Visualization; MS: Writing - original draft; All authors: Writing - review & editing.

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Figure 1. PRISMA flow diagram showing the procedure used for selection of studies for systematic review. Note that the additional papers identified from bibliographies did not contribute any papers to the quantitative synthesis

Figure 2. Forest plots of weighted response ratios (and 95% CI on log scale) for (A) abnormal swimming, (B) activity, (C) reproductive behaviours, (D) escape responses, (E) feeding behaviours, (F) hiding, (G) surface activity, and (H) velocity (where $n > 5$). We used weighted linear mixed-effects models with a unique identifier for each replicate and study fitted as a nested random effect (replicate within study), and intercepts were suppressed so that we could estimate separate coefficients. Grand means were estimated by removing contaminant-class as a factor from behavioural class-specific models. Numbers in brackets on the right indicate the number of response ratios within each data point, and grand means are boldface with percentage changes provided. PCB: polychlorinated biphenyl; Surfactant b.d.p.: Surfactant break-down product.

Figure 3. Forest plots of weighted response ratios (and 95% CI on log scale) for the rate at which amphibians were predated. We used weighted linear mixed-effects models with a unique identifier for each replicate and study fitted as a nested random effect (replicate within study), and intercepts were suppressed so that we could estimate separate coefficients. Grand means were estimated by removing contaminant-class as a factor from behavioural class-specific models. Numbers in brackets on the right indicate the number of response ratios within each data point, and grand means are boldface with percentage changes provided. For model details, see Figure 2 caption.

Figure 4. Forest plots of weighted response ratios (and 95% CI on log scale) of response to contaminants for seven behavioural endpoints comparing experiments with water (triangles) or solvent (squares) controls. We used weighted linear mixed-effects models with a unique identifier for each replicate and study fitted as a nested random effect (replicate within study), and intercepts were suppressed so that we could estimate separate coefficients. Numbers in brackets on the right indicate the number of response ratios within each data point.

Table 1. The initial behavioural endpoints that data were extracted for, and the subsequent behavioural endpoints used in the meta-analysis.

Endpoint used in analyses	**Endpoint extracted from study**
Abnormal swimming	Abnormal/irregular/erratic/dysfunctional swimming
	Convulsing
	Swirling
	Turns >90 degrees
	Twisting
Activity	Active (proportion individuals/time/detects)
	Line crosses
	Movement frequency
	Burst distance

	Distance moved
	Immobility duration*
	Periods of inactivity*
	Proportion immobile*
Reproductive behaviours	Approach (sexual)
	Arm waves
	Mating call duration
	Mating call latency
	Mating call rate
	Clasping
	Courtship time
	Fan duration
	Latency of female to respond to male
	Receptivity
	Time to breeding
	Touch
Escape response (simulated	Normal escape response

predator)	
	Number of stimuli to elicit response
	Prop move >4cm when prodded
	Responsiveness (prods per m)
	Startle response
Feeding behaviours	Attack prey frequency
	Feeding rate
	Prey consumed
	Proportion that ate
	Time feeding
	Time to capture prey
Hiding	Hiding
	In refuge
	Out in the open*
	Visible*
Predated**	Attacks by predator per encounter
	Average time till first attacked

	Eaten by predator
	Latency time to get ingested by predator
	Number consumed
	Time to be captured
Surface activity	Proportion at the surface
	Number of trips to the surface
Swimming speed	Average acceleration
	Average velocity
	Burst speed
	Max acceleration
	Max swim speed
	Max velocity
	Sprint speed
	Swimming speed

*measures for which the sign of the response ratios was reversed. This was done so the direction of effect was the same (e.g. reversing the sign of RRs for 'inactivity' so they can be grouped within 'activity').

**Rate at which amphibians were predated (i.e. a culmination of behaviours, but not a behaviour per se).

Table 2. Summary of the contaminant concentrations used in studies included in the meta-analysis. Provided are minimum, maximum, mean and median concentrations for each contaminant, in addition to the units of measurement (dictated by the most common unit specified by the study authors) and the number of replicates (n). For full reference details see Appendix A.

Contaminant	Min	Max	Mean	Median	Units	n	Species	Reference
Analgesic								
Acetaminophen	1	1000	337	10	ug/L	12	*Rana pipiens*	Fraker and Smith 2004
Antibacterial and antifungal								
Triclosan	0.23	230	63.9	12.7	ug/L	8	*Rana pipiens*	Fraker and Smith 2004
Anti-depression								
Fluoxetine	0.03	3	1.11	0.3	ug/L	9	*Bufo bufo*	Barry 2014
Anti-fouling								
Medetomidine	100	1000	700	1000	nM	12	*Anaxyrus americanus, Lithobates sylvaticus*	Fong et al 2018
Anti-Inflammatory Drugs								

	Naproxen	0.1	1000	222	10	ug/L	10	*Limnodynastes peronii*	Melvin 2016
EDC									
	17a-ethinylestradiol	0.296	296	66.45	2.96	ng/L	5	*Xenopus laevis*	Hoffmann and Kloas 2012b; 2012c
		1E-10	1E-10	1E-10	1E-10	M	24		
	4-Tert octylphenol	1E-08	1E-07	5.5E-08	5.5E-08	M	18	*Xenopus tropicalis*	Schwendiman and Propper 2012
	Dihydrotestosterone	100	100	100	100	ug/L	2	*Xenopus laevis*	Carr et al 2003
	Estradiol	100	100	100	100	ug/L	2	*Xenopus laevis*	Carr et al 2003
	Estradiol benzoate	1E-08	1E-08	1E-08	1E-08	M	9	*Xenopus tropicalis*	Schwendiman and Propper 2012
	Ethynylestradiol	1.65	179.5	40.39	1.65	ng/L	5	*Xenopus tropicalis*	Gyllenhammar et al 2009
	Fulvestrant	1E-07	1E-	1E-07	1E-07	M	8	*Xenopus laevis*	Hoffmann and Kloas

		07						2012b
Methyldihydrotestosterone	0.03	30.45	11.18	3.05	ng/L	54	*Xenopus laevis*	Hoffmann and Kloas 2012a
Tamoxifen	1E-10	1E-07	3.7E-08	1E-08	M	24	*Xenopus laevis*	Hoffmann and Kloas 2012b
Fertiliser								
Nitrogen	1.35	500	79.9	90	mg/L	163	*Bufo bufo*, *Discoglossus galganoi*, *Epidalea calamita*, *Hypsiboas faber*, *Lissotriton boscai*, *Lissotriton helveticus*, *Lithobates clamitans*, *Lithobates*	Burgett et al 2007, Egea-Serrano and Van Buskirk 2016, Egea-Serrano et al 2011, Garcia-Munoz et al 2011, Hatch and Blaustein 2000, Ilha and

							sylvaticus, *Pelobates cultripes*, *Pelodytes ibericus*, *Pelophylax perezi*, *Rana cascadae*, *Rana temporaria*, *Triturus vulgaris*	Schiesari 2014, Ortiz-Santaliestra et al 2009, 2010, Polo-Cavia et al 2016, Secondi et al 2009, 2013, Smith et al 2011, Watt and Oldham 1995, Xu and Oldham 1997
Fungicide								
	Fenpropimorph	2	11	6.5	6.5	ug/L	6	*Rana temporaria*	Teplitsky et al 2005
	Triphenyltin	1.25	20	8.75	5	ug/L	18	*Rana temporaria*, *Xenopus laevis*	Schriks et al 2006, Semlitsch et al

								1995
Vinclozolin	1E-10	1E-06	3.4E-07	1E-08	M	24	*Xenopus laevis*	Hoffmann and Kloas 2010
Herbicide								
Amitrole	0.01	10	2.78	0.55	mg/L	16	*Rana temporaria*	Mandrillon and Saglio 2007a
Atrazine	1	20000	424	40	ug/L	70	*Ambystoma barbouri*, *Ambystoma maculatum*, *Anaxyrus terrestris*, *Hyla chrysoscelis*, *Lithobates sylvaticus*, *Osteopilus septentrionalis*, *Rana*	Allran and Karasov 2001, Britson and Threlkeld 1998, Carr et al 2003, Davis et al 2012, Ehrsam et al 2016, Koprivnikar et al 2007, Mitchkash et al 2014,
	10	192	46.4	10	ppb	5		

							clamitans, *Rana pipiens*, *Xenopus laevis*	Rohr and Crumrine 2005, Rohr and Palmer 2005, Rohr et al 2003, 2004, Wood and Welch 2015
Glufosinate ammonium	3.55	15	8.22	7.38	mg/L	12	*Hypsiboas pulchellus*	Peltzer et al 2013
Glyphosate	0.007	7	2.08	1.56	mg/L	40	*Ambystoma maculatum*, *Anaxyrus terrestris*, *Eurycea wilderae*, *Hyla versicolor*, *Lissotriton vulgaris*, *Lithobates*	Gandhi and Cecala 2016, Katzenberger et al 2014, Levis et al 2016, McComb et al 2008, Miko et al 2017, Moore et al 2015,
	225	225	225	225	mg/kg	1		

							sylvaticus, *Rana clamitans*, *Rana dalmatina*, *Rana pipiens*, *Taricha granulosa*	Ujszegi et al 2015, Wojtaszek et al 2004, Wood and Welch 2015
	Hexazinone	100	100	100	100	ppm	48	*Rana catesbeiana*, *Rana clamitans*, *Rana pipiens*	Berrill et al 1994
	Imazapyr	4.8	9.7	7.25	7.25	ppm	122	*Rana pretiosa*	Yahnke et al 2013
	Triclopyr	37	37	37	37	mg/L	31	*Rana aurora*, *Rana catesbeiana*, *Rana clamitans*, *Rana pipiens*	Berrill et al 1994, Yahnke et al 2017
		0.6	4.8	1.5	1.2	ppm	125		
Humic acid									
	Humic acid	20	20	110	110	mg/	2	*Pelobates*	Polo-Cavia et

		0			L		*cultripes*	al 2016
Hydrocarbon								
Fluoranthene	10	60	32	40	ug/L	10	*Rana catesbeiana*	Walker et al 1998
PCBs	0.005	5	2.2	0.1	ug/L	58	*Rana clamitans*, *Rana pipiens*	Glennemeier and Denver 2011, Rosenshield et al 1999
Insecticide								
Abamectin	18	72	42	36	ug/L	15	*Lithobates catesbeianus*	do Amaral et al 2018
Bacillus thuringiensis var. israelensis	3	13.88	7.29	5	mg/L	9	*Physalaemus albonotatus*, *Rhinella arenarum*, *Rhinella fernandezae*	Junges et al 2017

Carbaryl	0.0005	8.3	3.28	3.5	mg/L	144	*Ambystoma barbouri*, *Ambystoma maculatum*, *Anaxyrus terrestris*, *Hyla versicolor*, *Notophthalmus viridescens*, *Rana berlandieri*, *Rana blairi*, *Rana catesbeiana*, *Rana clamitans*, *Rana sphenocephala*, *Xenopus laevis*	Bridges 1997, 1999a, 1999b, Davis et al 2011, Mitchkash et al 2014, Punzo 2005, Relyea and Edwards 2010, Relyea and Mills 2001, Rohr et al 2003, Wood and Welch 2015, Zaga et al 1998
Chlorpyrifos	0.001	1.5	0.2549	0.1	mg/L	1116	*Acris crepitans*, *Ambysto*	Richars and Kendall

	0.1	1000	135.8	10	uM	9	*ma mexicanum, Duttaphrynus melanostictus, Gastrophryne olivacea, Hyla chrysoscelis, Rana sphenocephala, Xenopus laevis*	2003, Robles-Mendoza et al 2011, Watson et al 2014, Widder and Bidwell 2006, 2008, Wijeshinghe et al 2011
Diazinon	0.5	1	0.75	0.75	mg/L	6	*Pseudocris regilla, Xenopus laevis*	Kerby et al 2012, Watson et al 2014
	0.1	100	27.775	5.5	uM	8		
Dichlorvos	0.1	100	27.775	5.5	uM	8	*Xenopus laevis*	Watson et al 2014
Endosulfan	0.0000 3	0.282	0.05	0.01	mg/L	2 5 5	*Ambystoma barbouri,*	Broomhall 2002, 2004,

	5	10	7.5	7.5	ppb	2	*Bufo bufo, Hyla citropa, Limnodynastes peronii, Lithobates sylvaticus, Litoria freycineti, Notophthalmus viridescens, Rana dalmatina, Rana temporaria*	Broomhall and Shine 2003, Brunelli et al 2009, Denoel et al 2012, 2013, Lavorato et al 2013, Park et al 2001, Rohr and Crumrine 2005, Rohr et al 2003
Esfenvalerate	0.8	10	4.1	3.6	ug/L	14	*Rana spp.*	Materna et al 1995
Fenitrothion	2.5	2.5	2.5	2.5	mg/L	1	*Hypsiboas pulchellus, Rana catesbeiana, Rana clamitans, Rana*	Berrill et al 1994, Junges et al 2010
	0.5	8	3.1	2	ppm	238		

							pipiens	
Fenvalerate	0.01	0.1	0.03	0.01	ppm	74	*Ambystoma maculatum, Rana clamitans, Rana pipiens*	Berrill et al 1993
Imidacloprid	0.25	0.5	0.38	0.38	ug/L	12	*Limnodynastes tasmaniensis*	Sievers et al 2018
Malathion	0.001	1	0.64	1	mg/L	11	*Hyla chrysoscelis, Hyla versicolor, Lithobates sylvaticus, Rana catesbeiana, Rana clamitans*	Krishnamurthy and Smith 2011, Mackey and Boone 2009, Relyea and Edwards 2010
Methoxychlor	0.001	1	0.23	0.03	mg/L	10	*Ambystoma macrodactylum, Xenopus*	Eroschenko et al 2002, Fort et al 2004, Verrell
	0.0	1	0.2	0.1	u	5		

		1		9		M		*laevis*	2000
	Permethrin	0.003	0.012	0.007	0.006	mg/L	9	*Ambystoma maculatum*, *Physalaemus albonotatus*, *Rana clamitans*, *Rana pipiens*, *Rhinella arenarum*, *Rhinella fernandezae*	Berrill et al 1993, Junges et al 2017
		0.01	2	0.1	0.01	ppm	81		
	Temephos	3.1	16	7.6	3.7	mg/L	9	*Physalaemus albonotatus*, *Rhinella arenarum*, *Rhinella fernandezae*	Junges et al 2017
Metal									
	Aluminium	222	222	222	222	ug/L	2	*Triturus helveticus*, *Triturus*	Brady and Griffiths

							vulgaris	1995
Arsenic	10	1000	336	150	ug/L	5	*Rana pipiens*	Chen et al 2009
Cadmium	0.002	1	0.23	0.02	mg/L	15	*Duttaphrynus melanostictus*, *Pelophylax nigromaculata*, *Rana luteiventris*	Huang et al 2018, Lefcort et al 1998, Ranatunge et al 2012
	1E-08	1E-06	1E-06	0.000001	M	15		
	0.1	0.1	0.1	0.1	ppm	2		
Copper	0.0185	0.1	0.056	0.06	mg/L	87	*Bufo bufo*, *Epidalea calamita*, *Limnodynastes tasmaniensis*, *Lithobates sylvaticus*, *Pelodytes ibericus*, *Rana pipiens*	Chen et al 2007, Garcia-Munoz et al 2009, 2011, Hayden et al 2015, Reeves et al 2011, Sievers et al 2018

	Lead	3	12 80	381	160	ug /L	1 1	*Pelophylax nigromaculata, Rana catesbeiana, Rana luteiventris, Rana pipiens*	Chen et al 2006, Huang et al 2014, Lefcort et al 1998, Rice et al 1999,
		0.01	25	12.5	12.5	ppm	4		
	Mercury	2.5	10	6.25	6.25	ug /g	4	*Bufo americanus*	Bergeron et al 2011
	Methyl mercury	0.4	0.4	0.4	0.4	mg/ kg	1	*Hyla chrysoscelis*	Britson and Threlkeld 1998
	Nickle	0.04	5.5	1.2	0.4	mg/ L	27	*Lithobates sylvaticus*	Klemish et al 2018
	Zinc	0.05	15	7.53	7.53	ppm	4	*Rana luteiventris*	Lefcort et al 1998
Molluscicide									
	Metaldehyde	0.25	0.5	0.375	0.375	mg/ L	8	*Rhinella arenarum*	Attademo et al 2016
	Triphenyltin	1	5	3	3	ug	1	*Ambystoma*	Rehage et al

						/L	2	*barbouri*	2002
Ozone									
	Ozone	0.6	0.6	0.6	0.6	ug/L	6	*Bufo bufo*	Dohm et al 2008
Salt									
	Salt	500	40000	1198	1000	mg/L	31	*Ambystoma jeffersonianum*, *Anaxyrus terrestris*, *Limnodynastes peronii*, *Lithobates sylvaticus*, *Litoria ewingii*, *Rana temporaria*, *Rhinela spinulosa*	Chambers 2011, Denoel et al 2010, Hall et al 2017, Kearney et al 2016, Milotic et al 2017, Sanabria et al 2018, Winkler and Forte 2011, Wood and Welch 2015
		4	4200	3001	4200	ppm	9		
		1200	1200	1200	1200	us/cm	3		

Stimulant								
Caffiene	0.6	600	202	6	ug/L	6	*Rana pipiens*	Fraker and Smith 2004
Surfactant breakdown product								
Octylphenol	5	500	185	50	ug/L	24	*Ambystoma barbouri*	Rohr et al 2003

Highlights

- Chemical contamination is driving amphibian declines, often at sub-lethal concentrations
- We used meta-analysis to quantify responses to a suite of contaminants
- Contaminants caused abnormal swimming and affected escape responses
- Behaviours were typically altered in meaningful ways
- Understanding behavioural alterations help predict ecological implications

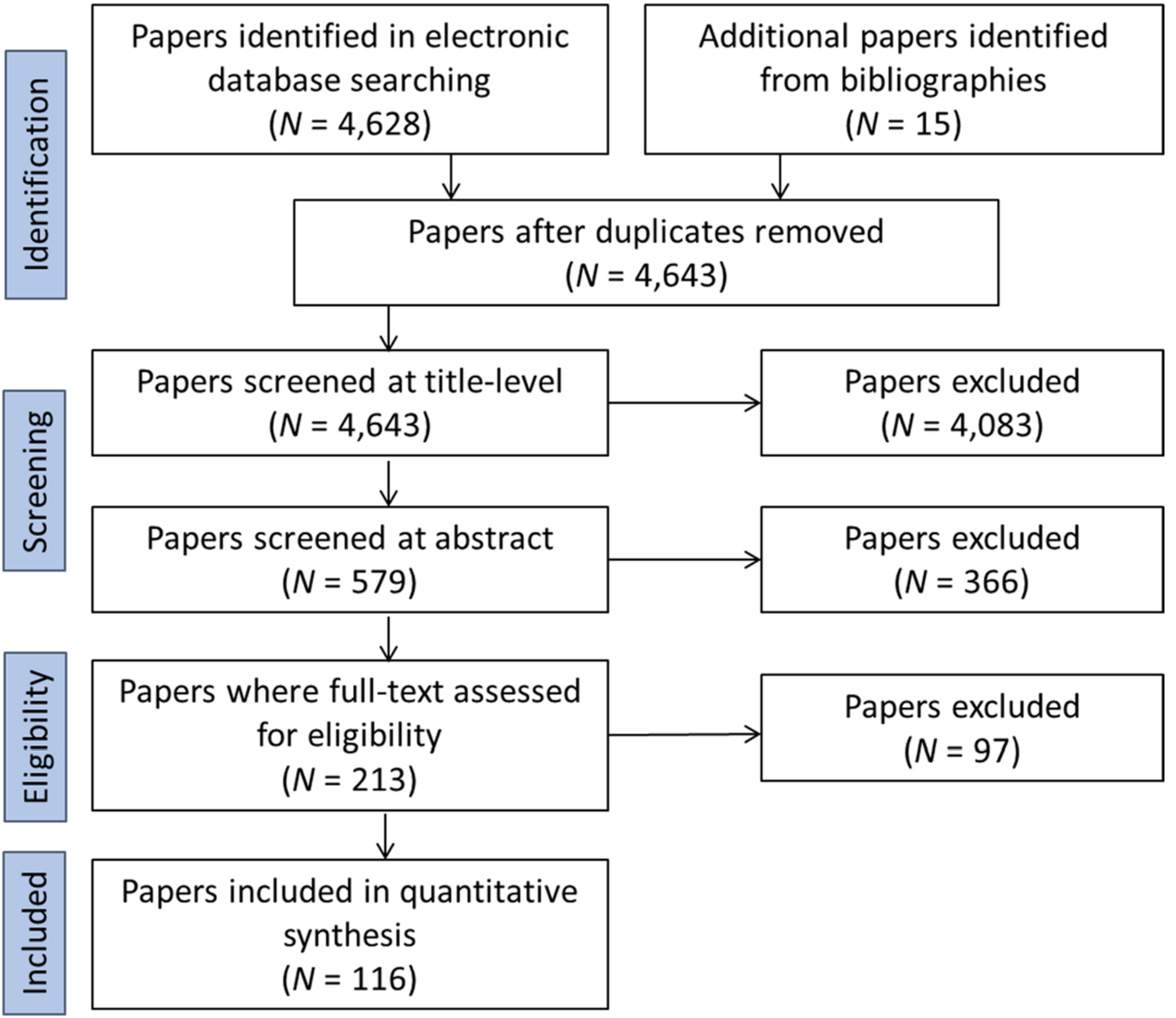
Identification
Papers identified in electronic database searching (N = 4,628)
Additional papers identified from bibliographies (N = 15)
Papers after duplicates removed (N = 4,643)
Screening
Papers screened at title-level (N = 4,643)
Papers excluded (N = 4,083)
Papers screened at abstract (N = 579)
Papers excluded (N = 366)
Eligibility
Papers where full-text assessed for eligibility (N = 213)
Papers excluded (N = 97)
Included
Papers included in quantitative synthesis (N = 116)

Figure 1

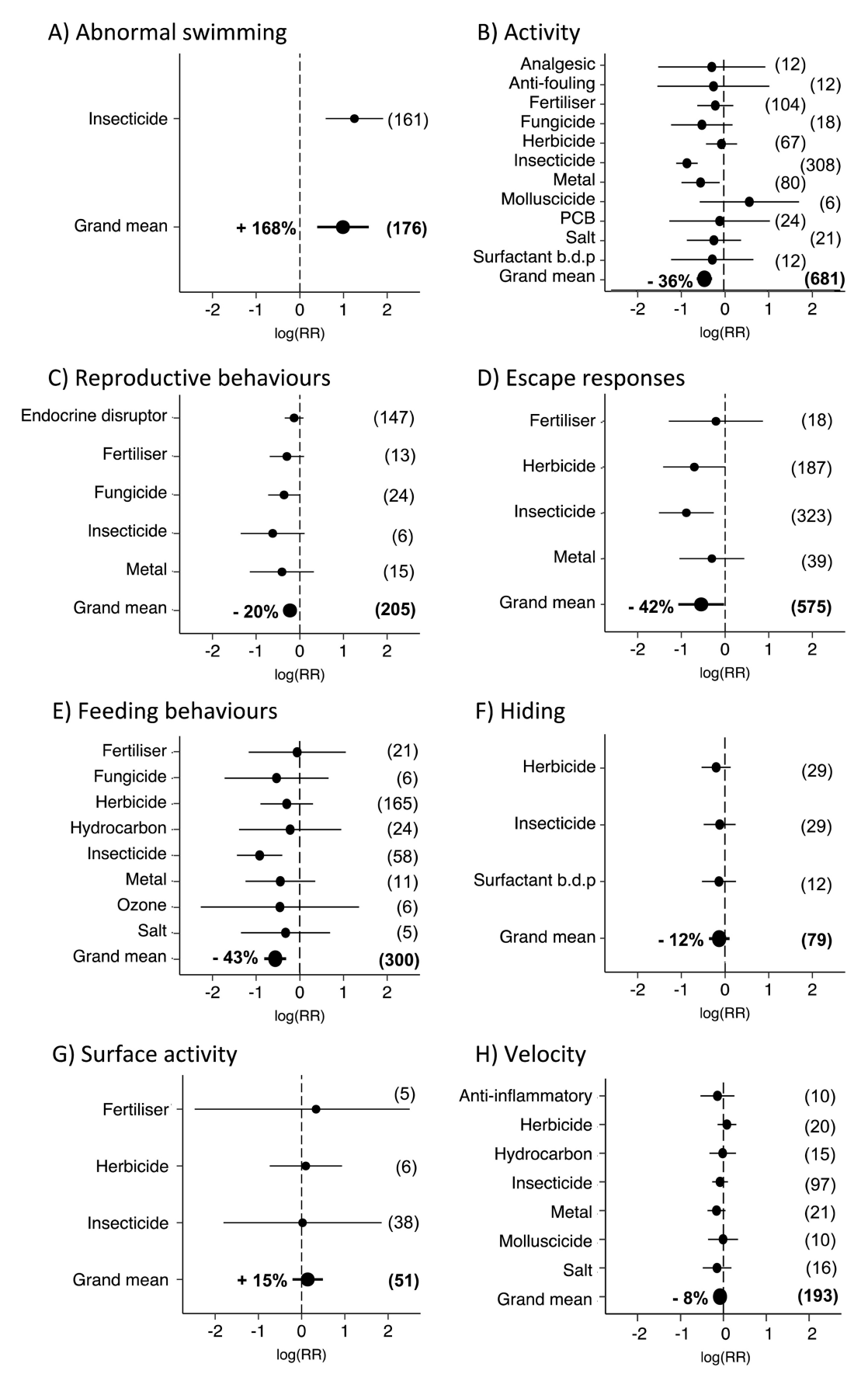
A) Abnormal swimming
Insecticide
(161)
Grand mean
+ 168%
(176)
-2 -1 0 1 2
log(RR)
B) Activity
Analgesic (12)
Anti-fouling (12)
Fertiliser (104)
Fungicide (18)
Herbicide (67)
Insecticide (308)
Metal (80)
Molluscicide (6)
PCB (24)
Salt (21)
Surfactant b.d.p (12)
Grand mean
- 36%
(681)
-2 -1 0 1 2
log(RR)
C) Reproductive behaviours
Endocrine disruptor (147)
Fertiliser (13)
Fungicide (24)
Insecticide (6)
Metal (15)
Grand mean
- 20%
(205)
-2 -1 0 1 2
log(RR)
D) Escape responses
Fertiliser (18)
Herbicide (187)
Insecticide (323)
Metal (39)
Grand mean
- 42%
(575)
-2 -1 0 1 2
log(RR)
E) Feeding behaviours
Fertiliser (21)
Fungicide (6)
Herbicide (165)
Hydrocarbon (24)
Insecticide (58)
Metal (11)
Ozone (6)
Salt (5)
Grand mean
- 43%
(300)
-2 -1 0 1 2
log(RR)
F) Hiding
Herbicide (29)
Insecticide (29)
Surfactant b.d.p (12)
Grand mean
- 12%
(79)
-2 -1 0 1 2
log(RR)
G) Surface activity
Fertiliser (5)
Herbicide (6)
Insecticide (38)
Grand mean
+ 15%
(51)
-2 -1 0 1 2
log(RR)
H) Velocity
Anti-inflammatory (10)
Herbicide (20)
Hydrocarbon (15)
Insecticide (97)
Metal (21)
Molluscicide (10)
Salt (16)
Grand mean
- 8%
(193)
-2 -1 0 1 2
log(RR)

Figure 2

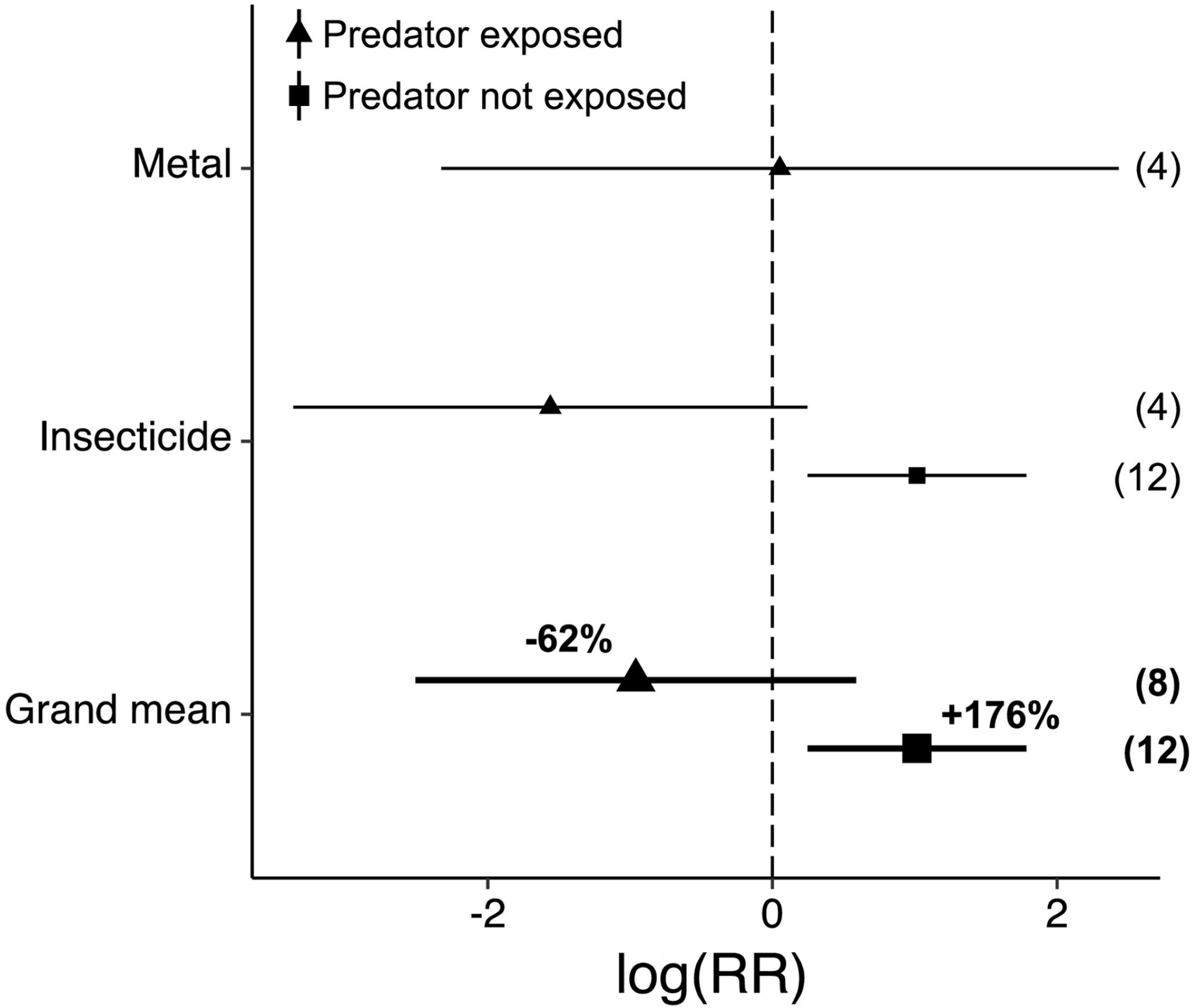
Predator exposed
Predator not exposed
Metal
Insecticide
Grand mean
(4)
(4)
(12)
-62%
(8)
+176%
(12)
-2
0
2
log(RR)

Figure 3

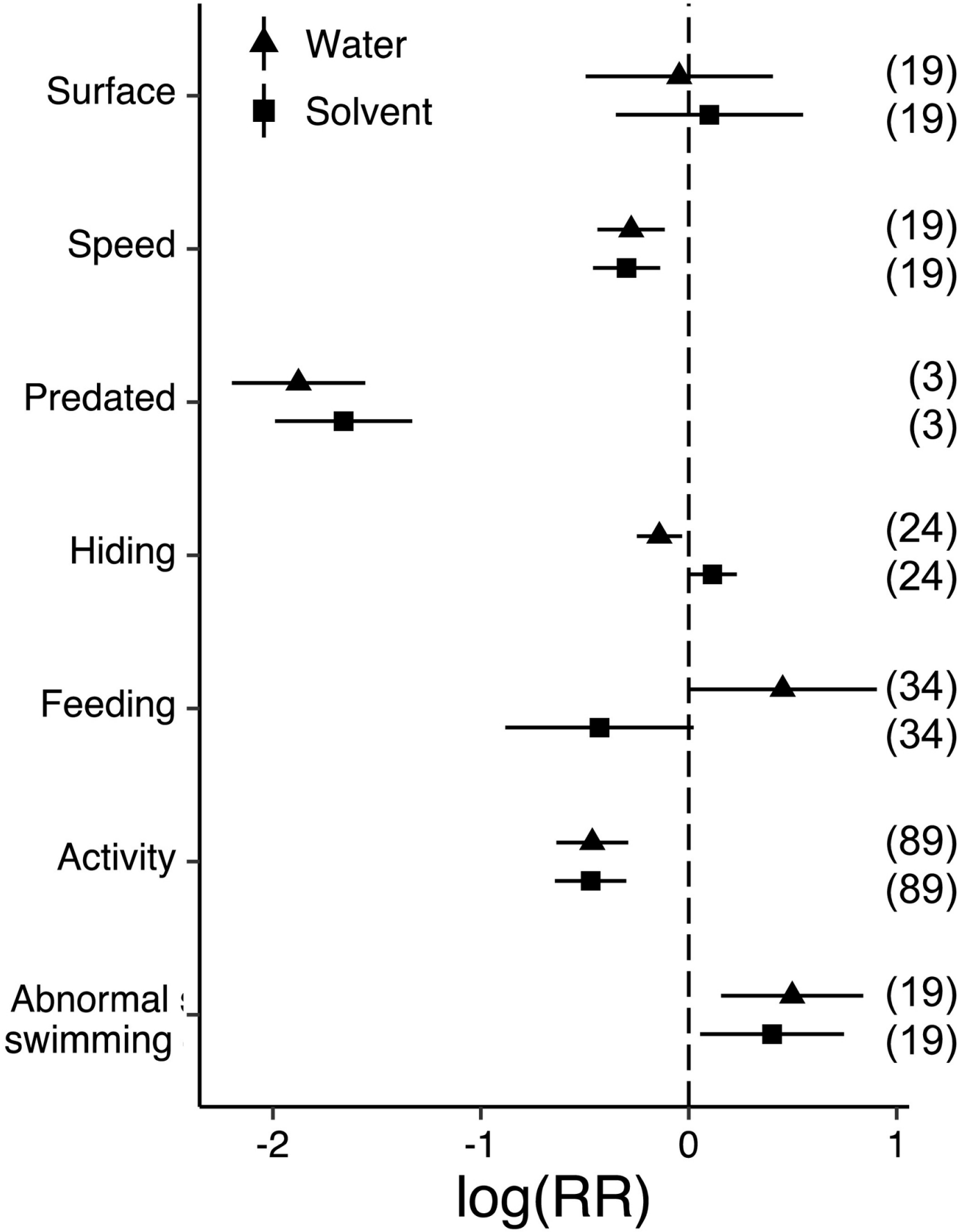
Water
Solvent
Surface
Speed
Predated
Hiding
Feeding
Activity
Abnormal
swimming
(19)
(19)
(19)
(19)
(3)
(3)
(24)
(24)
(34)
(34)
(89)
(89)
(19)
(19)
-2
-1
0
1
log(RR)

Figure 4