Author/s:
Rutkowski, NAJ;McNamara, KB;Jones, TM;Foo, YZ

Title:
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Date:
2023-08-01

Citation:
Rutkowski, N. A. J., McNamara, K. B., Jones, T. M. & Foo, Y. Z. (2023). Trans-generational immune priming is not mediated by the sex of the parent primed: a meta-analysis of invertebrate data. Biological Reviews, 98 (4), pp.1100-1117. https://doi.org/10.1111/brv.12946.

Persistent Link:
https://hdl.handle.net/11343/333832

BIOLOGICAL REVIEWS
Cambridge Philosophical Society
Biol. Rev. (2023), pp. 000–000.
doi: 10.1111/brv.12946

Trans-generational immune priming is not mediated by the sex of the parent primed: a meta-analysis of invertebrate data

Nicola-Anne J. Rutkowski[1,*], Kathryn B. McNamara[1], Therésa M. Jones[1] and Yong Zhi Foo[2]

[1]*School of BioSciences, University of Melbourne, Biosciences 4, Royal Parade, Parkville, VIC, 3052, Australia*
[2]*Centre for Evolutionary Biology & School of Biological Sciences, University of Western Australia, 35 Stirling Hwy, Crawley, WA, 6009, Australia*

ABSTRACT

Traditionally, only vertebrates were thought capable of acquired immune responses, such as the ability to transfer immunological experience vertically to their offspring (known as trans-generational immune priming, TGIP). Increasing evidence challenges this belief and it is now clear that invertebrates also have the ability to exhibit functionally equivalent TGIP. This has led to a surge in papers exploring invertebrate TGIP, with most focusing on the costs, benefits or factors that affect the evolution of this trait. Whilst many studies have found support for the phenomenon, not all studies do, and there is considerable variation in the strength of positive results. To address this, we conducted a meta-analysis to answer the question: what is the overall effect of TGIP in invertebrates? Then, to understand the specific factors that affect its presence and intensity, we conducted a moderator analysis. Our results corroborate that TGIP occurs in invertebrates (demonstrated by a large, positive effect size). The strength of the positive effect was related to if and how offspring were immune challenged (i.e. whether they were challenged with the same or different insult as their parents or not challenged at all). Interestingly, there was no effect of the ecology or life history of the species or the sex of the parent or the offspring primed, and responses were comparable across different immune elicitors. Our publication bias testing suggests that the literature may suffer from some level of positive-result bias. However, even after accounting for potential bias, our effect size remains positive. Publication bias testing can be influenced by diversity in the data set, which was considerable in our data, even after moderator analysis. It is therefore conceivable that differences among studies could be caused by other moderators that were unable to be included in our meta-analysis. Nonetheless, our results suggest that TGIP does occur in invertebrates, whilst providing some potential avenues to examine the factors that account for variation in effect sizes.

Key words: invertebrate immunity, ecological immunology, parental effects, paternal effects, host–pathogen interaction, evolution, meta-analysis.

CONTENTS

* Author for correspondence (Tel.: +61 13 63 52; E-mail: nicola.rutkowski@unimelb.edu.au).

I. INTRODUCTION

Parasites and pathogens present a persistent immune challenge throughout an organism's life, affecting their life history (Rolff & Siva-Jothy, 2003; Zuk & Stoehr, 2002), reproductive fitness (Little & Kraaijeveld, 2004; Tauber, 2017), and survival (Moret & Schmid-Hempel, 2000; Rolff & Siva-Jothy, 2003; Sadd & Schmid-Hempel, 2009*b*; Schulenburg *et al.*, 2009). In response, animals have developed a range of defences to mitigate these negative impacts (Janeway, 2001; Sadd & Schmid-Hempel, 2009*b*; Tauber, 2017). These defences include the innate and acquired responses of the immune system (Milutinovic & Kurtz, 2016; Roth *et al.*, 2018; Rowley & Powell, 2007). A key advantage of an acquired immune response is the ability of parents to transfer their immunological experience vertically to their offspring, termed trans-generational immune priming (TGIP) (Boehm, 2012; Swain & Nayak, 2009). One way this is achieved is through the production of pathogen-specific antibodies (Hasselquist & Nilsson, 2009) which can be transferred to offspring by the placenta, milk or egg yolk (Silverstein, 2001). TGIP provides a fitness advantage if offspring encounter a comparable immunological environment as their parents (Burgess & Marshall, 2014; Pigeault *et al.*, 2016). Presumably because of the limited support for an acquired immune response in invertebrates, it was initially believed that only vertebrates were capable of TGIP (Hasselquist & Nilsson, 2009; Pigeault *et al.*, 2016; Rinkevich, 1999; Rowley & Powell, 2007). This view has transformed in the last two decades: today the complexity and sophistication of the invertebrate immune system has been extensively documented and there is clear evidence for functionally equivalent intra- and trans-generational immune priming in invertebrates (Huang & Song, 1999; Moret, 2006; Sadd & Schmid-Hempel, 2006). However, whether there is a broad taxonomic distribution of TGIP in invertebrates, and the strength of its effect remain open questions.

(1) Evidence for invertebrate trans-generational immune priming

There is a body of evidence supporting TGIP in invertebrates and the experimental protocols for quantifying TGIP are broadly comparable: typically, an immune challenge (in the form of a parasite or pathogen) is administered to one or both parents, the parental generation is then mated and offspring immune parameters are measured. Despite the generality in approach, observations are somewhat

equivocal. A recent review found that 49 of 57 studies provided some evidence for TGIP in invertebrates (Tetreau *et al.*, 2019), but the remaining studies found either no, or inconsistent support for the phenomenon (Sadd & Schmid-Hempel, 2009*a*; Shikano, Hua & Cory, 2016; Vantaux *et al.*, 2014; Voordouw, Lambrechts & Koella, 2008). In their recent review, Tetreau *et al.* (2019) offer, as yet untested, insights into these discrepancies. First, they identified host-related moderating factors that could influence TGIP (for example, the ecology of the primed species, and the life stage or sex of the individual primed). Second, they highlighted the lack of concordance in experimental design (the method of priming, the type of pathogen and its host-specificity) or administration of the pathogen, which may affect estimates of both the prevalence and intensity of TGIP. Finally, they suggested that the field may suffer from publication bias, leading to the over-representation of statistically significant results (Lortie *et al.*, 2007; Rothstein, Sutton & Borenstein, 2005). Failing to consider one or all of these may obscure our understanding of TGIP.

(2) Host-related factors that moderate TGIP

(*a*) *Ecology of the species primed*

The occurrence and strength of TGIP appears to be related to the ecology of the chosen species. Recent theoretical models highlight species longevity and, to a lesser extent, levels of philopatry (the tendency for an individual to remain in its area of birth) as key factors selecting for the evolution of TGIP (Garnier, Boulinier & Gandon, 2012; Pigeault *et al.*, 2016). The predictions from these models are that shorter-lived species should invest in avoidance strategies (to avoid the parasite before infection), whereas longer-lived species should invest in acquired immune responses (such as TGIP) (Boots & Bowers, 2004; Garnier *et al.*, 2012). Consequently, long-lived, philopatric species should be better at protecting their offspring through TGIP than their shorter-lived, dispersing counterparts (Pigeault *et al.*, 2016). Moreover, increasing lifespan alters the prevalence of parasites, and increases the rate of infection (Miller, White & Boots, 2007), therefore, given the heightened risk for longer-lived species, it increasingly pays off to invest in TGIP with longer life expectancy. Given the above, identifying TGIP as a mechanism should be more likely in long-lived, philopatric species and thus it is perhaps not surprising that the majority of TGIP research has been conducted on the longer-lived (100+ days) philopatric order, Coleoptera (Dhinault, Chogne & Moret, 2018*a*; Khan, Prakash & Agashe, 2016; Moret, 2006; Roth *et al.*, 2010; Zanchi *et al.*, 2011). A potential problem with this taxon bias is it may provide a distorted view of immune priming, resulting in an unrepresentative overview of the generalisability of the phenomenon across invertebrates.

Additionally, the rate of metamorphosis (complete or incomplete/none) expressed by a species could impact TGIP expression. Species that undergo complete metamorphosis (holometabolous species) can experience considerable changes in their physiology, ecological niche, and immune system (Russell & Dunn, 1996). As a consequence, species undergoing metamorphosis are often exposed to different parasites at different life stages (Critchlow, Norris & Tate, 2019), as well as exhibiting large differences in infection prevalence of the same parasite across life stages (Clopton, Janovy & Percival, 1992). This variation in ecological niche and alteration of the immune system could impact the expression of TGIP, as TGIP should provide a greater fitness benefit when offspring and parent encounter similar parasites. Thus, we may see greater TGIP in hemimetabolous or non-metamorphic species, which generally remain in a comparable niche throughout their lifespan.

(*b*) *Sex of the parent primed*

Conventionally, maternal effects are considered to be more important than paternal effects as mothers typically invest more resources in the production and care of offspring (Mousseau & Fox, 1998). Consequently, studies of TGIP are biased towards exploring maternal effects (Dhinaut, Chogne & Moret, 2018*b*; Khan *et al.*, 2016; Littlefair, Laughton & Knell, 2017; Lopez *et al.*, 2014; Sadd & Schmid-Hempel, 2009*a*; Yue *et al.*, 2013). However, TGIP has been observed in both sexes (McNamara, van Lieshout & Simmons, 2014; Zanchi *et al.*, 2011), but the underlying mechanism and nature of the observed TGIP effect differs. This is exemplified by the yellow mealworm beetle, *Tenebrio molitor* (Zanchi *et al.*, 2011). In this species, maternal priming resulted in an increased concentration of haemocytes in offspring; while paternal priming increased the activity of offspring proenzymes (prophenoloxidase, proPO) (Zanchi *et al.*, 2011). However, paternal TGIP effects were transient: only offspring produced within the first 4 days following a mating exhibited enhanced immunity (Zanchi *et al.*, 2011). Nonetheless, paternally derived TGIP does occur, and ignoring the relative contributions of each parent limits our ability to understand TGIPs evolutionary history and the maintenance of this critical life-history trait.

(*c*) *The life stage primed*

The nature and strength of TGIP may also be influenced by the offspring life stage studied. Broadly speaking, TGIP is likely to be functionally more important for young offspring, as they have a less-developed immune system (Rowley & Powell, 2007). Accordingly, in vertebrates, TGIP is considered more valuable in early life stages, where mortality selection is at its highest (Rossiter, 1996; Roth *et al.*, 2018). Since young invertebrate larvae are also more susceptible to infection (Gillespie, Kanost & Trenczek, 1997), it seems the same selection pressures may be present, although this has never been quantified. Of further consideration is that offspring immunity likely differs both within and between the juvenile and adult life stages (Gillespie *et al.*, 1997; Laughton, Boots & Siva-Jothy, 2011; Tate & Rudolf, 2012), however,

TGIP studies typically observe offspring in one life stage only (juvenile or adult) (Khan *et al.*, 2016; Moret, 2006; Pigeault *et al.*, 2015; Roth *et al.*, 2010; Sadd & Schmid-Hempel, 2009*a*). Given the potentially transient nature of TGIP, particularly when paternally derived (Zanchi *et al.*, 2011), selecting one life stage may result in missing either the peak offspring immune response, or its presence altogether (Shikano *et al.*, 2016; Vantaux *et al.*, 2014). Where studies have observed offspring at multiple life stages (Trauer & Hilker, 2013; Yue *et al.*, 2013), the differences are clear. In the tobacco hornworm, *Manduca sexta*, larvae from immune-challenged parents had higher antibacterial and PO activity than offspring in the pupal or adult stages (Trauer & Hilker, 2013). Similarly, in the scallop, *Chlamys farreri*, response to a maternal bacterial infection (measured through offspring gene expression, protein levels, and mortality rates) differed across the four developmental stages measured (four-cell, blastula, gastrula and trochophore) (Yue *et al.*, 2013).

(*d*) *Which generation is challenged?*

The challenge status of the offspring – whether the immune insult was applied at both the parental and offspring stage – also has the potential to affect the prevalence and intensity of TGIP. Many studies challenged the parental but not the offspring generation (Cole *et al.*, 2020; Dubuffet *et al.*, 2015; McNamara *et al.*, 2014; Trauer-Kizilelma & Hilker, 2015). This may affect the strength of TGIP observed. As upregulation of the immune system is costly (Kraaijeveld & Godfray, 1997), in the absence of within-generation stimulation (i.e. *via* a subsequent offspring immune challenge), we should perhaps not expect to see the full effects of TGIP. Experiments on both challenged (parent and offspring immune challenged) and unchallenged offspring (only parents challenged) of *Manduca sexta* found weak evidence only for TGIP in the unchallenged offspring *via* enhanced PO levels until the fourth-instar stage (Trauer & Hilker, 2013). No effect of TGIP on antibacterial activity was found; this was attributed to the costs associated with potentially unnecessary immune upregulation (Trauer & Hilker, 2013). The opposite was found for challenged offspring, who had enhanced antibacterial activity but no changes to PO levels (Trauer & Hilker, 2013). The results of this study also demonstrate the importance of measuring multiple immune parameters in TGIP experiments, given the differential plasticity of immune traits (Martin *et al.*, 2021).

(*e*) *Generational challenges*

Theoretically, TGIP should provide a fitness benefit when offspring encounter a similar immunological environment as their parents (Burgess & Marshall, 2014; Pigeault *et al.*, 2016), thus the choice of challenge is expected to affect the strength of TGIP. Studies exploring TGIP do frequently challenge offspring with the same challenge as their parents (Norouzitallab *et al.*, 2015; Pigeault *et al.*, 2015; Vantaux *et al.*, 2014; Zanchi *et al.*, 2011) and typically observe positive results (Moret, 2006; Norouzitallab *et al.*, 2015; Zanchi *et al.*, 2011). However, some studies have used a different challenge on offspring (Dhinaut *et al.*, 2018*a*; Fuchs *et al.*, 2018; Littlefair *et al.*, 2017). For example, in the bumble-bee, *Bombus terrestris*, mothers were challenged with the bacteria *Arthrobacter globiformis* but their offspring were subsequently challenged with the parasite *Crithidia bombi* (Sadd & Schmid-Hempel, 2009*a*). Offspring of challenged mothers were more susceptible to *C. bombi* infection than controls (Sadd & Schmid-Hempel, 2009*a*), illustrating a potential cost of TGIP when the offspring–parent immune environments are mismatched. Contrastingly, in *T. molitor*, mothers were challenged with one of four Gram-positive or Gram-negative bacteria. Regardless of the immune challenge type (same or different to mother) all offspring of primed mothers exhibited similarly improved immunity (Dhinaut *et al.*, 2018*a*), demonstrating a lack of specificity. Clearly, there are contrasting reports in the literature and as the effects of offspring challenge status have never been measured, the role of this factor remains unknown.

(3) Variation in experimental design

(*a*) *The mode of administration and the type of immune challenge*

Host adaptation to an immune challenge may be contingent on infection route. In an experimental evolution study, fruit fly *Drosophila melanogaster* populations were infected with a pathogen *via* one of two infection routes. Subsequent infection using the alternate route (to the initial challenge) revealed that individuals were afforded no protection from their initial infection (Martins *et al.*, 2013), suggesting that TGIP may be route specific. Moreover, whether the infection route is biologically relevant may be important. For example, in the nematode, *Caenorhabditis elegans*, detection of TGIP following an insult from the Orsay virus depended on whether the pathogen was administered *via* injection (Ashe *et al.*, 2015) or provided orally (Felix *et al.*, 2011; Sterken *et al.*, 2014). Only the latter method (which mimics the natural route of infection) found evidence of TGIP (Felix *et al.*, 2011). An understanding of whether TGIP is stronger when elicited from natural infection routes may provide critical insights into the mechanisms underpinning its evolution. Specifically, it will allow us to determine the degree to which the evolution of TGIP is dependent on the predictability of a parasite using a known route and targeting a specific pathway. Moreover, understanding the relative impact of natural *versus* alternate infection routes will clarify the importance of using biologically valid infection methods.

(*b*) *The impact of pathogen specificity*

The extent of TGIP may depend on whether the immune elicitor used is one that is naturally encountered by the host parent. For example, in the red flour beetle, *Tribolium castaneum*, offspring antibacterial activity was greater when parents were challenged with the host-specific pathogen *Bacillus thuringiensis*, compared to when they were challenged with

Escherichia coli (Roth *et al.*, 2010). While TGIP should be greater when species-specific pathogens are used, the importance of this variable in determining patterns of TGIP across studies has never been quantified.

(c) *Immune pathways as a source of variation*

A source of potentially significant variation is that, even if the dose is comparable, different immune elicitors can activate different regulatory pathways of the immune system. For example, in *Drosophila melanogaster*, Gram-negative bacteria trigger the immune deficiency (Imd) pathway; while Gram-positive bacteria and fungi activate the Toll pathway (Lemaitre & Hoffmann, 2007). The activation times of these pathways differ: the Imd pathway is rapid and can occur in minutes (Paquette *et al.*, 2010); while the Toll pathway activates over hours (Lemaitre, Reichhart & Hoffmann, 1997). This temporal variation between pathways may impact the expression of TGIP. However, in other systems activation of the two pathways may work differently, with species such as the pea aphid, *Acyrthosiphon pisum*, having absent or undeveloped Imd pathways (Gerardo *et al.*, 2010). A systematic review which considers the relative impact of gram status on the prevalence and intensity of immune priming may provide insight into the mechanistic evolution of TGIP.

(d) *The type and concentration of the challenge*

The type (inactive or active) and concentration (high or sub-lethal) of immune elicitor used may also impact TGIP. Inactive immune elicitors [such as heat-killed bacteria, lipopolysaccharides (LPS), Sephadex beads or peptidoglycans (PGNs)] are typically administered in high concentrations (Tetreau *et al.*, 2019). The intent with inactive elicitors is to produce pathogen-associated molecular patterns that mimic a natural infection (Buchmann, 2014) without the confounding effects of physiological sickness (Milutinovic & Kurtz, 2016). By contrast, active immune elicitors (e.g. live bacteria, parasites, fungus or viruses) are administered at sub-lethal doses (Cole *et al.*, 2020; Littlefair *et al.*, 2017; Shikano *et al.*, 2016) to avoid selection for resistance in offspring (Littlefair *et al.*, 2017), whilst still stimulating the immune system. Inevitably these constraints result in variation in dosage between inactive and active immune elicitors which may in turn influence their relative strength.

(4) Publication bias as a driver of TGIP significance

A final, but significant, problem with our understanding of TGIP is that it may be subject to publication bias. Publication bias is a widespread problem in evolutionary ecology (Csada, James & Espie, 1996) and can lead to a distorted view of the prevalence of particular traits or phenomena (Moller & Jennions, 2001). Several literature reviews of TGIP (Roth *et al.*, 2010; Tetreau *et al.*, 2019) have highlighted the potential for significant publication bias in this field which can lead to the over-representation of statistically significant results in the published literature. Critically, failure to identify publication bias may provide false conclusions about the pervasiveness of TGIP among taxa and the moderating factors that determine its strength.

(5) Aims

In this study, we used a meta-analysis to quantify the presence and magnitude of TGIP in invertebrates and address the three untested insights proposed by Tetreau *et al.* (2019). Specifically, we explored: (*i*) what moderating factors influence the observed variation in effect sizes; (*ii*) to what degree does the diversity of experimental designs used to explore this phenomenon affect the experimental outcome; and (*iii*) what is the prevalence of publication bias in studies of TGIP? In order to determine that our meta-analysis was reported correctly, we completed the PRISMA-EcoEvo reporting checklist (O'Dea *et al.*, 2021) (see online Supporting Information, Table S1).

II. METHODS

(1) Literature search

Between April and August 2020, *ISI Web of Science* and *Scopus* were used to search for published studies that examined whether invertebrate species demonstrate evidence of TGIP. The following search terms were used: [('transgeneration*' OR 'trans-generation*' OR 'trans generation*' OR 'cross generation*' OR 'cross-generation*' OR 'multi generation*' OR 'multi-generation*' OR 'maternal effec*' OR 'paternal effec*' OR 'parental effec*' OR 'maternal transfer' OR 'paternal transfer' OR 'inheritance') AND ('immune prim*' OR 'immune challenge' OR 'immune defen*' OR 'immune protection' OR 'disease resistance' OR 'priming' OR 'immunisation' OR 'defen*' OR 'immune response' OR 'immune function' OR 'immunity' OR 'immunocompeten*' OR 'anti-viral response' OR 'immune memory') NOT ('human' OR 'people' OR 'mice' OR 'rat*' OR pipefish' OR 'great tit*' OR 'blue tit*' OR 'gull' OR 'barn swallow' OR 'partridge' OR 'tree*' OR 'nestling*' OR 'economics' OR 'cow' OR 'tomato' OR 'fish' OR 'bean' OR 'potato' OR 'tomato' OR 'chick*' OR 'seed*' OR 'cattle' OR 'bovine' OR 'rust' OR 'mildew' OR 'blight' or 'piglet*' OR 'children' OR 'soybean' OR 'sunflower*' OR 'rodent')]. A list of 31 exclusion terms (i.e. the 'NOT' terms) was used to reduce the large number of irrelevant references without excluding potentially relevant papers. Furthermore, search criteria were limited to include only articles, removing book chapters, book reviews and other irrelevant documents. We tested the validity of the search string by checking that most papers (91%) within the review by Tetreau *et al.* (2019) were found by the search.

In addition, five highly cited landmark papers in TGIP were identified (Huang & Song, 1999; Little & Kraaijeveld, 2004; Pigeault *et al.*, 2016; Sadd *et al.*, 2005; Tetreau *et al.*, 2019).

To prevent publication bias, meta-analyses incorporate 'grey literature' (unpublished post-graduate dissertations) (Siddaway, Wood & Hedges, 2019). We used an identical search string to search for unpublished 'grey' literature, from passed Masters' and PhD theses in *EBSCOhost* and *ProQuest*.

The total reference list of papers was then imported into the web application Rayyan, a reference management tool for systematic reviews (https://rayyan.qcri.org) (Ouzzani *et al.*, 2016). Duplicate papers were removed using a function on Rayyan (note that this process is not completely accurate due to typographical differences between databases). Rayyan was then used to conduct the initial screening.

(2) Inclusion/exclusion criteria

Studies were included if they fulfilled the following inclusion criteria: (*i*) the paper was written in English; (*ii*) the study was empirical; (*iii*) parents were immune challenged or primed using living or inactivated bacteria, fungus, parasites, viruses or Sephadex beads; (*iv*) the challenge was given *via* injection, ingestion, or topically/contact; (*v*) the studies were trans-generational, i.e. the effects of the immune challenge were studied beyond one generation; (*vi*) papers provided data on offspring immunity, such as physiological measures of innate immunity, or parasite load or survival data; (*vii*) papers provided descriptive statistics to calculate effect sizes, or figures or tables that allowed their calculation (Nakagawa & Cuthill, 2007). For papers that did not contain extractable data, but fulfilled the other criteria, authors were contacted for their original data sets. An author was given 2 weeks to respond, after which they were sent another request. If they again failed to respond, the effect sizes associated with their study(ies) were removed from analysis.

(3) Initial and full-text screening

Within Rayyan, studies were assessed for their initial inclusion ('papers screened' in the PRISMA chart) using an initial screening flowchart (Fig. S1). At this initial screening stage only the title, key words, and abstract of papers were looked at. If any of the inclusion/exclusion criteria were unclear at this stage, the paper remained for full-text screening. A pilot screening of 100 papers in Rayyan had a 94% agreement rate between N.-A. J. R. and two other screeners (K. B. M., Y. Z. F.). Agreement was 100% after discussion. The remainder of the initial screening was completed by N.-A. J. R.

After the initial screening, 130 papers remained for full-text screening. Again, this was performed using a screening flowchart (Fig. S2) on Google Forms. A pilot screening of 10 papers had a 70% agreement rate between N.-A. J. R. and two other screeners (K. B. M., Y. Z. F.). Agreement was 100% after discussion. If there was uncertainty about whether a paper should be included, it was marked as requiring double screening. K. B. M. then conducted a full-text screening.

(4) Effect size extraction/calculation

We chose Cohen's *d* as our measure of effect size. The descriptive statistics provided were used to generate standardised mean differences (Cohen's *d*) and their associated dispersion estimates. Where means and dispersion data were provided only in figures, these values were extracted using the R package *metaDigitise* (Pick, Nakagawa & Noble, 2019). For studies that expressed offspring immunity as the likelihood of survival across time, data were extracted for only one time point, that at which there was the largest difference in survival between the treatment and control groups. We assumed that this difference was most likely to be biologically significant. To calculate Cohen's *d* from proportion data (i.e. survival, proportion infected) the log odds ratio effect size was calculated and then was converted to Cohen's *d* following Borenstein (2009).

The directions of the effect sizes were standardised such that a greater effect size indicates a greater offspring immune response. Thus, effect sizes relating to parasite load, intensity of infection, proportion of offspring infected, colony forming unit (CFU) density and mortality rate, which are all negatively correlated with offspring immunity, were reversed in direction to align with other immune parameters, such as antibacterial activity, PO activity, and survival rates, which are positively correlated with immunity.

(5) Moderator variables and coding of papers

For each effect size, we recorded the species studied, sample size and the paper, study and sample identities. We defined a study as a group of individuals subject to different temporal or spatial conditions. Thus, a study could be individual experiments, or different sampling years. Sample identity refers to the sample used within a study. In some experiments, multiple samples were used within the same study, for example: in an experiment where individuals are subject to the same treatment (i.e. TGIP or control), but are taken from different populations, they will have the same study identity, but different sample identities. We also recorded the following moderator variables. Host-related variables: (*i*) sex of the immune-challenged parent: 'female', 'male' or 'both'; for full factorial designs (where both parents were challenged together and separately), the experimental treatment was where both parents were challenged, whilst the control treatment was where only one parent was challenged. (*ii*) Taxonomic class of the invertebrate studied; as most studies focused on insects, we classified species as 'Insecta' or 'not Insecta'. (*iii*) Offspring sex: the sex of the offspring tested ('male', 'female' or 'both'). (*iv*) Offspring developmental stage: whether offspring were 'juvenile' or 'adult' when assayed as recorded. (*v*) Offspring challenge status: this was classified according to whether the offspring were 'not challenged' or challenged with the 'same challenge' or a 'different challenge' to their parents. Variation in experimental design: (*vi*) parental immune challenge type was classified as 'inactive immune elicitors', including heat-killed bacteria,

LPS, Sephadex beads and PGNs, and 'active immune elicitors', including live bacteria, parasites, fungus or viruses; (*vii*) parental infection route classified as 'injection' or 'non-injection' (including ingestion, topical application or *via* contact). We were unable to differentiate the infection route at a finer scale as a disproportionate number of studies primed *via* injection. Unfortunately, data on specificity was not available for all hosts and parasites and was therefore excluded as a moderator variable. (*viii*) Metamorphic: this was classified as undergoing complete metamorphosis 'yes' or 'no'. (*ix*) Philopatry: classified as 'No or low dispersal' and 'Strong dispersal'.

A previous theoretical paper on the evolution of TGIP (Pigeault *et al.*, 2016) created a categorical variable for entire lifespan; short lifespan (< 60 days) and long lifespan (> 60 days). To determine if these categorical variables would be suitable for our meta-analysis, we plotted the distribution of lifespan for our 18 species (Fig. S3). Critically, where possible, we used the average complete lifespan (from emergence from the egg until death) of species at the temperature conditions explicitly reported in the relevant TGIP paper (Valenzano *et al.*, 2006), as rearing temperature typically has a significant impact on longevity. We determined that, given the considerable distribution in lifespan, it would be more accurate to include total longevity as a continuous variable. All longevity data were natural log transformed prior to analysis.

We used Pigeault *et al.*'s (2016) classification of philopatry within our analysis. They classified philopatry (or dispersal) as the average distance travelled by adults. Low dispersal is less than 500 m dispersal range and strong dispersal is greater than or equal to 500 m.

(6) Building phylogenies

R (v1.1.456) (R Core Team, 2020; RStudio Team, 2020) was used to create a phylogeny, using the package, *rotl* (Michonneau, Brown & Winter, 2016). *rotl* uses the Open Tree of Life database (Hinchliff *et al.*, 2015) to generate a tree using data from the National Center for Biotechnology Information taxonomy database. One tree was created, including all the species used within the TGIP experiments. We then generated a variance–covariance matrix among species using only the topology of the tree (i.e. the evolutionary relationships among the species without branch lengths) and incorporated this matrix as a random effect.

(7) Meta-analysis

The meta-analysis was conducted in R version 1.1.456, RStudio, Inc. (R Core Team, 2020). The *metafor* package (Viechtbauer, 2010) was used to run the meta-analysis using linear mixed models (Nakagawa & Santos, 2012; Viechtbauer, 2010). Linear mixed models are used when there is non-independence within the data. This non-independence occurs when effect sizes are derived from the same study, when a species has multiple effect sizes or when species have a shared ancestry. Non-independence arises from shared ancestry because closely related species may have more similar effect sizes compared to less closely related species (Foo *et al.*, 2017). To control for non-independence, we included the paper's unique identity, study identity, sample identity and the species studied in the model as random factors.

We first ran the model without any random effects to generate a heterogeneity (I^2) value using the *rma* function in *metafor*. The I^2 value indicates the percentage of variation that is due to heterogeneity rather than chance (Higgins & Thompson, 2002; Higgins *et al.*, 2003). A low I^2 (<25%) indicates that there is little variation between effect sizes. A high I^2 (>75%) means there is large variation between effect sizes (Higgins & Thompson, 2002; Higgins *et al.*, 2003), and thus moderator analyses are necessary.

We then tested the relative importance of the following random factors: species identity, paper identity, study identity, sample identity and effect size identity. These variables were entered into an intercept-only model to determine the effect of each variable in comparison to each other. Each random factor that accounted for a substantial proportion of the total I^2 value (i.e. $I^2 > 0\%$) was included in the meta-analysis. We also tested the necessity to control for species similarities by including phylogeny as a random factor within the intercept-only model. Controlling for phylogeny did not influence the overall effect size, therefore it was not included in later analyses. We then found the overall effect size of TGIP by running another intercept-only model [using the restricted maximum likelihood (REML) estimation] which included only the pre-determined random variables.

To conduct the moderator analyses, meta-regression models were run using the *rma.mv* function in *metafor*. First, we ran single-factor models (without the intercept), including only one categorical moderator as a fixed factor and the random factors. This allows determining the effect size estimates for each level within moderators. Then, final model selection (to determine which moderators would remain) was conducted using the package *MuMIn* (Bartoń, 2014). This model selection uses the Akaike Information Criterion with sample size correction (AICc) (Anderson, 2008), and uses the maximum likelihood (ML) estimation. The model selection only used effect sizes that had no missing data. This ensures that AICc values are comparable (Nakagawa & Freckleton, 2011). We created all the possible candidate models from the relevant random factors and moderator variables. We found all the models within two AICc units from the best (i.e. the model with the lowest AICc value), to average the model coefficients. This created the final list of moderators included in the model. We used conditional model averaging, following Nakagawa & Freckleton (2011). However, we note that conditional and full-model averaging yielded similar results, with the same variables being statistically significant. We then tested the significance of the moderators that were retained in the final model using an omnibus QM test, a Wald-type test for testing whether the inclusion of a moderator explains any heterogeneity in the data set.

(8) Sensitivity analysis

A sensitivity analysis was also conducted, on a subset of data, to examine the effect of bacteria type on TGIP. Here, we recorded whether studies using a bacterial challenge (either inactive or active) used, 'Gram-negative bacteria' or 'Gram-positive bacteria'. We then ran a single-factor model, using the *rma.mv* function in *metafor*. This variable was not included in subsequent analyses, given that it only applied to a subset of the papers included in our meta-analysis.

(9) Publication bias

Time-lag bias is a negative relationship between the year of publication and effect size (where earlier studies report larger effect sizes than later ones) (Moller & Jennions, 2001). To test for this bias in the literature, we ran a single-factor model, with year of publication entered as a fixed factor and all of the random factors included. Year of publication was then included in the model selection, as a moderator variable, to determine if it would be retained in the final model. Journal impact factor (JIF) was also included as an element of publication bias. JIF bias is a positive relationship between impact factor and effect size (journals with larger JIF report larger effect sizes) (Baker & Jackson, 2006; Murtaugh, 2002). To test for this bias, we used the same methods as for time-lag bias.

We also looked for any missing effect sizes by running a funnel plot asymmetry analysis using the residuals from our final model. Residuals were extracted using the *MCMCglmm* function in the *MCMCglmm* package (Hadfield, 2010). We used 130,000 iterations, 100 thinning, 30,000 burn-in, and inverse gamma prior for all residual distributions. We then ran an Egger's regression test (Egger *et al.*, 1997), using the *regtest* function in *metafor*. The Egger's test regresses the standardised residuals on precision, with publication bias signified by an intercept that is different from zero. Finally, a trim-and-fill analysis (Duval & Tweedie, 2000) was run on the residuals, to check for funnel plot asymmetry and identify missing studies. We did this using the *trimfill* function in *metafor*. This analysis assumes that the funnel plot is symmetric and tries to 'trim' the smaller studies that are causing asymmetry. It then fills the distribution with missing studies, making it symmetrical. We also checked the I^2 value reported by the *trimfill* function. This shows the amount of heterogeneity left after accounting for the random and fixed factors that were retained in the final model.

III. RESULTS

Before removing duplicates, the literature search resulted in 1016 papers from *ISI Web of Science* and 1042 papers from *Scopus*. Forward and backward searches of the five landmark papers in the field subsequently identified an additional 546 papers. Our grey literature search identified 92 chapters, although none of these remained in our database after full-text screening. After duplicate removal, Rayyan was used to conduct the initial screening of 2383 papers. The outcomes of the literature search are shown using a Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) chart (Moher *et al.*, 2009) in Fig. 1.

(1) Description of data set

We retained 37 papers for the meta-analysis; the papers included in our meta-analysis are identified with asterisks in

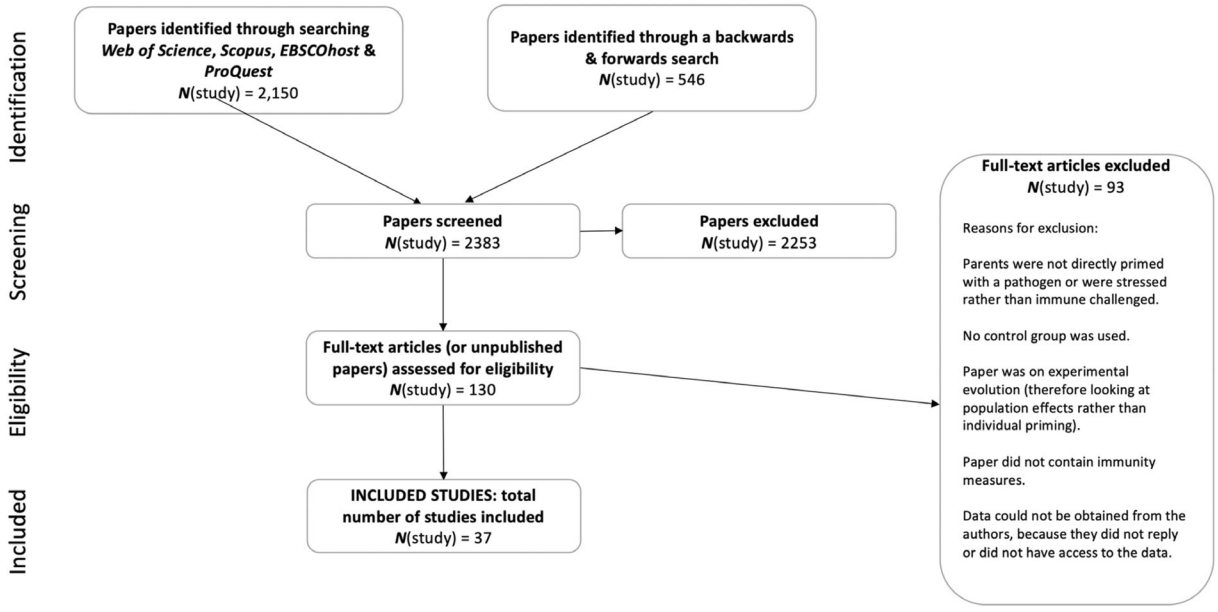

Fig. 1. PRISMA flowchart (Moher *et al.*, 2009) showing the methods and outcomes of the literature search.

the reference list. All data were extracted by N.-A. J. R. and checked by a second author (either K. B. M. or Y. Z. F.) for quality assurance. We extracted 346 effect sizes from 18 different species of invertebrates (see Fig. 2 for the species included in the meta-analysis), and 19,776 individuals. Heterogeneity and parameter estimates of the meta-analytic and meta-regression models are presented in Tables 1 and 2 and Fig. 3.

(2) Evidence for trans-generational immune priming in invertebrates

First, we determined the total variation explained by a given random effect. 9% of the variation in TGIP was explained by consistent differences among species, while 31% was due to paper identity, 6% was accounted for by the sample identity (Table 1) and 5% was due to the study identity (Table 1). Effect size identity explained 47% of the variation in TGIP and phylogeny only accounted for less than 0.001%. Including phylogeny in the model did not alter the overall effect size (Table 2; Fig. 3), consequently we omitted phylogeny from all subsequent analyses. All other random effects were retained. Overall, we found evidence of TGIP, expressed *via* an increased immune response in the offspring generation ($d = 1.07$; 95% CI = 0.22, 1.92; $P = 0.01$) (Table 2; Fig. 3). The data set had large total heterogeneity (98.12%), motivating moderator analyses.

(3) Moderator analysis

The final AICc model retained the following moderators: offspring developmental stage, offspring challenge status, parental immune challenge type, parental infection route, taxonomic class, philopatry, metamorphic and longevity. The year of publication and impact factor were also included in the final model. See Table S2 for the AICc results.

Despite being included in the full model, the effect of offspring developmental stage was non-significant ($Q = 1.45$; $P = 0.23$). However, if, and how, the offspring were challenged had a significant effect on TGIP ($Q = 10.28$; $P = 0.006$). Challenging them with a different challenge (compared to their parents) had no effect (Table 2, Fig. 3), while using the same challenge as parents had a large positive effect ($d = 1.78$; 95% CI = 0.75, 2.8; $P < 0.001$). For offspring that were not challenged there was also a significant positive effect on TGIP ($d = 1.27$; 95% CI = 0.35, 2.18; $P = 0.006$).

For the parental immune challenge type the effect was non-significant ($Q = 1.57$; $P = 0.21$), as was the overall effect of parental infection route ($Q = 0.97$; $P = 0.32$). Although taxonomic class was retained in the final model, the overall effect of this moderator was also non-significant ($Q = 1.31$; $P = 0.25$); similarly, while philopatry was included in the full model, the moderator was non-significant ($Q = 0.82$, $P = 0.36$). Whether a species undergoes complete metamorphosis (or not) had no impact on TGIP, despite it being included in the full model ($Q = 0.52$, $P = 0.47$), and the effect of longevity also was not significant ($Q = 0.54$; $P = 0.46$).

Two other moderators were included in our analysis but were not included in the final model, suggesting that they did not impact the effect sizes: sex of the immune-challenged parent, and offspring sex.

(4) Sensitivity analysis

Whether parents were primed with Gram-positive bacteria or Gram-negative bacteria was not significant ($Q = 0.99$, $P = 0.32$).

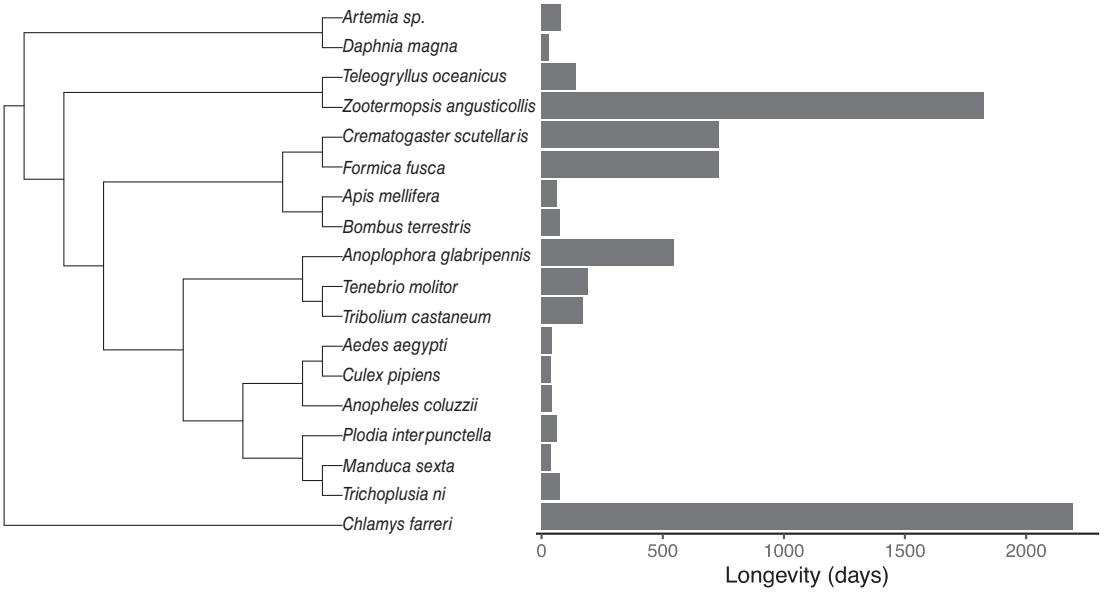

Fig. 2. Phylogenetic tree of the species included in the meta-analysis (left) and longevity in days of each species (right).

Table 1. Heterogeneity (I^2) values for each of the random effects.

	Total	Species	Paper	Sample	Study	Effect size	Phylogeny
I^2	98%	9%	31%	6%	5%	47%	<0.001%

Table 2. Parameter estimates and *P*-values for the effect of trans-generational immune priming (TGIP) in invertebrates. *d* is mean effect size; CI.lb and CI.ub are the lower and upper bounds of the 95% confidence interval, respectively; * indicates the moderators that were retained in the final model.

	Cohens *d*	CI.lb	CI.ub	*P*
Meta-analytic mean	1.07	0.22	1.92	0.01
Phylogenetic mean	1.07	0.22	1.92	0.01
Sex of immune-challenged parent				
Both	0.89	−0.44	2.21	0.19
Female	1.07	0.17	1.97	0.02
Male	1.43	0.30	2.56	0.01
Offspring sex				
Both	1.22	0.32	2.13	0.007
Female	0.46	−1.07	1.99	0.56
Male	0.53	−1.12	2.18	0.53
Offspring developmental stage*				
Adult	0.76	−0.25	1.77	0.14
Juvenile	1.32	0.36	2.27	0.006
Offspring challenge status*				
Different challenge	0.33	−0.63	1.29	0.50
Not challenged	1.27	0.36	2.18	0.006
Same challenge	1.78	0.75	2.80	0.0007
Parental immune challenge type*				
Active immune elicitor	0.93	−0.19	2.06	0.10
Inactive immune elicitor	1.17	0.15	2.18	0.02
Parental infection route*				
Injection	0.86	−0.13	1.85	0.09
Non-injection	1.50	0.17	2.82	0.03
Taxonomic class*				
Insecta	0.85	−0.04	1.74	0.06
Not insecta	2.43	0.26	4.59	0.03
Philopatry*				
No or low dispersal	1.41	0.40	2.41	0.006
Strong dispersal	0.31	−1.22	1.84	0.69
Metamorphic*				
Yes	0.92	−0.03	1.86	0.06
No	1.79	−0.23	3.77	0.08
Longevity*	0.13	−0.62	0.88	0.74
Year published*	−0.15	−0.36	0.05	0.14
Impact factor*	0.29	−0.15	0.73	0.20

(5) Publication bias

The model estimate for year of publication was negative (d = −0.16, 95% CI = −0.37, 0.05, P = 0.14, Fig. 4A), although the effect was not significant (Q = 2.32; P = 0.13). The model estimate for impact factor was positive (Fig. 5, d = 0.29; 95% CI = −0.15, 0.73; P = 0.20) but non-significant (Q = 1.19; P = 0.28).

For the funnel plot analysis (Fig. 4B), an Egger's regression test indicated significant asymmetry in the funnel plot of residuals (t = 4.22, P < 0.0001). The trim-and-fill method added eight effect sizes to the left (negative) side of the funnel plot, and gave an adjusted effect size value of d = 0.83 (95% CI = 0.21, 1.43), i.e. slightly smaller than our initial estimate of d = 1.07 but is still a large positive effect. High heterogeneity still remained in the residuals (I^2 = 97.9%), suggesting that the effect of TGIP may be moderated by other variables not considered in our analysis. Since this paper was written, new developments in publication bias tests have become available (Nakagawa *et al.*, 2022), which we include as additional analysis in Appendix S1. After that analysis, our effect size was reduced further (d = 0.73; 95% Cl = −2.35, 3.82), which represents a non-significant effect. However, for a discussion on the relevance of this analysis, see Section IV.1.

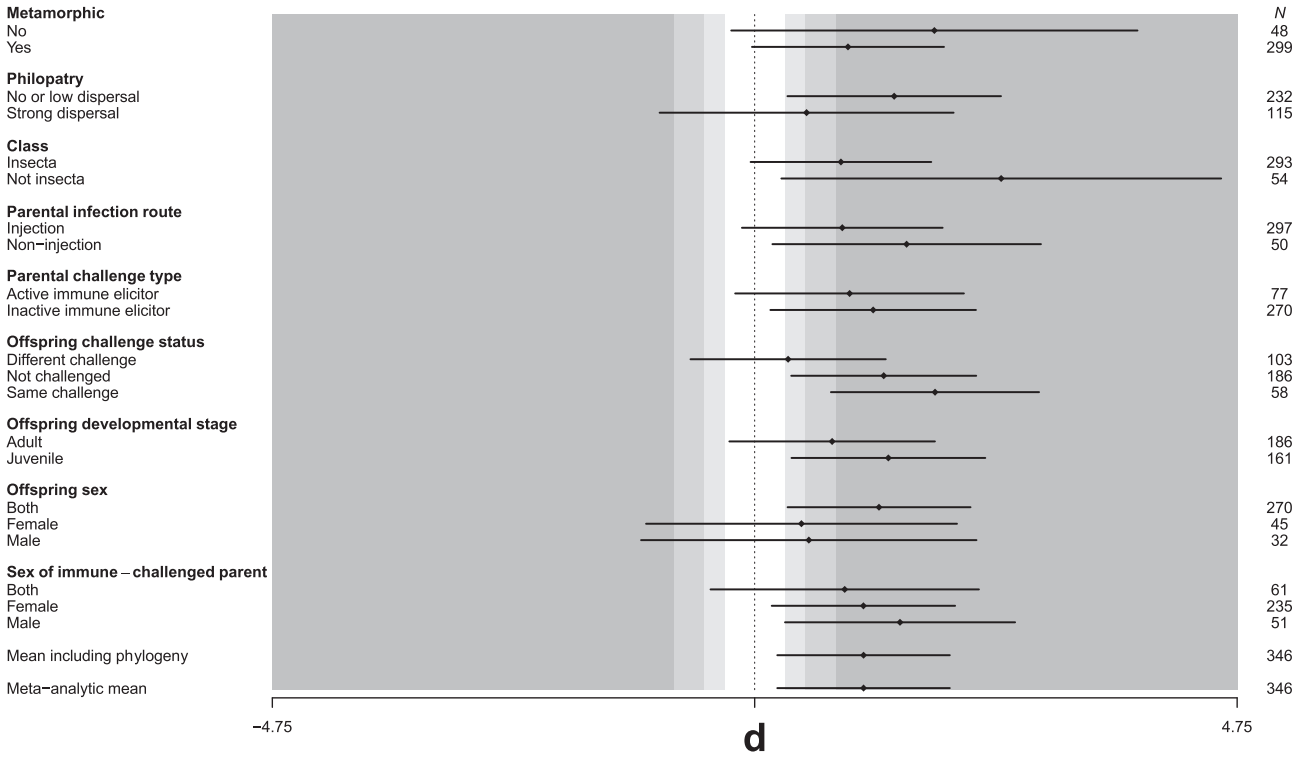

Fig. 3. Parameter estimates for studies investigating trans-generational immune priming (TGIP) in invertebrates. Diamonds represent the mean, and the error bars indicate the 95% confidence intervals. *N* is the number of effect sizes. White, light-grey, medium-grey and dark-grey spaces represent the regions for small, small-to-medium, medium-to-large, and large effect sizes (Cohen, 1988).

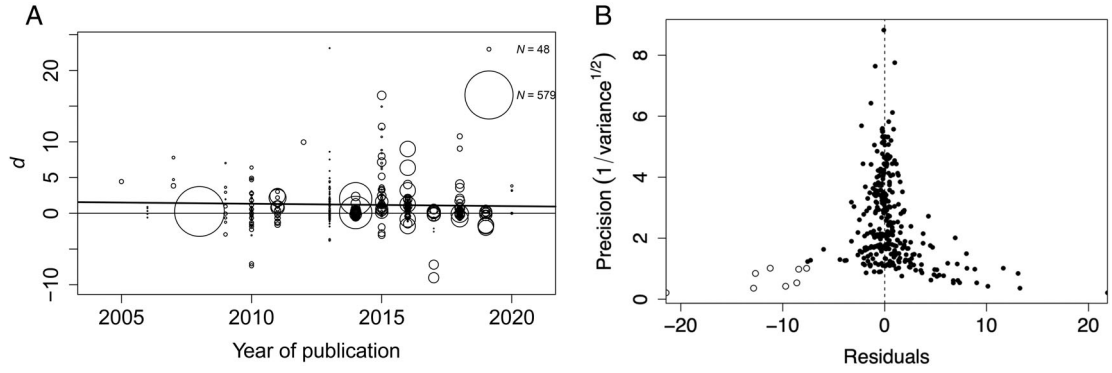

Fig. 4. Publication bias plots. (A) Relationship between effect size and year of publication for trans-generational immune priming (TGIP) studies. Solid line indicates the overall relationship, note the relationship is not significant. Circle size indicates the sample size for each effect size. (B) Funnel plot of the residuals from the final model plotted against precision. Filled circles are the actual effect sizes and empty circles are missing effect sizes estimated by the trim-and-fill analyses. The dashed line is at zero.

IV. DISCUSSION

The mechanism of trans-generational immune priming (TGIP) suggests that immune-challenged parents should be able to transfer immunological experience vertically to their offspring. Although studies have investigated this phenomenon in invertebrates (Dhinaut *et al.*, 2018*a*; Garnier *et al.*, 2012; Roth *et al.*, 2018; Tetreau *et al.*, 2019), the biological significance of the observed variation in the nature and strength of responses remains poorly understood. We conducted a quantitative synthesis on TGIP in invertebrates to understand its overall effects and the factors that affect its variation. The results from the meta-analysis support the, now prevailing, view that TGIP occurs in invertebrates. Overall, TGIP had a significant strong positive effect on the upregulation of immune parameters in the offspring

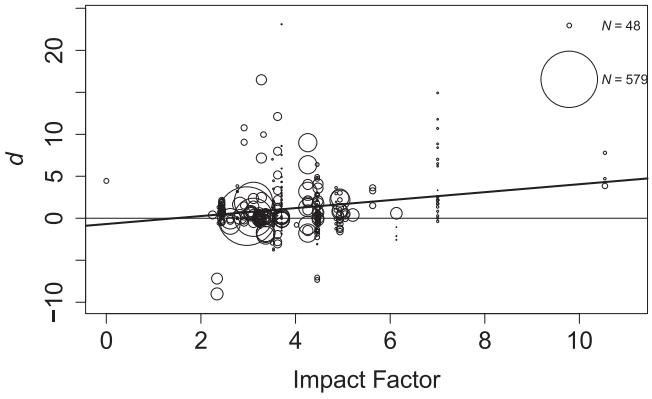

Fig. 5. Relationship between effect size and journal impact factor (JIF) for trans-generational immune priming (TGIP) studies. Solid line indicates the overall relationship, note the relationship is not significant. Circle size indicates the sample size of each effect size.

generation ($d = 1.07$). Although phylogeny did not influence our results, species identity accounted for a substantial proportion of the heterogeneity in our data set. Furthermore, there was a high amount of heterogeneity in the reported effect sizes. This suggests that much of the variation in effect sizes is likely due to experimental differences among studies, and/or to other biologically relevant variables. Our moderator analyses revealed that differences in offspring challenge status (if the offspring were challenged at all, or if they were challenged with the same or different parasite as parents) explained significant variation in TGIP studies. Contrary to our predictions, the sex of the parent (or whether one or both parents were primed), the longevity of the species studied, as well as the parental immune challenge type, did not affect the intensity or prevalence of TGIP. Finally, publication bias tests found some evidence of bias for positive results in TGIP papers.

According to Cohen (1988), an effect size of 0.2 represents a small effect, 0.5 is medium, and 0.8 is large. Our effect size of 1.07 thus represents a large effect size. However, Cohen's values are arbitrary and should not be interpreted rigidly (Lakens, 2013; Thompson, 2007). A meta-analysis by Moller & Jennions (2002) of ecological and evolutionary studies (encompassing 43 studies on invertebrates, vertebrates and plants) found that the average reported effect size was between $d = 0.63$ and 0.72. Again, our average effect size exceeds these values, suggesting that our result is biologically significant.

(1) Publication bias

Our single-factor models for both year of publication and JIF revealed no evidence of publication bias due to these factors. However, the trim-and-fill analysis indicated a potential positive-results bias, suggesting that eight negative-result studies were missing from our analysis. Such publication bias occurs when authors are more likely to submit, and editors to accept, positive over negative or inconclusive results (Begg & Berlin, 1988; Higgins *et al.*, 2019). Although we took steps to account for bias, by including grey literature in our search, none were retained after the full-text screening.

The publication bias analysis suggests that the effect size should be adjusted to $d = 0.83$. This effect size still represents a large positive effect, confirming that TGIP is present in invertebrates. Additional testing for publication bias (see Appendix S1) further adjusted our effect size down to $d = 0.73$ (95% CI = −2.35, 3.82). Note that these adjustments represent a form of sensitivity analysis rather than estimates of the true effect size. The additional publication bias test, in particular, represents an estimated effect size that we might get if a study has infinitely large sample size. Although the results point to a reduced effect, indicating possible publication bias, we urge readers to not overinterpret the findings as they are essentially extrapolations from our model results. We also note that care should be taken when interpreting publication bias analyses, as the results may reflect factors other than publication bias (Jennions *et al.*, 2013; Thornhill, Moller & Gangestad, 1999). For example, heterogeneity in effect sizes can also lead to asymmetry in funnel plots (Foo *et al.*, 2017). To control for heterogeneity, we ran the trim-and-fill analysis on the residuals from the final model. After controlling for random and moderator variables, we found that I^2 remained high in the residuals (97.9%), which indicates that the differences among studies may be caused by other moderators. There were a number of moderators that we identified as important, but for which we were unable to extract complete data, such as whether the priming parasite was host specific or novel, and whether the experimental infection protocol followed the natural route of infection for each host species. We suggest that future papers endeavour to record such information.

(2) Challenge status as a moderator for TGIP expression

Offspring challenge status (i.e. whether they were challenged with the same or different insult as their parents or were not challenged at all) positively affected TGIP expression. The strength of TGIP was higher both when offspring were challenged with the same challenge as their parents ($d = 1.78$), and when they were not challenged ($d = 1.27$) compared to when they were challenged with a different pathogen ($d = 0.33$). For within-generation priming, there is evidence of pathogen-specific immunity (Roth *et al.*, 2010; Sadd & Schmid-Hempel, 2006), where individuals exposed to a pathogen are more likely to survive a subsequent exposure to that same pathogen. Our results suggest that TGIP can be pathogen-specific too. A study on the Asian longhorned beetle, *Anoplophora glabripennis*, found that for the fungus *Metarhizium brunneum*, TGIP only occurred when both mother and offspring were challenged with the same pathogen (Fisher & Hajek, 2015). However, the same study found that maternal exposure to the bacteria *Serratia marcescens* was able to provide non-specific pathogen protection to offspring

(Fisher & Hajek, 2015). This may explain the low, positive result found in our meta-analysis; i.e. priming *via certain* parasites may be able to provide non-specific protection. Additional studies on pathogen-specific/non-specific immunity are required to assess this pattern.

We assumed that the effect of TGIP would be weaker in unchallenged offspring. Yet, even when offspring were not challenged, there was a large positive effect size of TGIP. This provides evidence of increased constitutive immunity in the unchallenged offspring of primed parents, despite the known costs associated with immune upregulation (Schmid-Hempel, 2003). However, increased immunity in unchallenged offspring also could be the result of selection in the parental generation. If there is significant parental post-infection (pre-mating) mortality, then any increase in offspring immune response could be due to selection acting on the parental generation rather than TGIP, as immune responses are likely to be heritable (Melillo *et al.*, 2018; Whitfield *et al.*, 2017). Unfortunately, we were unable to include parental pre-mating mortality as a moderator in our analysis, as this was only reported in 11 of the 37 studies. Given its potential impact on TGIP, we suggest that all future papers report parental pre-mating mortality or pathogen virulence.

(3) Variation in TGIP is not explained by longevity

Contrary to our hypothesis, ecology and life history did not affect the likelihood of detecting TGIP. This is inconsistent with the expectation that longevity should promote the evolution of TGIP (Boots *et al.*, 2009; Garnier *et al.*, 2012; Muller *et al.*, 2018; Pigeault *et al.*, 2016). Consequently, our finding suggests that TGIP is generalisable across invertebrates and is not a feature of longer-lived species (our range of species included lifespans from 32 to 2192 days; Fig. S3). One explanation is that, whilst modelling suggests that shorter-lived species (here, less than 60 days) should invest in avoidance strategies (mechanical barriers or behavioural mechanisms) (Boots & Bowers, 2004; Pigeault *et al.*, 2016), all invertebrates are capable of acquired immunity. Indeed, 70% of the papers included in our meta-analysis that considered 'short-lived' species found evidence for TGIP, suggesting that species longevity is unlikely to influence TGIP expression.

Contrary to a previous theoretical modelling study (Pigeault *et al.*, 2016), we found no influence of philopatry (philopatric species did not have a higher TGIP response than dispersing species). There are two potential explanations for this result. First, TGIP may offer benefits for dispersing species at risk of vertically transmitted pathogens (Roth *et al.*, 2018). Second, TGIP could help species dispersal into new regions, by providing them with upregulated immunity to fight pathogens (Shi, Lin & Hou, 2014). Of the 37 papers included in this meta-analysis, 73% were conducted on low- or non-dispersing species. Therefore, whilst philopatry theoretically could affect TGIP, more studies on dispersing species are needed to investigate whether it is relevant.

(4) Variation in TGIP is not explained by the sex of the parent or offspring

Although there was no overall effect of the sex of the immune-challenged parent, there was a similarly large significant effect size for maternal and paternal studies (maternal $d = 1.07$, paternal $d = 1.43$; Table 2). Maternal effects have traditionally been considered more important (Mousseau & Fox, 1998), given their greater investment in, and unilateral exposure to, growing embryos in vertebrates. Our results corroborate previous (albeit limited) findings that suggest that fathers also have the ability to transfer immunity (McNamara *et al.*, 2014; Roth *et al.*, 2010; Schulz *et al.*, 2019; Zanchi *et al.*, 2011) and that the strength of this effect is equal to that of maternal TGIP. In our meta-analysis, the magnitude of the average effect size was larger for paternal than maternal TGIP. There are four hypothesised mechanisms for stimulating TGIP: the transfer of signals, the transfer of messenger RNAs (mRNAs), the transfer of effectors and epigenetic modification (Table 3). However, as most male investment into offspring is restricted (i.e. they only provide sperm and seminal proteins) the number of mechanisms for enabling paternal TGIP may be reduced.

Males may transfer bacterial or antimicrobial peptides *via* the ejaculate (Avila *et al.*, 2011; Lung, Kuo & Wolfner, 2001; Otti *et al.*, 2009; Peng *et al.*, 2016). However, recent research has focused on paternal effects *via* epigenetic modification (Wang, Liu & Sun, 2017). Here, offspring development is influenced by the transfer of epigenetic factors in the sperm and ejaculate (Macartney, Crean & Bonduriansky, 2018).

Table 3. Descriptions of the four hypothesised mechanisms for eliciting TGIP.

Mechanism	Example	Evidence
Transfer of signals	Parents transmit a 'signal' to offspring e.g. bacterial substances transferred from the mother's gut to the eggs.	Freitak *et al.* (2014)
Transfer of messenger RNAs (mRNAs)	Mothers provide eggs with mRNA coding for immune effectors, which embryos can then produce.	Biczkowski & Dittmann (1995); Huttenhuis *et al.* (2006)
Transfer of effectors	Transfer of effector proteins (e.g. antimicrobial peptides) to the eggs (passively or actively using specialised cells).	Dubuffet *et al.* (2015); Esteves *et al.* (2009)
Epigenetic modification	Transfer of epigenetic factors (small non-coding RNAs, DNA methylation and chromatin structure) to offspring.	Schulz *et al.* (2022)

There is a growing body of evidence in support of transgenerational epigenetic effects, associated with a range of stressors experienced by parents (Ashe *et al.*, 2012; Macartney *et al.*, 2018; Ost *et al.*, 2014; Rechavi, Minevich & Hobert, 2011; Seong *et al.*, 2011; Wang *et al.*, 2017). For example, in *Drosophila melanogaster*, there is evidence for epigenetic inheritance after parental heat stress. In parents, this stress leads to the disruption of heterochromatin, which controls gene silencing epigenetically (Seong *et al.*, 2011). The phenotypic changes caused by this disruption were observable in successive generations, and was found to be transferred through the germline (Seong *et al.*, 2011). Despite evidence of epigenetic modification in within-generation priming (Castro-Vargas *et al.*, 2017) there is limited evidence for it enabling TGIP. However, recent work suggests DNA methyltransferase as a putative epigenetic mechanism underpinning paternal TGIP (Schulz *et al.*, 2022). The equivocal nature of these findings demonstrates a need for further studies on the mechanisms underlying paternal TGIP.

Similarly, offspring sex did not influence TGIP. Both male and female offspring had small positive effect sizes (Table 2), although these did not reach significance. As females typically invest more in immunity than males (Nunn *et al.*, 2009; Rolff, 2002), we hypothesised that female offspring would show higher levels of TGIP. However, both Zanchi *et al.* (2011) and McNamara *et al.* (2014) demonstrated sexually dimorphic responses to immune priming, with sons displaying higher levels of immunocompetence than daughters. This lack of concordance in the data regarding offspring sex-specific differences in TGIP emphasises the need for studies to report data for offspring sexes separately.

(5) Variation in TGIP is not explained by the parental immune challenge type

Perhaps surprisingly, TGIP was unaffected by the parental immune challenge type (active or inactive). We hypothesised there would be a difference between these two types, as inactive immune elicitors may be administered at higher concentrations and are non-pathogenic. However, our results demonstrate that inactive immune elicitors (heat-killed bacteria, LPS, Sephadex beads and PGN) lead to similar TGIP responses as active immune elicitors (live bacteria, parasites, fungus or viruses), despite lacking pathogenicity. Moreover, the sensitivity analysis found no difference according to the Gram strain of bacteria (positive or negative) used in the parental priming (Gram strain of LPS and PGN were also included in these analyses). As different types of bacteria can trigger different pathways (responses to Gram-negative bacteria involve the Toll pathway, responses to Gram-positive bacteria involve the Imd pathway) we predicted that a difference in effect size could arise if TGIP had evolved *via* a specific pathway or if temporal variation plays a role in TGIP expression. Given that the Imd and Toll pathways are highly conserved in holometabolous insects, such as *T. castaneum* and the honeybee, *Apis mellifera* (Nishide *et al.*, 2019), it may be the case that both pathways are involved in TGIP. Additionally, some antimicrobial peptides have the ability to activate both pathways (Kounatidis & Ligoxygakis, 2012; Nishide *et al.*, 2019; Valanne *et al.*, 2010), while some species (such as *A. pisum*) have absent or undeveloped Imd pathways (Gerardo *et al.*, 2010). Functional redundancy between pathways (Nishide *et al.*, 2019) could explain the similar effect sizes. A TGIP study that targets species lacking the Imd pathway, such as the bedbug, *Cimex lectularius* (Benoit *et al.*, 2016; Zumaya-Estrada *et al.*, 2018) could provide further insight.

(6) Limitations and future directions

This study provides corroborating evidence for TGIP in invertebrates and revealed factors that significantly affect its intensity. However, it is clear that large heterogeneity still remained in the model residuals after taking account of the moderators, and therefore that other moderators, such as pathogen species specificity and natural infection routes should be considered in future work. Importantly, we were unable to include parental pre-mating mortality as a moderator in our analysis, as it was typically unreported. This was surprising, as information on the virulence of the parasite is critical in the interpretation of TGIP. Fortunately, over 50% of studies used inactive immune elicitors, which stimulate an immune response without a direct infection cost, and for which parental mortality may be negligible. Indeed, for ontogenic priming (a form of within-generation priming where individuals are challenged first as juveniles and then as adults), mortality was low (3/160 individuals died) after injection of *T. castaneum* with the heat-killed bacteria *Bacillus thuringiensis* (Khan *et al.*, 2016). Although not direct evidence that inactive immune elicitors lead to low mortality in TGIP studies, this does suggest that mortality can be negligible for certain immune challenges. It is vital that future empirical TGIP studies report parental mortality data, in the form of mortality rates or lethal doses.

V. CONCLUSIONS

(1) Despite lacking adaptive immunity, we found evidence that TGIP occurs in invertebrates *via* the upregulation of immunity in the offspring generation. Our overall effect size was higher than effect sizes commonly reported in ecological and evolutionary studies, suggesting that our result is biologically significant.
(2) We identified a significant positive effect size for both challenges of offspring with the same agent as parents, as well as for offspring that were not challenged.
(3) We found evidence for paternal as well as maternal effects on TGIP. We suggest that more emphasis should be placed on the study of epigenetics as a mechanism of TGIP.
(4) We report some evidence of publication bias for positive results, using indirect tests. However, there was no relationship between effect size and JIF or year of publication.

(5) We found large heterogeneity in effect sizes after controlling for random and fixed variables. This suggests that other factors in addition to those included in this meta-analysis may be moderating the effects of TGIP. We advise the consistent reporting of parental mortality or parasite virulence, as well as whether the experimental infection protocol followed the natural route of infection and whether the parasite was host specific or novel.

(6) Finally, we highlight topics for future research. First, the effects of parental age (i.e. older *versus* younger adults) are likely to be important. As older parents have lower residual reproductive value (expectations of future offspring), priming may lead them to increase their investment in reproduction. This investment may result in older parents producing offspring with higher levels of immunocompetence. Second, we suggest a focus on species with prolonged parental care, such as the burying beetle, *Nicrophorus vespilloides*. In this species, parents protect offspring from threats, and feed them directly in early instar stages, both of which improve offspring survival and growth. If parental care is coupled with an immune challenge, we should observe higher levels of immune function and survival in such offspring, compared with those receiving no parental care or whose parents are not immune challenged.

VI. ACKNOWLEDGEMENTS

K. B. M. was supported by the Australian Research Council (DE160100097 and DP200101615). Open access publishing facilitated by The University of Melbourne, as part of the Wiley - The University of Melbourne agreement via the Council of Australian University Librarians.

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VIII. SUPPORTING INFORMATION

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

Table S1. Completed PRISMA checklist from O'Dea *et al.* (2021).
Fig. S1. Initial screening flowchart, used for the initial screening of the paper title, key words and abstract.
Fig. S2. Final screening flowhcart, used for the full-text screening of papers.
Fig. S3. Distribution of longevity (based on the number of effect sizes) for the 18 species included in our meta-analysis.
Table S2. Table of Akaike Information Criterion with sample size correction (AICc) results.
Appendix S1. Supplementary analysis for publication bias.

(*Received 28 July 2022; revised 21 February 2023; accepted 23 February 2023*)