The Role of Abiotic and Biotic Factors in Infection Outcomes



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Declaration

I declare that this thesis was composed by myself and that the work contained herein is my own except where explicitly stated in the text. This work has not been submitted for any degree or professional qualification except as specified.

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"So, naturalists observe, a flea
Hath smaller fleas that on him prey;
And these have smaller fleas to bite'em,
And so proceed ad infinitum."

- Jonathan Swift, *On Poetry: a Rhapsody* (1733)

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Publications and Contributions

The following published (and *in Prep*) papers have arisen from this thesis, and are presented in Chapters 2, 3, 4 and 5:

Chapter 2

Pike V.L., Lythgoe K.A.* and King K.C.* (2019) On the diverse and opposing effects of nutrition on pathogen virulence. *Proceedings of the Royal Society B*: 286: 20191220

*Denotes equal contribution.

V.L.P., K.A.L. and K.C.K designed the study. V.L.P. collected the data and carried out the statistical analysis. All authors contributed to writing the manuscript.

Chapter 3

Pike V.L. & King K.C., A meta-analysis of experimental work on the virulence of coinfections (*in Prep*)

V.L.P. and K.C.K. conceived and designed the study. V.L.P. collected the data and carried out the statistical analysis, with guidance from K.C.K. Both authors contributed to writing the manuscript.

Chapter 4

Pike V.L., Griffin A.S., King K.C., Within- and between-host dynamics of producer and non-producer pathogens (*in Prep*)

V.L.P, K.C.K and A.S.G conceived and designed the study. V.L.P. performed the experiments and analysed the data, with guidance from K.C.K. All authors contributed to writing the manuscript.

Chapter 5

Pike V.L., Cornwallis C.K.*, Griffin A.S.*, 2021 Why don't all animals avoid inbreeding? *Proceedings of the Royal Society B* (Accepted 12st July 2021, due to be published on 4th August 2021)

*Denotes equal contribution.

V.L.P and A.S.G designed the study. V.L.P collected the data, C.K.C and V.L.P carried out the statistical analysis. All authors contributed to writing the manuscript.

I also contributed to two other projects during my DPhil:

Pike V.L., Ford S.A, King K.C.*, Rafaluk-Mohr C.* (2019) Fecundity compensation is dependent on the generalized stress response in a nematode host. *Ecology and Evolution*, 9: 11957– 11961.

*Equal contribution

Hoang K.L., Salguero-Gómez R., **Pike V.L.**, and King K.C. (2021) Symbiont fitness across variable environments (*in Prep*)

Thesis abstract

Hosts, parasites, and the environment are interlinked in the disease triangle. Thus, environmental changes can lead to shifts in the ecological and evolutionary dynamics of host-parasite interactions. In this thesis, I combine meta-analytical and experimental approaches to explore the role of abiotic and biotic factors, specifically nutrition and within-host parasite-parasite interactions, on infection outcomes. Firstly, I conducted a literature search to gather data from a wide range of host-parasite systems and carried out a meta-analysis to investigate whether changes to host nutrition affect virulence. Among other findings, this study reveals that changes to host nutrition may have diverse effects across host and parasite species, depending upon a balance between the effect of host nutrition on the immune system and on parasite resources. Subsequently, using a similar meta-analytic approach, I investigate the extent to which coinfections affect virulence relative to single infections. Across host-parasite systems, I find that coinfections are overall more virulent than single infections, with phylogenetic divergence of coinfecting parasites and their transmission strategies key determinants. Extending on this analytical work, I experimentally test the role of specific pathogen traits (a 'producer' and two 'non-producers' for siderophore production and quorum sensing) on within-and-between-host dynamics in single infection and coinfections, using a nematode host (*Caenorhabditis elegans*) and pathogenic bacterium (*Pseudomonas aeruginosa*). I find that producer strains are consistently better at both colonising and transmitting between nematode hosts than non-producers in single infections and coinfections. On a different theme to the preceding research, in the final data chapter, I ask the broad evolutionary question: why don't all animals avoid inbreeding? I demonstrate that inbreeding avoidance only evolves when there is a risk of inbreeding depression and when related sexual partners frequently come into contact. In this thesis, I use the disease triangle as a tool to investigate abiotic and biotic drivers of host-parasite interaction outcomes and

dynamics, extending on previous research highlighting the importance of studying host-parasite dynamics within an ecological context at both the level of within-host and between-host dynamics. In a changing world, this work is a timely contribution to our understanding of the broad and varied effects of environmental change on infection outcomes.

Chapter 1

Introduction

Introduction

1.1 The Disease Triangle

The natural world is highly variable with a range of abiotic (Lazzaro and Little 2009; Wolinska and King 2009) and biotic (Leggett et al. 2014; Betts et al. 2016) environmental factors which can affect the ecological and evolutionary dynamics of host-parasite interactions (Wolinska and King 2009). Here the term parasite is used to broadly include any infectious disease causing agent, as is common practice within evolutionary ecology [e.g.(Eswarappa et al. 2012; Alizon et al. 2013; Rynkiewicz et al. 2015)]. This link between parasite, host and environment was first formalised with the disease triangle (McNew 1960) (Figure 1). Although the disease triangle was initially developed for considering plant pathology, it has since been applied to a diverse range of animal and human diseases (Scholthof 2007), which will be the focus of this thesis**.**

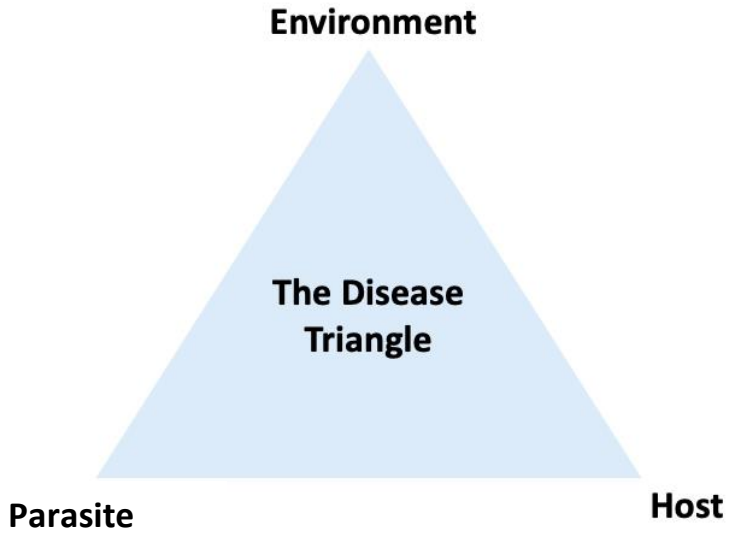


Figure 1: The disease triangle. Developed by McNew (1960), the disease triangle shows the host, parasite and environment at the vertices.

Currently, anthropogenic-induced climate change is driving vast fluctuations across a range of environmental conditions (Cahill et al. 2013; Altizer et al. 2013). Both abiotic conditions,

such as extreme weather events (McMichael 2015), and biotic conditions, such changes in species interactions due to climate-induced host shifts (Hance et al. 2007; Longdon et al. 2015), have been impacted by climate change (Cahill et al. 2013), highlighting a timely need for the application of the disease triangle. To date, studies have revealed that environmental changes are affecting host-parasite interactions (Wolinska and King 2009; Altizer et al. 2013), including parasite-imposed host harm (virulence) (Pike et al. 2019b). Whether and the degree to which parasite virulence is affected by external biotic and abiotic factors is an outstanding question within ecology and evolution (Lively et al. 2014), and one I tackle in this thesis.

1.2 Virulence

The harm parasites cause a host is known as its virulence [reviewed by (Frank 1996)]. Virulence is highly variable across parasite species (Ewald 1994; Frank 1996). Some species cause little harm to their host, such as Henipavirus infections in grey-headed fruit bats (*Pteropus poliocephalus*) (Middleton et al. 2007). In contrast, infection with other parasites can be fatal. For example, the fungus *Batrachochytrium dendrobatids* is the causal agent of chytridiomycosis, and thought to be responsible for declines (Voyles et al. 2009) and extinctions (Wilber et al. 2017) in amphibian populations across the world.

A parasite is dependent on its host for survival, but to be successful, parasites must transmit between new hosts (Anderson and May 1986). This counterbalancing relationship is described as a trade-off. For parasites, a trade-off is predicted to occur between within-host replication and between-host transmission (Anderson and May 1982; Ewald 1983; Koella and Antia 1995; Walther and Ewald 2004). To replicate, parasites consume host resources, which increases the harm caused to the host, reduces the hosts lifespan and the increases the risk of

host mortality. The increased host mortality associated with replication is hypothesised to decrease pathogen transmission (Anderson and May 1982; Ewald 1983; Koella and Antia 1995; Walther and Ewald 2004; Acevedo et al. 2019), resulting in conflict between within-host growth and between-host transmission. This is an analogous trade-off to that experienced by multicellular organisms (Reece et al. 2009) between reproduction and growth (Bell 1980; Clutton-Brock 1984; Greischar et al. 2016). Overall, theory predicts that optimal virulence is that which maximises transmission (Anderson and May 1982; Frank 1996).

The virulence-transmission trade-off may be influenced by transmission mode (Antonovics et al. 2017). For environmentally transmitted parasites, where transmission may be airborne or utilise vectors (indirectly-transmitted, (Antonovics et al. 2017), high virulence is predicted to evolve as parasites do not rely on hosts for transmission (Ewald 1983; Walther and Ewald 2004; Antonovics et al. 2017). Extreme high virulence is found in the indirectly transmitted bacterium *Bacillus anthracis*, the causal agent of Anthrax, whose spores can persist for years in soil (Dragon and Rennie 1995; Rafaluk-Mohr 2019). Alternatively, parasites which are transmitted through direct contact between hosts (direct transmission (Antonovics et al. 2017)) rely on a live host for transmission. Thus, as these parasites rely on their host to successfully transmit to new hosts, theory predicts that extreme virulence is less likely to evolve (Boldin and Kisdi 2012; Antonovics et al. 2017).

Although empirical studies across a range of species find support of the virulence-transmission trade-off hypothesis [e.g. (Messenger et al. 1999; Jensen et al. 2006; Fraser et al. 2007; de Roode et al. 2008)] other studies find contradicting evidence (Ebert and Bull 2003; Alizon et al. 2009). Thus, the broad relevance and application of the virulence transmission trade-off has been extensively debated and challenged (Ebert and Bull 2003; Alizon et al.

2009; Leggett et al. 2013). A recent meta-analysis across empirical studies found strong support for a positive correlation between within-host replication and virulence and between within-host replication and transmission (Acevedo et al. 2019). However, the authors also highlighted the need for further studies to be conducted in order to relieve the uncertainty and more accurately assess the relationship between virulence and transmission (Acevedo et al. 2019).

From the application of the disease triangle (McNew 1960), we know that host-parasite interactions and particularly virulence can be affected by environmental factors. I will now explore the role abiotic and biotic factors in infection outcomes, with a focus on virulence and transmission throughout.

1.3 Abiotic factors and disease

1.3.1 Range of abiotic factors

A diverse range of non-living (abiotic) factors can affect host-parasite interactions (Lazzaro and Little 2009; Wolinska and King 2009). Factors include, but are not limited to, nutrition [e.g.(Cornet et al. 2013; Conroy et al. 2016)], temperature [e.g.(Thomas and Blanford 2003; Mitchell et al. 2005)], pH [e.g. (Asplund et al. 2014; Fuhrmann et al. 2019)], water availability [e.g.(Sebastian et al. 2015; Ruggeri et al. 2018)] and pollution levels [e.g. (Arkoosh et al. 1998; Pietrock and Marcogliese 2003)]. For example, even small temperature fluctuations can enhance parasite development and thus host exploitation, which in turn may affect virulence (Thomas and Blanford 2003). From the host's perspective, temperature has been shown to influence the quality of their immune defences (Thomas and Blanford 2003). In the fruit fly *Drosophila melanogaster* (Lazzaro et al. 2008; Linder et al. 2008), fluctuations

100 from the host's normal local environmental temperature have a strongly deleterious effect on
101 host immunity (Lazzaro et al. 2008).
102
103 However, abiotic factors are often excluded from studies, dismissed as noise, or highly
104 simplified relative to the natural world despite their crucial role in influencing disease
105 dynamics (Lazzaro and Little 2009; Wolinska and King 2009). Now, more than ever,
106 with anthropogenic activities causing fluctuations across a range of abiotic conditions
107 (Lebarbenchon et al. 2008; Cahill et al. 2013; Oro et al. 2013; Becker et al. 2015;
108 Civitello et al. 2018), it is important that we improve our understanding of the role of abiotic
109 factors in disease outcomes. One particular abiotic factor, nutrition, has until recently been
110 broadly overlooked in disease ecology (Beck et al. 2004) despite being shown to be a strong
111 driver of disease dynamics in wild animal host populations (Becker et al. 2015; Murray et al.
112 2016; Civitello et al. 2018).
113

114 **1.3.2 Nutrition**

115 Agricultural intensification, overfishing and urbanisation have depleted food availability for
116 many species (Jackson et al. 2001; Lotze et al. 2006; Fischer and Lindenmayer 2007). On the
117 other hand, humans also provide supplementary nutrition either by intentional [e.g. bird-
118 feeders (Robb et al. 2008)] or unintentional means [e.g. waste (Ciach and Kruszyk 2010)]
119 [for a full review see (Oro et al. 2013; Becker et al. 2015; Altizer et al. 2018)]. These
120 subsidies can be substantial (Altizer et al. 2018), with estimates of between 43-78% of
121 households in the UK and USA regularly feeding urban birds (Robb et al. 2008) and 30-40%
122 of all food produced being wasted (Parfitt et al. 2010). Resources being provided at such a
123 scale has resulted in some species altering their behaviours, for example the foraging patterns
124 of the American white ibis (*Eudocimus albus*) (Murray et al. 2016). Although subsidies are

125 readily accessible, they are often of low quality (Murray et al. 2016). In the case of the
126 American white ibis, their supplemented diet is predominantly made up of carbohydrates, in
127 contrast to their natural protein-rich diet (Murray et al. 2018).
128
129 Nutrition can affect pathogen virulence (Bedhomme et al. 2004; Cornet et al. 2013). From the
130 hosts perspective, launching an immune response is costly (Sheldon and Verhulst 1996), thus
131 changes in nutritional quality and quantity affect the resources for hosts to invest in their
132 immune response (Beck et al., 1994; Chandra, 1996; Cornet et al., 2013). Furthermore,
133 changes in host nutrition have knock on effects for the pathogen directly, affecting their
134 resource availability for growth and reproduction, which may lead to an increase or decrease
135 in virulence (Tseng and Myers 2014; Cressler et al. 2014). Even small changes to
136 micronutrients, such as manganese (Mn), have been shown to affect virulence (Beck et al.
137 2004; Juttukonda et al. 2017). When dietary manganese was given to Mice (*Mus musculus*)
138 infected with the bacterium *Staphylococcus aureus*, the bacteria were able to utilize Mn to
139 detoxify reactive oxygen species and as a defence against the hosts immune response
140 (Juttukonda et al. 2017).
141
142 Furthermore, nutrition-induced behavioural changes may affect the transmission of diseases
143 (Oro et al. 2013; Altizer et al. 2018; Civitello et al. 2018). Behavioural changes may arise
144 from natural fluctuations in nutritional availability, such as seasonal changes (Leroy et al.
145 2004, 2005). For instance, in populations of great apes Ebola virus infections increase during
146 the dry season when there is increased contact between apes due to higher competition for
147 food (Leroy et al. 2004, 2005). Similarly, nutritional supplements provided at specific sites
148 may increase the contact rate of individuals as has been identified at feeding stations within
149 elk (*Cervus elaphus*) populations (Cross et al. 2007).

1.4 Biotic factors and disease

1.4.1 Range of biotic factors

Taking an ecological approach to investigating host-parasite interactions involves an understanding of the ecological context in which infections occur. So far, I have discussed abiotic factors which influence host-parasite dynamics, but hosts and parasites can also be affected by interspecific and intraspecific biotic (live) interactions which occur within their ecosystem (Collinge and Ray 2006; Holt and Roy 2007). Taking the example of top-down predator interactions, parasite-induced morbidity has been shown to increase the risk of host predation (Packer et al. 2003). Increased predation has been demonstrated in snowshoe hare (*Lepus americanus*) populations, where hares treated with anthelminthic drugs were less vulnerable to predation (Murray et al. 1997). Predatory interactions can be beneficial for parasites in cases where parasites have complex life cycles involving multiple host species (Poulin 2010; Hughes et al. 2012). Some parasites are even capable of manipulating the host phenotype such as the protozoan *Toxoplasma gondii* which causes rodents to be attracted to the odour of predatory cat urine (Berdoy et al. 2000). The infected rodents are consumed by the cat, which is the definitive host (host in which parasite sexual reproduction occurs) of *T .gondii* (Berdoy et al. 2000). Phenotypic alterations also include some incredible morphological changes, such as the resemblance of a red berry in the ant *Cephalotes atratus* induced by the nematode *Myrmeconema neotropicum*, making the ants attractive to the definitive bird host (Yanoviak et al. 2008).

Earlier, I discussed increased parasite transmission associated with hosts gathering feeding sites [e.g. (Leroy et al. 2004; Cross et al. 2007)]. Hosts may also interact as for other reasons such as mating (Sheldon 1993; Lockhart et al. 1996; Altizer et al. 2003; Knell and Webberley

2004) or if they live in groups (Altizer et al. 2003; Ezenwa 2004; Ezenwa et al. 2016; Ezenwa and Worsley-Tonks 2018). Mating may facilitate the transmission of sexually transmitted parasites, such as infection with the intracellular bacteria *Chlamydia sp* in the koala (*Phascolarctos cinereus*) (Polkinghorne et al. 2013), with exposure risk increasing with the number of sexual partners (Anderson and May 1992). Likewise, in social species increased parasite transmission may occur between hosts as in wild Grant's gazelles (*Nanger granti*) where group living comes with an increased risk of infection with a myriad of gastrointestinal nematodes (Ezenwa and Worsley-Tonks 2018). However in the latter example, group living is associated with an increased feeding rate and so alleviates the parasite-induced suppression of host food intake (Ezenwa and Worsley-Tonks 2018).

Taken together, the web of interactions within ecosystems which may affect host-parasite interactions are complex. Yet further complexity can arise depending on the host and the parasite within an infection (Ewald 1994; Longdon et al. 2015). The same parasite may have diverse outcomes across different host species, for example, when bats are infected with Ebola virus they are largely asymptomatic (Swanepoel et al. 1996; Leroy et al. 2005) whereas for humans and other primates infection can be fatal (Swanepoel et al. 1996; Leroy et al. 2005). Parasites are highly diverse (Poulin and Morand 2000) and infections are often made up of multiple parasite species (Cox 2001; Griffiths et al. 2011; Alizon et al. 2013; Betts et al. 2016). Furthermore, when multiple parasites infect a host, how they interact can have important consequences for disease outcomes such as infection virulence and transmission dynamics (Frank 1996; Mideo 2009; Telfer et al. 2010; Eswarappa et al. 2012; Leggett et al. 2014).

1.4.2 Coinfections

When hosts are simultaneously infected by multiple parasites it is known as a coinfection (Cox 2001). Most infections in nature are made up of multiple parasite species (Petney and Andrews 1998; Cox 2001; Pedersen and Fenton 2007; Rynkiewicz et al. 2015; Betts et al. 2016; Hoarau et al. 2020). Similarly, coinfections frequently occur in humans (Griffiths et al. 2011) with two of the most prevalent human infections, malaria and helminthiases, regularly arising in coinfections due to their similar geographic distribution (Mwangi et al. 2006). Despite their ubiquity, few guidelines exist for the treatment of coinfections (Griffiths et al. 2015). They are difficult to effectively treat (Pedersen and Antonovics 2013; Clerc et al. 2019; Ezenwa et al. 2021) because coinfection treatments can have unexpected effects on non-target species (Pedersen and Antonovics 2013; Clerc et al. 2019; Ezenwa et al. 2021). For example, the decline associated with treating one parasites may lead to reciprocal increases another species, which has been demonstrated in wild mice populations (white-footed mice (*Peromyscus leucopus*) and deer mice (*Peromyscus maniculatus*)) (Pedersen and Antonovics 2013).

The outcomes of coinfections can be diverse and unpredictable, because they are not necessarily additive (Rigaud et al. 2010; Alizon et al. 2013). In some coinfections, the combined virulence of the interacting parasites is higher than that in single infections, for example snail hosts (*Biomphalaria glabrata*) coinfected with the trematode parasites *Schistosoma mansoni* and *Echinostoma caproni* experience higher virulence than in single infection (Sandland et al. 2007). The increase in virulence is thought to be driven by competition and exploitation of host resources (Sandland et al. 2007). However, in other coinfections, the overall virulence may be lower than in single infection. In multi-strain coinfections of the causal agent of human African sleeping sickness *Trypanosoma brucei* within a mouse model (Balmer et al. 2009), the strains mutually supress each other via

competition resulting in a decrease in the cost of infection for the mouse host (Balmer et al. 2009).

Coinfection outcomes may also vary depending on the order with which parasites infect a host, so called priority effects (Goodman and Ross 1974; de Roode et al. 2005; Clay et al. 2018). The parasite which is first to infect a host may have a competitive advantage over subsequent strains for limited resources (Goodman and Ross 1974; de Roode et al. 2005; Clay et al. 2018). Alternatively, a parasite that arrives after the initial infection may benefit from a weaker host immune response (Goodman and Ross 1974; de Roode et al. 2005; Clay et al. 2018). Utilising an ecological approach (Pedersen and Fenton 2007; Rynkiewicz et al. 2015; Hassell et al. 2021), we can improve our understanding of these within-host interactions thereby enhancing our ability to predict infection outcomes (Handel and Rohani 2015).

1.4.3 Within-host interactions between parasites

Parasites interact via a range of mechanisms which may be direct, such as competition for resources (Mideo 2009), or indirect via the hosts immune system (Graham 2008). Additionally, interactions between parasites may be cooperative and allow successful colonisation of a host, for example via forming biofilms (Griffin et al. 2004; Kreft 2004; Foster 2005; Diggle et al. 2007; West et al. 2007). The mechanisms by which parasites interact within a host can drive changes in a variety of infection outcomes, including parasite virulence and transmission (May and Nowak 1995; Frank 1996; Rigaud et al. 2010; Alizon et al. 2013). Theory suggests that within-host competition favours rapid growth to achieve greater relative success and thus is likely to drive an increase the overall virulence of a coinfection (Nowak and May 1994; Frank 1996; Ferguson and Read 2002). Yet, if the

interaction mechanism between parasites is cooperative, virulence is predicted to positively correlate with the relatedness between coinfecting parasites (Brown et al. 2002; West and Buckling 2003; Griffin et al. 2004).

Compared to within-host interactions, fewer studies have investigated between-host dynamics meaning less is known about how within-host processes contribute to variation in pathogen transmission between hosts (Brown et al. 2009; Handel and Rohani 2015; VanderWaal and Ezenwa 2016; Stephenson et al. 2017). This may be a result of some of the logistical challenges which arise from investigating transmission, requiring high numbers of potential hosts which is also results in high experimental costs (Handel and Rohani 2015). Studies have shown a range of factors to influence between-host dynamics including parasites with different modes of transmission, which can lead to within-host conflict and altered between-host dynamics (Thomas et al. 2002; Ben-Ami et al. 2011). In addition, evidence from empirical studies has suggested some social traits may affect between-host dynamics (Brown et al. 2009; Jiricny et al. 2010, 2014), which I will now explore in more detail.

1.4.3.1 Cheating

A central aspect of interactions in the local environment between microbial parasites is the production and secretion of public goods (Leggett et al., 2014; West et al., 2007). Public goods benefit all the cells in the local environment and are costly to produce and thus are susceptible to exploitation by cheats (Velicer 2003; West et al. 2007; Ghoul et al. 2014). Cheats utilise the costly public goods without contributing to their production themselves. Cooperative behaviours are regulated by using small autoinducer molecules in a process known as quorum sensing (Rumbaugh et al., 2009). Cheats have been shown to evolve in

natural populations, such as the quorum-sensing cheats found in the lungs of cystic fibrosis patients (Diggle et al. 2007).

It has been hypothesised that cheats may affect disease dynamics (Brown et al. 2009; Jiricny et al. 2010, 2014). Infections of *Pseudomonas aeruginosa* are often initiated by highly cooperative strains and when populations are sampled at random cheats are rare (Köhler et al. 2009; Andersen et al. 2015), suggesting they could be poor at within-host colonisation or transmitting between hosts (Jiricny et al., 2014). Within-host cheating could therefore negatively impact the transmission dynamics of an infection and thus could be potentially utilized as a disease intervention strategy (Brown et al. 2009; Jiricny et al. 2014; Rezzoagli et al. 2020), emphasising the medical relevance of understanding the social lives of parasites (Foster, 2005; Harrison, 2013).

1.5 Experimental and meta-analytic approaches to tackle ecological and evolutionary questions

In this thesis, I used analytical and experimental approaches to ask evolutionary questions. I will now introduce the meta-analytical approach and the experimental system.

1.5.1 Meta-analysis

When asking broad evolutionary questions, it can be beneficial to use large datasets incorporating a variety of species (Koricheva et al. 2012). A good way to approach this is via a meta-analysis, a quantitative method that combines results from different studies to draw general conclusions and evaluate the consistency amongst study findings (Hedges and Olkin 1985). Meta-analyses have been hugely important across scientific fields, but have been particularly significant within ecology and evolutionary biology (Koricheva et al. 2012;

300 Gurevitch et al. 2018) as they allow the detection of any broad-scale patterns across species
301 (Gurevitch et al. 2018). For example, a meta-analytic approach was used to investigate
302 variation in kin discrimination across cooperatively breeding vertebrates (Griffin and West
303 2003).
304
305 To maintain a robust and comparable approach across meta-analyses, there are a set of items
306 which are required to be reported known as the Preferred Reporting Items for Systematic
307 Reviews and Meta-Analyses (PRISMA) (Moher et al. 2009; Page et al. 2021). There are four
308 stages of the systematic review process; 'identification', 'screening', 'eligibility' and
309 'included' (Moher et al. 2009). The first stage involves using search terms to identify
310 potentially relevant literature ('identification'), forward and backward citation searches are
311 also conducted on any relevant studies. A screening process is then conducted with the
312 identified literature ('screening') from which studies which do not meet any inclusion criteria
313 are excluded ('eligibility') leaving the final set of relevant studies for analysis ('included').
314 This formal methodology makes a meta-analytic approach transparent and reproducible
315 (Nakagawa et al. 2017).
316
317 **1.5.1.1 Effect sizes**
318 In a meta-analysis the quantitative measure extracted from each study is known as an effect
319 size. Effect sizes enable comparisons between the outcome of each separate study (Nakagawa
320 and Santos 2012; Nakagawa et al. 2017; Gurevitch et al. 2018). There are three broad groups
321 of effect sizes with corresponding measures ((Nakagawa et al. 2017), Table 1). The type of
322 effect size statistic used depends on the data available across studies. Some measures may
323 also be used in specific contexts, for example Hedges' g (Hedges 1981) weights effect sizes

by sample size, meaning that an overall effect is not biased by studies with smaller sample sizes (Borenstein 2009; Koricheva et al. 2012).

Table 1: Effect sizes and Statistics.

Type of effect size	Corresponding effect size statistic	References
The difference between the means of two groups	Standard mean differences: *Cohen's d, Hedges'd or Hedges'g, response ratio*	(Hedges 1981; Hedges and Olkin 1985; Koricheva et al. 2012)
The correlation between two variables	Fisher's z-transformed correlation coefficient (*Zr*)	(Hedges et al. 1999; Koricheva et al. 2012)
The incidence of two outcomes in two groups	Natural logarithm of the odds ratio (ln*OR*) and relative risk (ln*RR*)	(Koricheva et al. 2012)

1.5.2 Experimental Host: *Caenorhabditis elegans*

Caenorhabditis elegans is a free-living hermaphroditic nematode species, present in most temperate regions of the world (Hope 1999; Schulenburg and Félix 2017). This animal is a useful model organism due to its rapid life cycle which occurs in around 3.5 days (at 20ºC) and is composed of four distinguishable larval stages (L1 to L4) (Brenner 1974; Hope 1999) (Figure 2). Furthermore, nematodes produce offspring at a high rate, with a single nematode producing around 300 offspring (Hodgkin and Barnes 1991). Although they primarily reproduce via self-fertilization of hermaphrodites, males do arise (androdioecy) at low frequencies within laboratory populations (around 0.2%) (Hodgkin and Doniach 1997; Frézal and Félix 2015) which are morphologically distinguishable from hermaphrodites via their tail shape. This production of males along with the selfing facilitates populations without inbreeding depression (Frézal and Félix 2015), although they are susceptible to outbreeding depression (Dolgin et al. 2007).

Furthermore, it is relatively cheap and easy to maintain large population sizes of *C. elegans*. The maintenance protocols are well defined and involve rearing at around 20^{O}C on Nematode Growth Medium (NGM) with a bacterial food source, typically *Escherichia coli* (OP50) (Brenner 1974; Stiernagle 1999). Experimental work is normally carried out on the canonical laboratory reference strain N2, but there are many strains and large mutant libraries available (archived by the Caenorhabditis Genetics Center (CGC)).

The gut of *C. elegans* can be colonised by a wide variety of microbe species making it a good model for host-pathogen interactions (Gravato-Nobre and Hodgkin 2005; Clark and Hodgkin 2016; Schulenburg and Félix 2017) (Figure 3). *C. elegans* has been infected with a range of human pathogens including *Psedomonas aeruginosa* and *Salmonella enterica* (Marsh and May 2012). They rely on innate immune defences (Sifri et al. 2005; Gravato-Nobre and Hodgkin 2005; Marsh and May 2012) and are also capable of behavioural avoidance of pathogens (Zhang et al. 2005; Pradel et al. 2007). *C.elegans* have also been shown to alleviate fitness consequences of infection by increasing their offspring production (Pike et al. 2019a), a process known as fecundity compensation (Minchella and Loverde 1981; Minchella 1985). There is variation in the ability of bacterial strains to colonise the nematode gut (Portal-Celhay et al. 2012) but those which colonise the host are transmitted between nematodes in their faeces – akin to faecal oral transmission within larger animals (Diaz and Restif 2014).

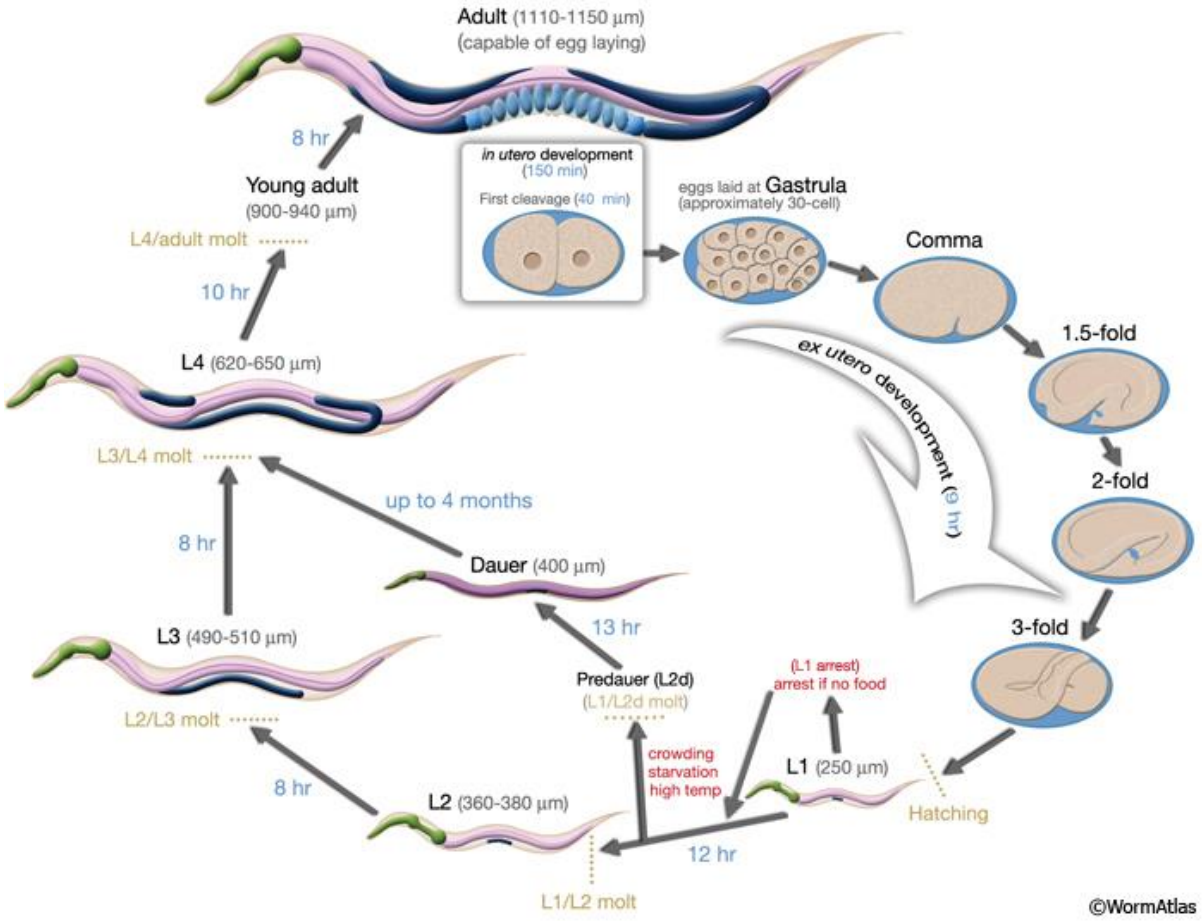


Figure 2 – *Caenorhabditis elegans* life cycle. *C.elegans* develop from eggs to adults producing their own fertilized eggs in around 3 days. The first larval stage (L1) will arrest their development if insufficient food is available and enter into a stress-resistant larval stage known as the dauer stage (Golden and Riddle 1984). The nematode then goes through 3 more larval stages (L2, L3 and L4) each lasting around 8-12 hours before becoming an adult. Adults can live for several weeks. In the figure 0 hours represents fertilization and the blue numbers correspond to the duration of each developmental stage. The length of the nematode is noted in brackets. Note this life cycle is for 22^{0}C and the image is taken from wormatlas.org.



Figure 3: Colonisation of the *Caenorhabditis elegans* gut. The image shows *C. elegans* colonised by mCherry-tagged PaO1 strain of *Pseudomonas aeruginosa.* The image is taken from Figure 1B of (Rezzoagli et al. 2019).

1.5.2.1 Experimental pathogen: *Pseudomonas aeruginosa*

The gram-negative bacterium, *Pseudomonas aeruginosa,* is a common opportunistic pathogen of *C.elegans* as well as of plants and animals, including humans (Tan et al. 1999; Hirsch and Tam 2010). *P. aeruginosa* causes chronic infections in immunocompromised patients (Hirsch and Tam 2010), notably in patients with cystic fibrosis (Köhler et al. 2009) where frequent infections arise and lead to high levels of mortality (Smith et al. 2006).

Pseudomonas aeruginosa is a well-established model system for studying social traits [e.g. (Griffin et al. 2004; Buckling et al. 2007; Diggle et al. 2007)]. These traits include quorum sensing (QS) and siderophore (pyoverdin) cheats. Siderophores, such as pyoverdine, are compounds produced by microorganisms that are used to bind to and uptake iron (Buckling et al. 2007). The production of siderophores can benefit all bacteria in the local area and so is an example of a cooperative trait. Thus, cheats can emerge which benefit from the other local bacteria producing siderophores without producing them themselves (Griffin et al. 2004). Pyoverdin production has also been suggested to contribute to the virulence of *P. aeruginosa* (Meyer et al. 1996). Quorum sensing is a means of communication for bacteria which involves releasing small diffusible molecules into the local environment that can aid in the coordination of cooperative behaviours such as biofilm formation which can affect the virulence of the bacterium (Diggle et al. 2007). Both QS-regulated virulence factors and siderophores have been shown to be involved in *C.elegans* mortality (Tan et al. 1999; Zaborin et al. 2009; Kirienko et al. 2013; Rezzoagli et al. 2019), making them interesting focal traits for investigating within-and-between host dynamics.

P.aeruginosa has not been isolated from wild nematodes, but has been reported to coexist with *C.elegans* in mushroom farms (Grewal 1991). The majority of our ecological knowledge

of *C.elegans-P.aeruginosa* interactions comes from lab studies [e.g. (Diaz and Restif 2014; Rezzoagli et al. 2019)]. For example, the finding that *P.aeruginosa* is transmitted between hosts in the faeces of *C.elegans* came from a lab based study (Diaz and Restif 2014). In *C.elegans,* the virulence of *P. aeruginosa* depends upon the media (Tan et al. 1999). On standard NGM plates it nematode mortality occurs in several days, but on high osmolarity media host mortality occurs within a few hours of exposure (Tan et al. 1999). Nematodes are able to recover from infection if exposure to *P. aeruginosa* is brief (12 hours or fewer) (Marsh and May 2012). Defence against *P.aeruginosa* is dependent upon p38 mitogen-activated protein kinase (MAPK) cascade (Kim 2002), which has also been linked to other stress responses in *C.elegans* (Pike et al. 2019a).

1.6 Inbreeding avoidance

As part of my thesis, I conducted a project investigating inbreeding avoidance across the animal kingdom. I will introduce this chapter separately below:

1.6.1 Inbreeding

In this thesis, we use the term inbreeding broadly to describe mating with relatives which encompasses a range of definitions (Jacquard 1974; Keller and Waller 2002). There is often inconsistency in the definition of the term ‘inbreeding’ (Jacquard 1974; Templeton and Read 1994) which can lead to confusion in the literature (Templeton and Read 1994). Thus, using a broad definition can help to compare inbreeding across populations.

1.6.2 Inbreeding depression

Mating with relatives (inbreeding) is often deleterious for the fitness of organisms (Keller and Waller 2002; Charlesworth and Willis 2009). The reduced survival and fertility of inbred

429 offspring is known as inbreeding depression (Charlesworth and Charlesworth 1987;
430 Charlesworth and Willis 2009). These harmful effects of inbreeding were first considered by
431 Darwin (Darwin 1876) when experimenting with self-fertilization and outcrossing in plants.
432 Inbreeding depression is caused by an increase in offspring homozygosity (Charlesworth and
433 Charlesworth 1987). This can lower fitness via increasing homozygosity for partially
434 recessive deleterious mutations and by increasing homozygosity for alleles at loci with
435 heterozygote advantage (Charlesworth and Willis 2009). Inbreeding depression is a particular
436 concern for small population conservation, such as captive breeding of endangered species
437 (Hedrick and Kalinowski 2000). Inbreeding depression is measured in a variety of ways,
438 which are often difficult to compare across taxa (Keller and Waller 2002). The standard
439 measure of inbreeding within-populations is Wright's inbreeding coefficient (f) (Wright
440 1921). The value of f represents the probability that two alleles in an individual are identical
441 by descent (Wright 1921; Charlesworth and Willis 2009).
442
443 Inbreeding depression is an issue for both wild (Crnokrak and Roff 1999; Keller and Waller
444 2002) and captive populations (Hedrick and Kalinowski 2000), including laboratory species
445 such as the commonly used insects *Aedes aegypti* (Ross et al. 2019) and *Drosophila*
446 *melanogaster* (Briscoe et al. 1992). Studies have revealed a high degree of variation in the
447 levels of inbreeding depression across species (Keller and Waller 2002). In some species,
448 including collared flycatchers (*Ficedula albicollis*) (Kruuk et al. 2002) and grey wolves
449 (*Canis lupus*) (Liberg et al. 2005), the result of inbreeding can be severe (Keller and Waller
450 2002; Charlesworth and Willis 2009). Whereas in others species, for instance the ground tit
451 *Parus humilis* (Wang and Lu 2011), there are limited consequences or even benefits of
452 mating with relatives (Kokko and Ots 2006; Szulkin et al. 2013; Duthie and Reid 2016). In
453 these cases, indirect fitness benefits may outweigh any direct fitness costs associated with

454 inbreeding, thus increasing the inclusive fitness of the focal individual under some conditions
455 (Bengtsson 1978; Kokko and Ots 2006; Szulkin et al. 2013; Duthie and Reid 2016). It is
456 usually considered advantageous to avoid inbreeding (Szulkin et al. 2013) and it is widely
457 assumed that inbreeding depression will drive the evolution of inbreeding avoidance
458 strategies (Blouin and Blouin 1988; Pusey and Wolf 1996). However, inbreeding avoidance
459 is not ubiquitous across the animal kingdom (de Boer et al. 2021).
460

461 **1.6.3 Inbreeding avoidance**

462 There are a diverse array of mechanisms which animals utilise to avoid mating with relatives
463 (Pusey and Wolf 1996). This can involve basing mate selection choices on distinguishing
464 between related and non-related mates (Blouin and Blouin 1988). Additionally, inbreeding
465 avoidance can occur via mechanisms that reduce the rate of encountering related individuals,
466 for example sex-biased dispersal (Geffen et al. 2011; Li and Kokko 2019). Furthermore,
467 mechanisms may be employed after breeding in some form of post mating avoidance (Pizzari
468 et al. 2004; Kokko and Ots 2006). I will outline some of the most commonly used
469 mechanisms in the animal kingdom below [for a full review see (Blouin and Blouin 1988;
470 Pusey and Wolf 1996)]:
471

472 **1.6.3.1 Active mate choice**

473 Some species are able to recognise and avoid choosing related mates (Blouin and Blouin
474 1988; Pusey and Wolf 1996). There are a range of genetic cues which may be used to
475 distinguish between related and unrelated individuals. For instance, evidence for active
476 inbreeding avoidance has been found in populations of long-tailed tits (*Aegithalos caudatus*)
477 (Leedale et al. 2018, 2020) which are cooperative breeders and live in groups where relatives
478 are frequently encountered. It has been hypothesised long-tailed tits are able recognise kin

through vocal cues (Sharp et al. 2005; Leedale et al. 2020), and thus can actively choose non-related mates on the basis of recognition of vocal cues (Leedale et al. 2020).

1.6.3.2 Sex-biased dispersal

When one sex is more likely to disperse from their natal area prior to breeding, it is known as sex biased dispersal (Pusey 1987). This phenomenon is widespread across mammals and birds (Pusey 1987; Trochet et al. 2016). For birds, dispersal is more likely to be female biased whereas for mammals dispersal is more likely to be male biased (Pusey 1987; Li and Kokko 2019). For fish and reptiles, both male and female biased dispersal is common (Trochet et al. 2016; Li and Kokko 2019). These dispersal patterns reduce the encounter rate of relatives (Geffen et al. 2011) and thus can reduce the risk of related individuals mating (Bengtsson 1978; Pusey 1987; Li and Kokko 2019). For example, within populations of the communally breeding Bechstein's bat (*Myotis bechsteinii*) there are extreme rates of male-biased dispersal which is strongly driven by inbreeding avoidance (Kerth et al. 2002). Although, the question of whether sex-biased dispersal has evolved as an inbreeding avoidance mechanism is debated, as inbreeding avoidance is not the only driving force of dispersal [see (Trochet et al. 2016) and references therein].

1.6.3.3 Post-mating avoidance

Some mechanisms of inbreeding avoidance occur after mating, usually termed post-mating inbreeding avoidance. Post-mating avoidance may occur in species which lack precopulatory avoidance mechanisms, or when forced matings occur (Kokko and Ots 2006). Post-mating inbreeding avoidance has been documented across the animal kingdom, in species including guppies (*Poecilia reticulata*) (Fitzpatrick and Evans 2014), field crickets (*Gryllus bimaculatus*) (Bretman et al. 2004) and red junglefowl (*Gallus gallus*) (Pizzari et al. 2004). In

504 the latter example, female red junglefowl retained less sperm from related males than
505 unrelated males (Pizzari et al. 2004). However, mechanisms of post-mating avoidance may
506 be variable between sexes, as the cost of inbreeding incurred by females is higher than for
507 males (Pizzari et al. 2004). In red junglefowl, males counteract female avoidance by
508 inseminating related females with more sperm than unrelated females (Pizzari et al. 2004).
509 Post-mating inbreeding avoidance can also occur when females mate with multiple males
510 with different levels of relatedness. This can arise when females are promiscuous with high
511 levels of extra-pair copulations, as is the case for red-backed fairy-wrens (*Malurus*
512 *melanocephalus*) where females paired with related mates were more likely to sire young
513 from extrapair males (Varian-Ramos and Webster 2012).
514

515 **1.7 Thesis Outline**

516 In this thesis I will develop on the above themes. Specifically:
517

518 In **Chapter 2,** I examine the role of host nutritional quality and quantity on pathogen
519 virulence in a meta-analysis across the tree of life. I did not find broad support across species
520 for a one-way effect of nutrition on pathogen virulence. Thus, I go on to conduct a qualitative
521 analysis where I discuss the hypothesis that there is a balance between the effect of host
522 nutrition on the immune system and on pathogen resources, with the pivot point of the
523 balance differing for vertebrate and invertebrate hosts. Overall, the results suggest that
524 variation in nutrition, caused by natural or anthropogenic factors, can have diverse effects on
525 infectious disease outcomes across species.
526

527 In **Chapter 3**, I tackle the impact of biotic interactions on parasite virulence. Specifically, I
528 investigate the role of coinfection on virulence across a range of experimental systems using

529 a meta-analytic approach. I find that overall, coinfections are more virulent than single
530 infections, but the magnitude of this effect varies depending on the divergence between
531 coinfecting parasites and their mechanism of interaction, as well as mode of transmission. I
532 highlight the need for further experimental work investigating how mechanisms of
533 interactions affect disease dynamics.
534
535 In **Chapter 4**, I extend the work of Chapter 3 by taking an experimental approach to consider
536 the role of pathogen traits on within-and between-host dynamics in single infections and
537 coinfections. Specifically, testing producer and non-producer strains of *P. aeruginosa* for
538 siderophore production and quorum sensing within the nematode host *C. elegans*. I find that
539 producer strains are consistently better at colonising and transmitting between hosts than non-
540 producers in both single infections and coinfections. These results highlight the importance of
541 understanding parasite dynamics across multiple levels to control and treat infectious
542 diseases.
543
544 In **Chapter 5**, on a slightly different theme, I investigate why inbreeding avoidance is not
545 ubiquitous across the animal kingdom. Taking a phylogenetic approach, I test whether the
546 frequency with which related males and females encounter each other can explain the
547 variation in inbreeding avoidance we observe. I find that inbreeding avoidance is only found
548 in species where there is a risk of inbreeding depression and related sexual partners regularly
549 encounter each other.
550
551 Finally, in **Chapter 6**, I summarise the main findings from Chapters 2-5 and discuss the
552 wider implications of investigating the role of abiotic and biotic factors in infection
553 outcomes. I conclude by suggesting some future research directions.

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Chapter 2

On the diverse and opposing effects of nutrition on pathogen virulence

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On the diverse and opposing effects of nutrition on pathogen virulence

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Abstract

Climate change and anthropogenic activity are currently driving large changes in nutritional availability across ecosystems, with consequences for infectious disease. An increase in host nutrition could lead to more resources for hosts to expend on the immune system or for pathogens to exploit. In this paper, we conducted a meta-analysis of studies on host-pathogen systems across the tree of life, to examine the impact of host nutritional quality and quantity on pathogen virulence. We did not find broad support across studies for a one-way effect of nutrient availability on pathogen virulence. We thus discuss a hypothesis that there is a balance between the effect of host nutrition on the immune system and on pathogen resources, with the pivot point of the balance differing for vertebrate and invertebrate hosts. Our results suggest that variation in nutrition, caused by natural or anthropogenic factors, can have diverse effects on infectious disease outcomes across species.

1. Introduction

Dramatic shifts in global and local environmental conditions are currently being driven by climate change with major impacts on food security and nutritional availability in ecosystems (1,2). Additionally, humans have a direct influence on the nutrition of animal and plant populations through, for example, supplementary feeding and fertilizer use (3–6). In the context of infectious disease, nutrition is hypothesized to be an important factor in infection outcomes by affecting pathogen virulence, which we define broadly here as disease severity caused by an infecting organism [e.g. (7,8)], as well as in other aspects of host-pathogen interactions [e.g., host reproduction (9)]. The need to understand the general effects of nutrition on infection outcomes is a pressing issue for species conservation (11), as well as for the study of human disease (12), in a changing world.

Host nutrition can affect infection outcomes by driving separate changes in the host immune system [e.g.(7,13,14)] and pathogen resource availability [e.g. (15),(16)(Fig 1)]. From the host's perspective, it is energetically demanding to maintain an active immune response (17,18). A decline in their nutritional status could leave hosts less able to suppress infection and, all else being equal, increase the harm caused by the pathogen (7,19). For example, in domestic canaries (*Serinus canaria*) fed diets supplemented with protein and vitamins, *Plasmodium relictum* exhibited lower parasitemia and virulence (i.e., measured as reduced weight and haematocrit loss) relative to non-supplemented hosts (7). Alternatively, from the pathogen's perspective, changes in host nutritional quantity (food amount) and quality (specific nutrient content) might affect the availability and type of resources available for pathogen growth during infection (8,20,21). In this case, the assumption is that faster-growing pathogens, or a higher pathogen fitness/load, would necessarily lead to greater virulence. This assumption, however, is not always valid, such as when hosts are tolerant to the infecting pathogen [e.g. (22) and references therein].

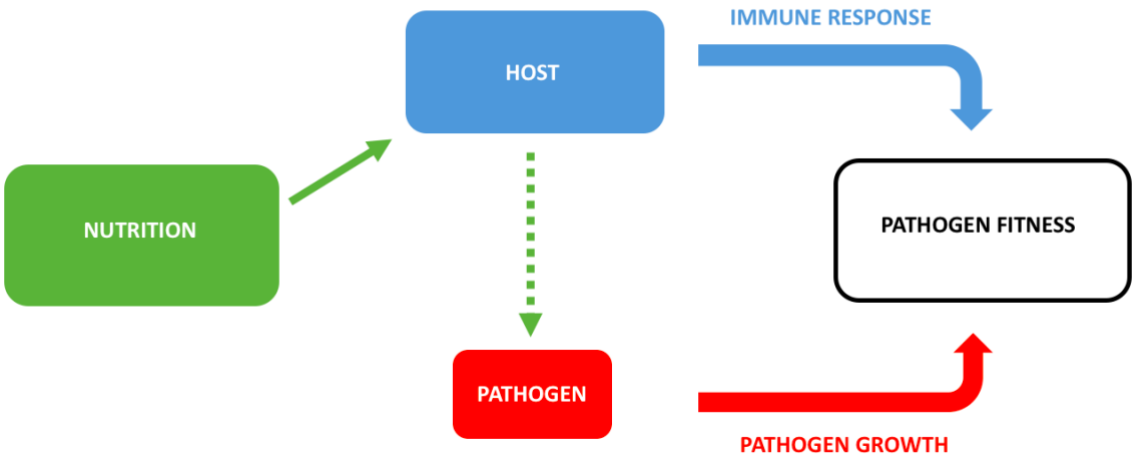


Figure 1: A schematic showing how host nutrition could affect pathogen fitness and disease severity. Nutrition (green) provides energy for the host to use for the immune response (blue) and/or directly provides resources for pathogen growth (red).

In some systems, there may be other factors which make the impact of nutrition on infection outcomes more complex. For immunopathological diseases, a major contributor of damage to the host is not pathogen load, but the host's own immune system (23). One hypothesis suggests that providing improved nutrition, and so more energy for the immune system, is thus likely to worsen, not improve, the outcome of these diseases (24). For pathogens that infect cells of the immune system, such as HIV and its relative simian immunity deficiency virus (SIV), a strong immune response (to HIV, SIV, or other pathogens) can result in more targets for infection rather than controlling the infection (25–27). Beyond the immune system, variation in nutrition can also have an impact on host behaviours. An abundance of food can decrease foraging time, and thus increase the time available for behavioural defences against pathogens, such as grooming (4,28). Grooming is an effective way of removing ectoparasites (28), such as ticks, which can act as vectors for pathogens such as *Rickettsia sp.*

95

96 The energetic demands of the host's immune system might determine the dominant
97 mechanism underlying the link between nutrition and virulence. Given immune systems are
98 costly to maintain (18,29), reductions in host nutrition could affect the ability of the host to
99 launch an immune response. Despite the large number of similarities between vertebrate and
100 invertebrate immune systems (30), there are also some major differences, such as a lack of
101 acquired immunity in invertebrates (31). The impact of nutritional changes on the immune
102 system might vary between vertebrates and invertebrates, with the former requiring more
103 resources for an effective immune response. We thus predict that host nutrition will drive
104 changes in virulence via impacts on immune systems in vertebrate host species, but via
105 resource availability to pathogens in invertebrates.
106

107 We conducted a meta-analysis to get an overall picture of how nutrition might affect
108 pathogen virulence. We separately considered nutritional quantity (for non-human animals)
109 and quality (for non-human animals, plants and humans). The quantity analysis assessed the
110 changes in pathogen virulence driven by an increase or decrease in host food availability,
111 whereas the quality analysis considered more specific changes to diet that are targeted at the
112 particular host or pathogen, such as variation in vitamins [e.g. (14)] or minerals [e.g. (15)].
113 To understand the mechanism by which nutrition affects pathogen virulence, we also tested
114 for differences between systems with invertebrate or vertebrate hosts. Subsequently, we
115 discuss the studies in more detail to consider the balance between the effects of host nutrition
116 on the strength of the immune system and the resources available to the pathogen. The
117 immune system is highly complex, but here we simply refer to its 'strength' or ability to act
118 to reduce pathogen fitness, enabling us to address this broad evolutionary question across
119 host-pathogen relationships. Overall, the meta-analysis does not reveal a consistent effect

across systems. Bringing these studies together highlights how natural or anthropogenic changes in nutrient availability might cause diverse effects on virulence across species.

2. Meta-analyses

a) Literature search

To gather quantitative evidence of the impact of host nutrition on pathogen virulence, we conducted a literature search on Web of Science extracting data from papers up to and including 4th February 2019 (See Supplementary Figure 1 for PRISMA flow chart (34)). We then performed backwards and forwards citation searches on the papers of interest. We contacted authors to obtain unpublished data from relevant studies. We used the following selection criterion for inclusion in the meta-analysis:

1) The study involved an alteration of host nutrient quality or quantity.
2) The study measured pathogen virulence, in terms of host survival or mortality, at different nutrition levels.
3) The study was experimental rather than observational. This restriction allowed us to collate controlled tests of the effects of nutrition.

In total, we found 35 papers that fulfilled these criteria. These papers incorporated a variety of hosts: 32 non-human animal species, 2 plant species, 1 unicellular ciliate, 3 studies on human patients, enabling us to obtain 52 effect sizes. Of these effect sizes, 33 (from 23 studies) altered the quality of the host diet, and 19 (from 12 studies) altered the quantity of the host diet.

b) Statistical analysis

We used Hedges' g (35) as our measure of effect size, as it is not biased by small sample sizes (36). We first determined Cohen's d, using the formula below and later we converted this into Hedges' g.

$$Cohen's\ d = \frac{M_1 - M_2}{S_{pooled}} \qquad where\ S_{pooled} = \sqrt{\frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{(n_1 - 1) + (n_2 - 1)}}$$

Here, M_1 and M_2 refer to the mean virulence resulting from each of the treatment groups, and $s_1$and s_2 refer to the standard deviations of the mean virulence for the treatment groups. In all comparisons, M_1 refers to the higher nutrition treatment group, and M_2 to the lower nutrition treatment group. In our analysis we have three different 'treatment types': High v Control, Control v Low and High v Low. For the treatments of *High v Control*, M_1 refers to the high treatment group and M_2 to the control group; for *Control v Low*, M_1 refers to the control group and M_2 to the low treatment group; and for *High v Low* (studies which compared high and low treatments without a control), M_1 refers to the high treatment group and M_2 to the low treatment group.

We converted $Cohen's\ d$ into Hedges' g using the following formula:

$$Hedge's\ g = J\ x\ Cohen's\ d$$

where

$$J = 1 - \frac{3}{4(n_1 + n_2 - 2) - 1}$$

Next, we calculated the variance of Hedges' g (V_g) using:

168 $$V_g = J^2 \; x \; Cohen's \; d$$

169

170 Values of Hedges' g equal to zero represent no difference in pathogen virulence between
171 treatments. Positive Hedges' g values represent cases where the pathogen virulence is greater
172 in the higher nutrition treatment group than the lower nutrition group. Conversely, negative
173 Hedges' g values represent cases where the pathogen virulence is greater in the lower
174 nutrition treatment group than the higher nutrition group.

175

176 We then split the data into studies according to whether they investigated nutritional quality
177 or quantity. We used R version 3.4.3 (37) and R studio version 1.0.153 (38) for statistical
178 analyses of the quality and quantity data. We constructed multi-level meta-analytic models
179 using the package "metafor" (39). This multi-level model was required as there were
180 multiple effects sizes from some studies (40)**.** We included two random effects on the model
181 intercept: 1) a study-level random effect (the level of the model at which variation between
182 studies is distributed), and 2) an observation-level random effect (the level of the model at
183 which variance within studies is distributed)(40). We used a Restricted Maximum Likelihood
184 estimation method (REML) for estimating parameters. The overall relationship between host
185 nutrition (quality or quantity) and pathogen virulence could be affected by a number of other
186 variables such as: treatment type (High v Control, Control v Low, High v Low), host type
187 (broad taxonomic groupings and invertebrate vs vertebrate hosts) and pathogen type (broad
188 taxonomic groupings). Thus, we performed univariate moderator analyses to investigate the
189 effects of these additional variables (40)**.** To determine the significance of these moderator
190 variables we carried out Analysis of variance (ANOVA). Any groups with sample sizes
191 lower than three were excluded from the moderator analyses. To test for publication bias, we
192 tested for funnel plot asymmetry and then carried out a trim and fill analysis using the

193 package “metafor” (39). We also used the packages “ggplot2” (41) and “cowplot” (42) for
194 producing figures and “dplyr” (43) for data manipulation.
195
196 In our data, we identified four extreme values that could be considered as outliers, two in the
197 quality data (see supplementary table 1) and two in the quantity data (see supplementary table
198 2). To investigate whether or not these outliers were driving our findings, we re-ran all the
199 analyses (outlined above) with the outliers removed.
200
201 ***c) Results of the meta-analyses***
202 Changes to host nutritional quality or quantity did not have a significant effect on pathogen
203 virulence (Fig 2A**,** Quality: overall effect size = 1.086 (Standard error = 1.349), t(32)= 0.805,
204 p = 0.427, Fig 2B Quantity: = -1.353 (Standard error = 1.470), t(18)= -0.929, p = 0.370). For
205 the quality moderator analyses, we found no significant moderating effects of treatment type
206 (Fig 3A, High v control/ control v low/ high v low: F(2, 30) = 0.429, p = 0.616), host type
207 (Fig S2A, 4A: broad taxonomic grouping: fish/ insect/ mammal /plant: F (3, 25) = 0.317, p =
208 0.813 or invertebrate vs vertebrate: F (2,30) = 0.044, p = 0.957) or pathogen type (Fig S3A
209 bacteria/ fungi/ nematode/ virus: F (3, 29) = 0.782, p = 0.514). Similarly, for the quantity
210 moderator analyses we found no significant moderating effects of treatment type (Fig 3B
211 High v control/ control v low/ high v low: F(2, 16) = 0.877, p = 0.435), host type (Fig S2B,
212 4B broad taxonomic grouping: Insect / mammal / mollusc: F (1, 14) = 2.060, p = 0.164 or
213 invertebrate vs vertebrate: F (2,16) = 2.646, p = 0.102) or pathogen type (Fig S3B broad
214 taxonomic grouping: bacteria / fungi / protist / trematode /trypanosome/virus: F (2,11) =
215 0.233, p = 0.796).
216

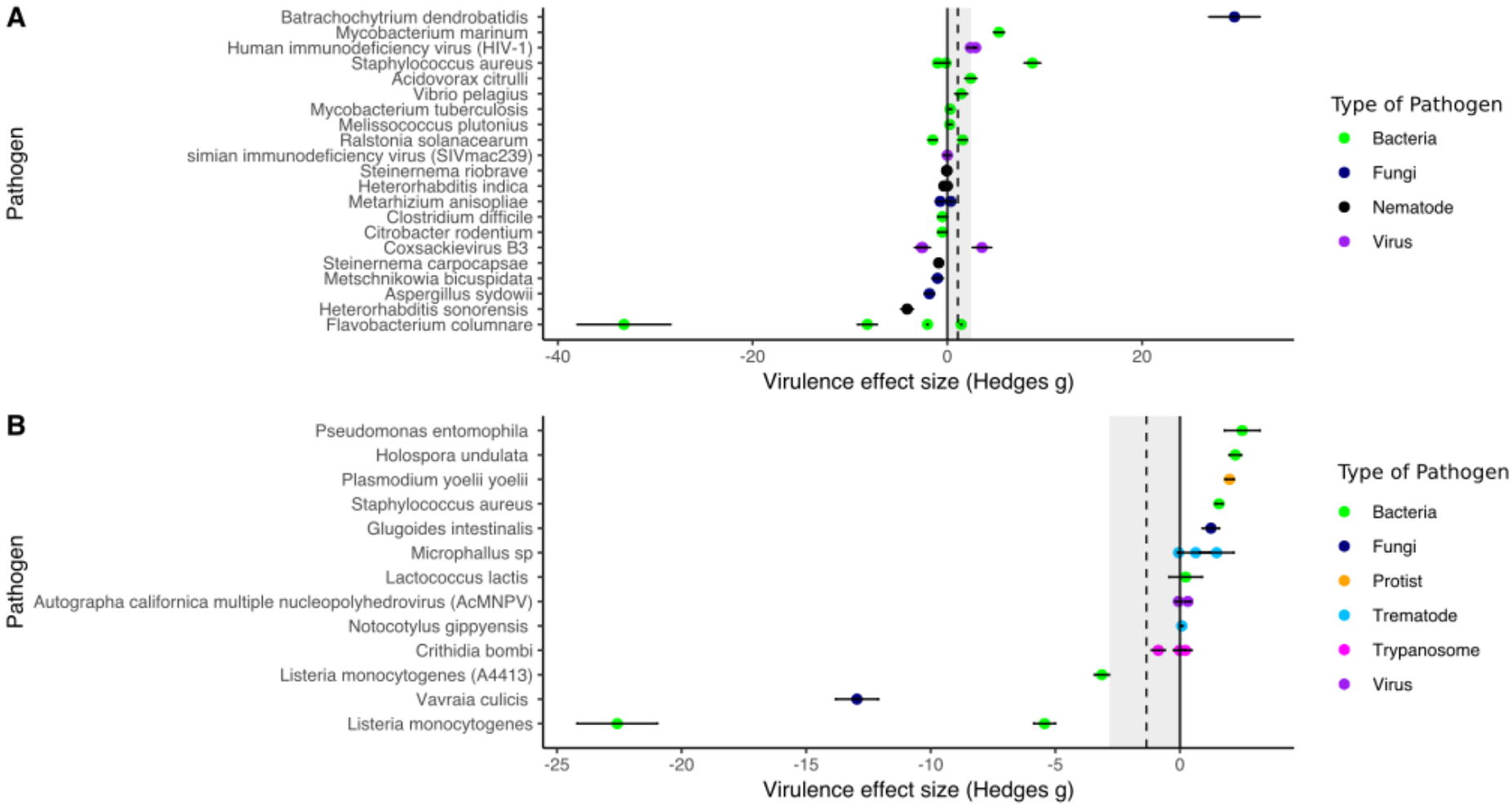


Figure 2. Forest plots of nutrient quality (a) and quantity (b) and their effects on virulence effect size (Hedges's g ± 1 s.e.) for each pathogen species. Dotted line shows the overall effect size with the grey area demarcating the margin of error. Values of Hedges' g equal to zero represent no difference in pathogen virulence between treatments. Positive Hedges' g values represent cases where the pathogen virulence is greater in the higher nutrition treatment group than the lower nutrition group. Negative Hedges' g values represent cases where the pathogen virulence is greater in the lower nutrition treatment group than the higher nutrition group.

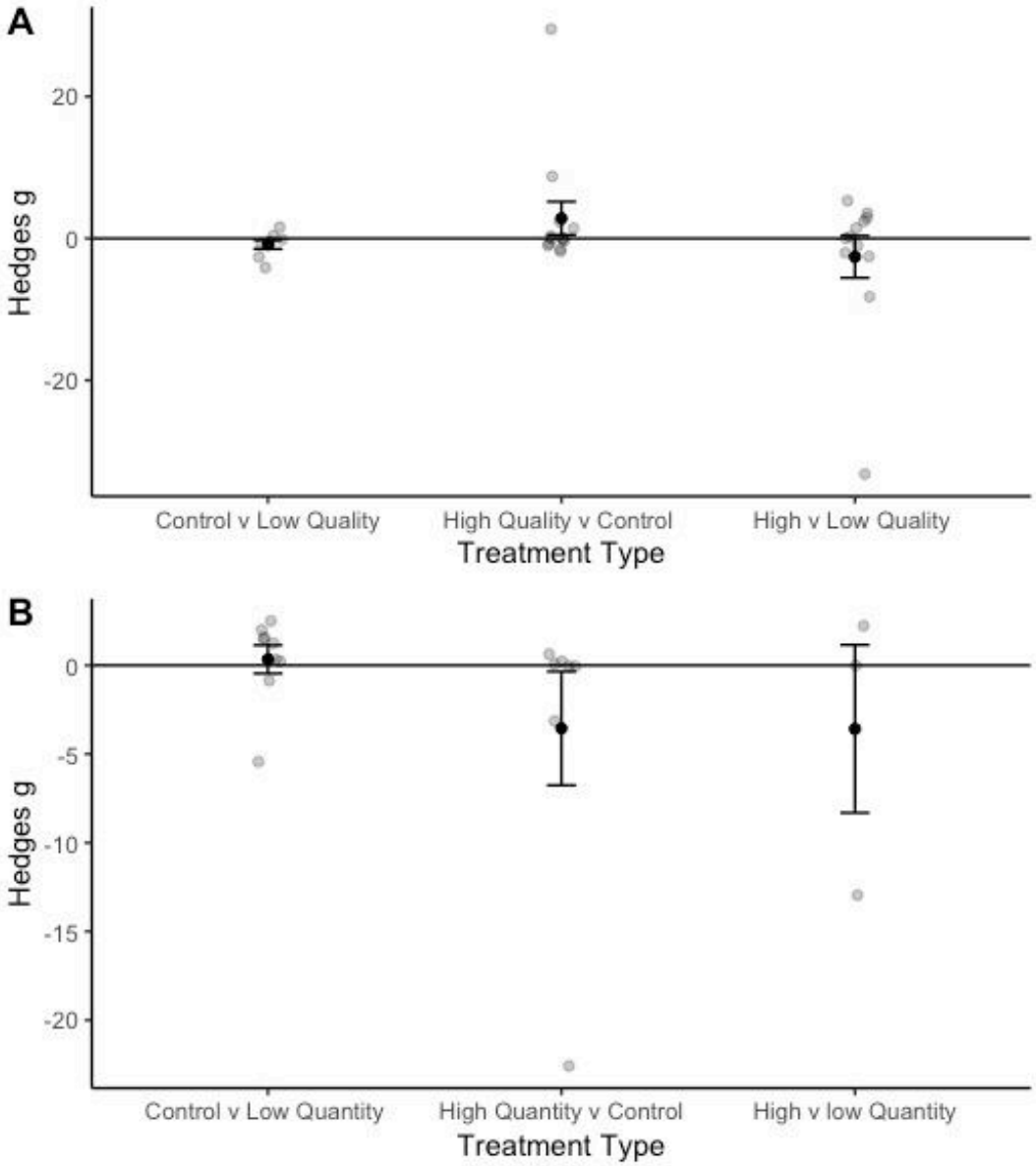


Figure 3. The effect of nutrient quality (a) and quantity (b) on virulence effect size (Hedges' g). Black points represent mean values ± 1 s.e., and raw data are displayed as jittered points.

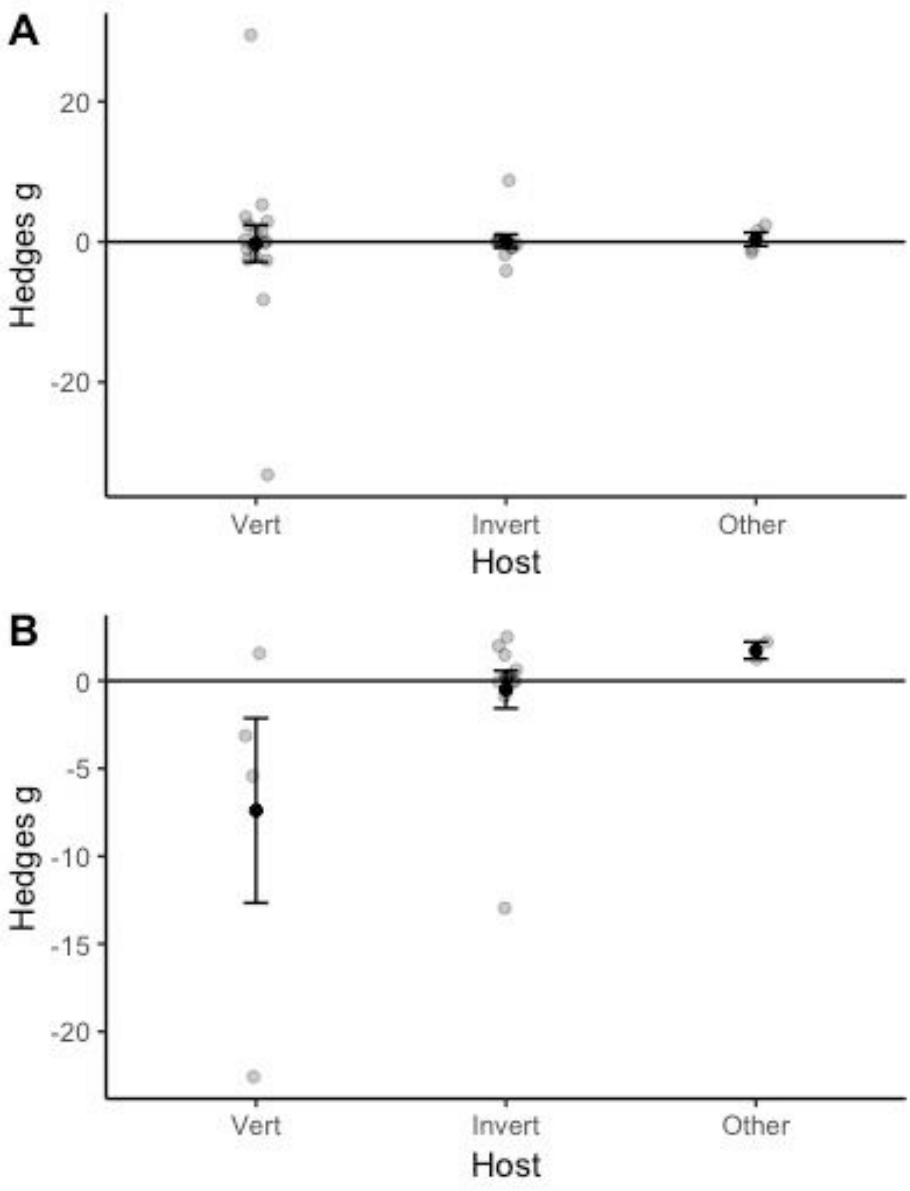


Figure 4. Virulence effect size (Hedges' *g*) for vertebrate and invertebrate hosts as well as 'other' hosts (including plants, zooplankton and ciliates) for treatments manipulating nutrient quality (*a*) and quantity (*b*). Black points represent mean values ± 1 s.e. The raw data are displayed as jittered points.

Removing the outliers from our analyses caused changes in individual effect sizes, but crucially did not affect the findings of our study. There was still no overall effect of nutrition on pathogen virulence (see supplementary table 3), and the moderator analyses did not produce a significant result (see supplementary table 4).

For the quality analysis, there was no evidence for a significant publication bias (See S4A: Regression test for funnel plot asymmetry: $z = -1.4822$, $p = 0.1383$), but bias was found for the quantity meta-analysis (See S4B: Regression test for funnel plot asymmetry: $z = -4.8127$, $p < .0001$). However, when we removed the outliers from our analyses, we found no publication bias for either quality (See S4C: Regression test for funnel plot asymmetry: $z = -0.2857$, $p = 0.7751$) or quantity (See S4D: Regression test for funnel plot asymmetry: $z = 0.2275$, $p = 0.8200$).

3. Discussion: Nutrition and the balance between host immunity and pathogen resources

We hypothesise that the lack of a significant effect of host nutrition on pathogen virulence is because of the different, and potentially opposing, mechanisms at play. Nutrition can alter both pathogen resources and/or the strength of the host immune response, with these acting to increase and decrease virulence, respectively. We considered the effect of nutrition on pathogen fitness, measured as either as pathogen growth rate or load in a given host environment. Better nutrition might boost the immune system and so reduce pathogen fitness in that environment [e.g. (19)]. Conversely, better nutrition might provide more resources to the pathogen, and thus directly increase pathogen fitness [e.g. (8)]. Specifically, we hypothesise that the outcome of changes of host nutrition on pathogen virulence depends on the balance between these two effects, with differences in the biology of the host and

242 pathogen systems determining where the equilibrium point lies. For example, energetic
243 requirements of the immune system are likely less for invertebrates than for vertebrates, and
244 consequently nutrition might have less of an effect on invertebrate immunity (31,44).
245
246 For those studies used in the meta-analysis that measured immunity and/or pathogen fitness,
247 as well as virulence (rather than just virulence), we broadly determined whether nutrition
248 affected the strength of the immune response based on what was measured. Where possible,
249 we considered the effect of changing host nutrition on both host immunity and pathogen
250 fitness (Table 1). For studies where changes in host immunity were not measured, we only
251 considered the effect of nutrition on pathogen fitness (Table 2). We discuss these studies in
252 the context of vertebrate and invertebrate hosts.
253
254 Six of the studies considered the host's immune system (Table 1), five of which were in
255 mammalian hosts (14,45–48) and one in birds (7). In all but one of the studies, a decrease in
256 pathogen fitness was observed with an increase in the immune response (7,14), or vice versa
257 (45). The exception was a study of Rhesus macaques (*Macaca mulatta*) infected with simian
258 immunodeficiency virus (SIV) (46). The supplemented macaques experienced both an
259 increase in the number of immune cells and in virus pathogenicity (46), probably because the
260 supplements increased the susceptibility of target (memory CD4+ T) cells to SIV infection
261 (46). Thus, in this SIV study the nutritional supplements increased resource availability to
262 the virus, outweighing any increase in immune response. The outcome was greater disease
263 severity.
264
265 Within these six studies, dietary supplements did not uniformly enhance the immune
266 response (Table 1). No effect on immunity was observed when HIV infected individuals were

Table 1: Table of pathogen fitness considering changes to host immune system			
	Increase in Pathogen Fitness	Decrease in Pathogen Fitness	No Change
Increase in Host Immunity		Coxsackie virus in selenium deficient mice (*Mus sp*) (14) Avian malaria (*Plasmodium relictum*) in canaries (*Serinus canari*) with supplemented diet (7)	
Decrease in Host Immunity	Copper deficiency in mice (*Mus sp*) infected with Coxsackie virus B3 (45)	Simian immunodeficiency virus in Rhesus macaques (*Macaca mulatta*), decline in non-supplemented hosts (46)	
No change	*Staphylococcus aureus* infection in mice (*Mus sp*) with increased Manganese (47)		Micronutrient supplementation on humans with HIV (48)

Table 2: Pathogen Fitness without considering changes to Host immune system			
	Increase in Pathogen Fitness	**Decrease in Pathogen Fitness**	**No change**
Increase in Host/Pathogen resources	*Metschnikowia bicuspidate* growth in *Daphnia dentifera* with higher quality diet (50) AcMNPV virus production with increased food availability in the Cabbage looper (*Trichoplusia ni*)(15) *Daphnia magna* infected with *Pasteuria ramosa* with high quantities of food (49)	*Clostridium difficile* growth in mice (*Mus sp*) with high protein diet (53) *Ralstonia solanacearum* in Tomato plants (*Lycopersicon esculentum*) with higher calcium (33) HIV patients supplemented with antioxidant micronutrients (54)	
Decrease in Host/Pathogen resources		Starved *Bombus impatiens* infected with *Crithidia* trypanosome parasite (51) Starved *Daphnia magna* infected with *Glugoides intestinalis* (20)	Diet quality on bacterial infection of *Drosophila melanogaster* (52)

given micro supplements (48), or when mice infected with the bacteria *Staphylococcus aureus* received manganese supplements (47). In this latter case, the bacteria in the supplemented hosts had a higher fitness than the un-supplemented hosts because the bacteria was able to utilize manganese to protect itself against reactive oxygen species and neutrophil killing (47). Finally, in one case, a deficient diet enhanced the immune response: mice given a selenium deficient diet showed an increase in host immune markers driving down the fitness of the infecting coxsackie virus (14).

Nine of the studies considered how changes to host resources affected pathogen fitness but did not consider the host's immune system. In these studies, we noticed opposite pattern for vertebrate and invertebrate hosts (see Table 2). For invertebrates, (*Daphnia magna* (20,49), *Daphnia dentifera* (50), the cabbage looper (*Trichoplusia ni*) (15), and the bumble bee (*Bombus impatiens*) (51)) improved host nutrition was associated with an increase in pathogen fitness (15,21,49) and reduced host nutrition was associated with a decrease in pathogen fitness (20,51). No effect on virulence was seen in *Drosophila melanogaster* when its nutrition was decreased (52). These studies support the hypothesis that an increase in host nutrition can increase host resources available for exploitation by pathogens. In contrast, for mouse (53) and human (54) hosts, an improvement in host nutrition was associated with a decrease in pathogen fitness. This relationship might be indicative of an enhanced host immune response or other defence mechanisms in vertebrates. Finally, for the only plant species considered here, the tomato plant (33), improved host nutrition was associated with decreased pathogen fitness.

4. Conclusions

Our study revealed that host nutrition did not have a significant effect on pathogen virulence across systems. We hypothesise that the lack of a general pattern emerges from a balance of both host and pathogen responses to host nutritional status, specifically between impacts on the host immune system [e.g.(7,14)] and resources available to the pathogen (8,20,21).

We predicted there would be differences in the response of the immune system to changes in nutrition for vertebrates compared to invertebrates. Specifically, there may be differences between animal groups in energy requirements for the immune system, with the less 'complex' immune system of invertebrates (31,44) requiring fewer resources. In our meta-analysis, we found no significant difference in the effect of dietary quantity or quality on pathogen virulence for vertebrate compared to invertebrate hosts. However, when we looked at a subset of the studies more closely, we found different patterns in vertebrate and invertebrate host organisms (see Table 1). For the most part, we find that invertebrate hosts display what we would expect under a *pathogen-centric* mechanism – a decrease in fitness with reduced resources for the host, and therefore also the pathogen. Conversely, vertebrate hosts in these studies display what we would expect under a *host-centric* mechanism – a decrease in pathogen fitness with increased resources, due to enhanced host immune responses. Similarly, Cressler et al (16) found increased host nutrition was more likely to lead to an increase in pathogen load for invertebrate, rather than vertebrate, hosts. Further studies are needed to increase the number and types of hosts and pathogens for which we have data, to fully test this hypothesis.

The patterns we discuss here are broad, and on closer inspection there are exceptions. However, importantly, the exceptions we observe make sense. For example, in SIV where the memory CD4+ T are the targets of infection, we observed a decrease in pathogen growth

316 with a decrease in host immunity. Our interpretation of these data may be limited by the
317 fewer studies measuring immune responses (rather than, for example, survival or mortality)
318 in invertebrates in the context of host nutrition. This limitation may highlight a system bias
319 for choosing vertebrate hosts when considering immune function in the context of infection
320 and nutrition. More work is required to study the role of host nutrition on virulence in
321 invertebrate hosts. Moreover, careful consideration of how host nutrition is altered in specific
322 systems is needed. For example, very specific changes to nutritional quality, such as the
323 addition or removal of certain micronutrients, for example selenium (14,55), can have
324 targeted effects on pathogen virulence, which may also help to explain the lack of a
325 directional pattern when examining host nutritional quality and pathogen virulence.
326
327 Globally, organisms are facing large changes in nutritional availability (1). Theoretical work
328 has shown these changes can have far reaching ecological (58) and epidemiological (59)
329 consequences. For example, the constant supply of resources provided by anthropogenic
330 activities can decrease the propensity of species to migrate and consequently affect infection
331 dynamics within those populations (58). In addition, resource subsidies can affect the
332 evolution of pathogen virulence and transmission(59). It is important for us appreciate how
333 widespread the knock-on effects of nutrition induced changes on host-pathogen dynamics and
334 evolution are likely to be. Understanding the long-term consequences of host nutrition on
335 infectious disease will require moving beyond single-generation experiments (as included in
336 this meta-analysis) towards long-term data collection from natural populations [e.g. ((60))],
337 evolution experiments [e.g. (57)] or, theoretical approaches considering within-host and
338 between-host evolution [e.g.(59,63)].
339

Understanding the impact of host nutrition on infection outcomes is critical as climate change and human activities are dramatically altering food availability (1–6). Altering host diet has a complex relationship with infection, either inhibiting or enhancing disease severity and pathogen proliferation across animal and plant species. We think this complexity precludes a general, one-way pattern from emerging across all studies to date. Nonetheless, a greater understanding of the general mechanisms underlying links between nutrition and virulence are needed, with data on both host immunity and pathogen fitness merged. We hypothesize a balance of two factors, the strength of the host immune response and the resources available to the pathogen, shapes the link, but more studies are needed to pin this down. Such efforts will allow us to fully grasp the extent to which the nutritional changes we observe in nature will impact host-pathogen interactions, now and over time.

Ethics

The study does not include any work on humans or animal research.

Data accessibility

Data is available at Dryad (DOI: https://doi.org/10.5061/dryad.2qt04nm). The code used in this study is archived at Figshare Digital Repository: https://doi.org/10.6084/m9.figshare.8160239.v1

Authors Contributions

V.L.P, K.A.L and K.C.K designed the study. V.L.P collected the data and carried out the statistical analysis. All authors contributed to writing the manuscript.

Competing Interests

We have no competing interests.

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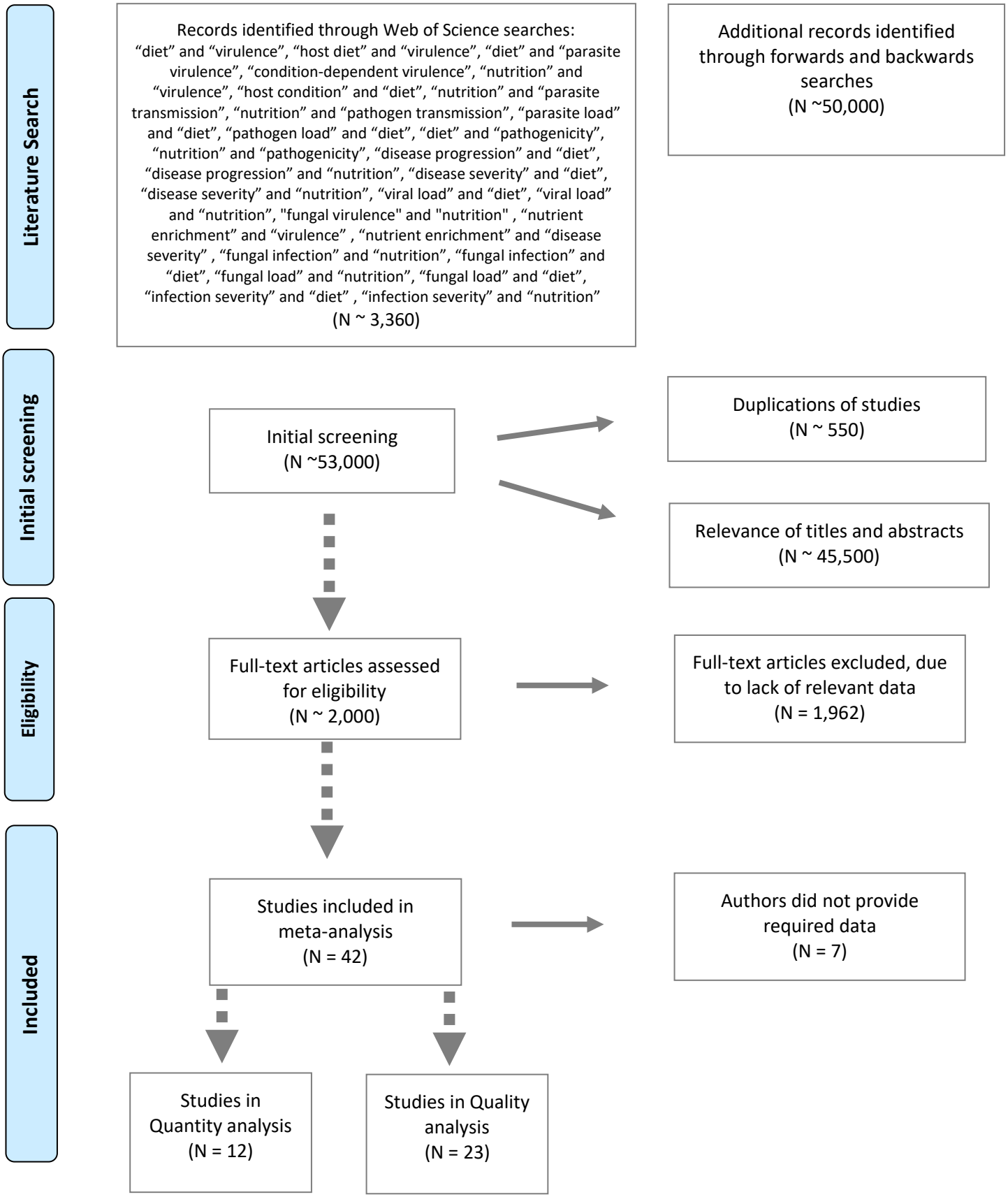
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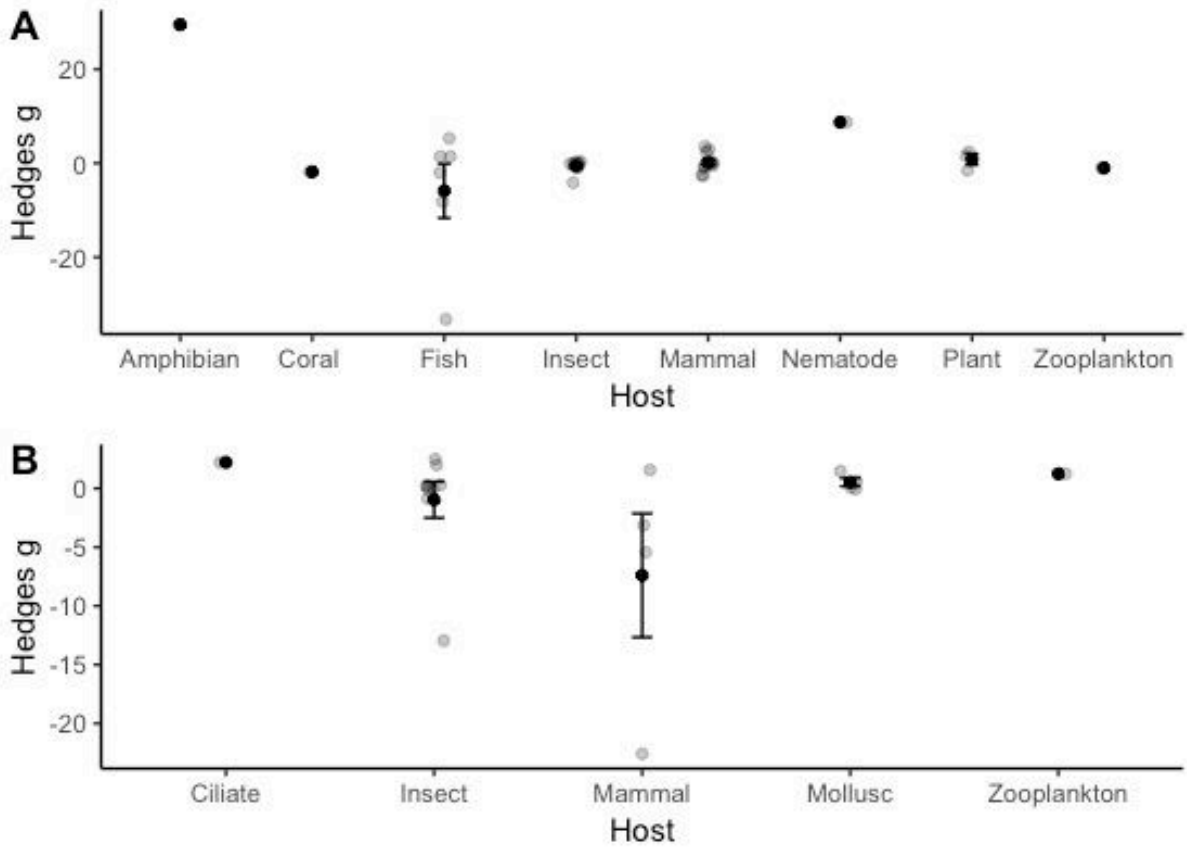
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Supplementary Material

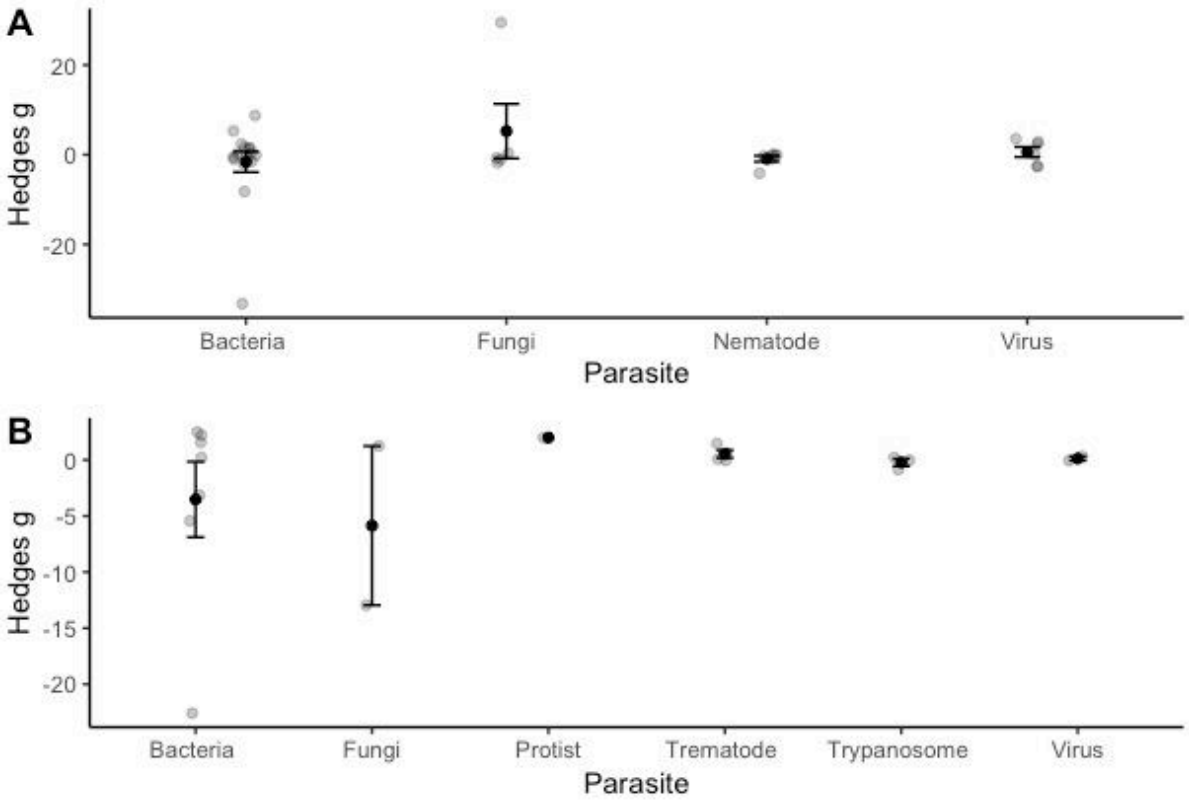
Chapter 2



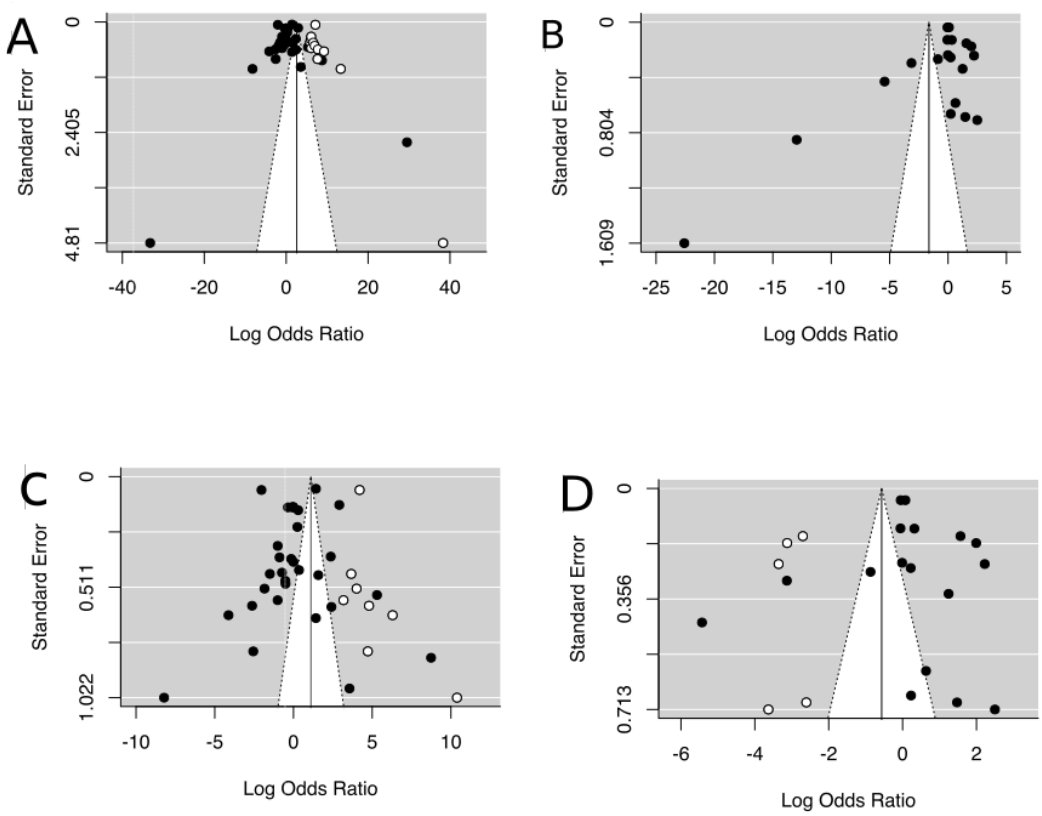
Supplementary Figure 1: PRISMA flow chart detailing data collection.



Supplementary Figure 2: Virulence effect size (Hedge's g) for different host types for treatments affecting nutrient quality (A) and quantity (B). Black points represent mean values +/- 1 S.E. Raw data are shown as jittered points.



Supplementary Figure 3: Virulence effect size for different pathogen types for treatments affecting nutrient quality (A) and quantity (B). Black points represent mean values +/- 1 S.E. Raw data are shown as jittered points.



Supplementary Figure 4: Trim and fill plots for the nutrient quality (A, C) and quantity (B, D) meta-analyses, with outliers included (A and B) and removed (C and D).

Supplementary Table 1: A table showing the studies used in the nutrient quality meta-analysis.' * ' denote outliers.

Host (vertebrate (V)/invertebrate (In)/plant (P))	Pathogen	Treatment type	Specific change to nutrition	Change in virulence (+ increase, - decrease, = no change)	Hedge's g	Refs
Caenorhabditis elegans (In)	Staphylococcus aureus (E)	High v Control	Glucose	+	8.739	(64)
Mus sp (V)	Coxsackievirus B3	High v Low	Vitamin E	+	-2.553	(32)
Mus sp (V)	Staphylococcus aureus (E)	High v Control	Manganese	+	3.559	(47)
		Control v Low	Manganese	+	-1.002	
Plutella xylostella (In)	Metarhizium anisopliae (E)	High v Control	Carbon and Nitrogen	-	-0.732	(65)
		Control v Low	Carbon and Nitrogen	-	0.352	
Mus sp (V)	Coxsackievirus B3	Control v Low	Selenium	-	-2.630	(14)
Mus sp (V)	Citrobacter rodentium (E)	Control v Low	Selenium	-	-0.518	(55)
Tenebrio molitor (In)	Heterorhabditis indica (E)	High v Control	Lipid	+	-0.352	(66)
			Protein	-	0	
Tenebrio molitor (In)	Steinernema riobrave (E)	High v Control	Lipid	=	-0.075	(66)

			Protein	=	-4.128	
Manduca sexta (In)	Heterorhabditis sonorensis (E)	Control v Low	Lower quality feed	=	-0.886	(67)
Manduca sexta (In)	Steinernema carpocapsae (E)	Control v Low	Lower quality feed	+	1.424	(67)
Ictalurus punctatus (V)	Flavobacterium columnare (E)	High v Low	Lower $CuSO_4$ supplementation	-	-2.033	(68)
			Higher $CuSO_4$ supplementation	=	0.247	
Apis mellifera (In)	Melissococcus plutonius (E)	High v Low	Potassium	=	0.247	(69)
Daphnia dentifera (In)	Metschnikowia bicuspidate (E)	High v Low	Environmental nutrients	=	-1.007	(50)
Morone saxatilis (V)	Mycobacterium marinum (Int)	High v Low	Lower quality feed	+	5.306	(70)
Macaca mulatta (V)	Simian Immunodeficiency Virus (SIV) (Int)	High v Low	Micronutrient supplements	+	-0.002	(46)
Homo sapien (V)	Mycobacterium tuberculosis (Int)	High v Control	Vitamin A and Zinc	=	0.298	(71)

Homo sapien (V)	Human immunodeficiency virus HIV-1 (Int)	High v Control	Vitamin E and C	-	2.368	(54)
Mus sp (V)	Clostridium difficile (E)	Control v Low	Protein	=	-0.505	(53)
Cucumis melo (P)	Acidovorax citrulli (E)	High v Low	Potassium	-	2.409	(72)
Lycopersicon esculentum (P)	Ralstonia solanacearum (E)	Control v Low	Calcium	-	1.579	(33)
		High v control	Calcium	-	-1.500	
Homo sapien (V)	Human immunodeficiency virus HIV-1 (Int)	High v Low	Micronutrient supplements	-	2.910	(48)
Dicentrarchus labrax (V)	Vibrio pelagius	High v Control	Shrimp protein hydrolysate	-	1.424	(73)
Oncorhynchus mykiss (V)	Flavobacterium columnare	High v Low	Growth medium (Shieh broth)	+	-33.212 *	(74)
Danio rerio (V)	Flavobacterium columnare	High v Low	Growth medium (Shieh broth)	+	-8.219	(74)
Gorgonia ventalina (In)	Aspergillus sydowii	High v Control	Fertilizer	+	-1.839	(75)
Pseudacris regilla (V)	Batrachochytrium dendrobatidis	High v Control	Nitrogen and Phosphorus	-	29.498 *	(76)

Supplementary table 2: A table showing all of the studies used in the nutrient quantity meta-analysis. ' * ' denote outliers

Host (Vertebrate (V)/ Invertebrate (In))	Pathogen	Treatment type	Change in virulence (+ increase, - decrease, = no change)	Hedge's g	Study
Mus sp (V)	*Staphylococcus aureus* (E)	Control v Low	+	1.568	(77)
Paramecium caudatum (ciliate)	*Holospora undulata* (Int)	High v Low	+	2.224	(78)
Aedes aegypti (In)	*Vavraia culicis*	High v Low	+	-12.959*	(8)
Potamopyrgus antipodarum (In)	*Microphallus sp* (E)	High v Control	=	-0.051	(79)
Potamopyrgus antipodarum (In)	*Notocotylus gippyensis* (E)	High v Control	+	0.075	(79)
Bombus impatiens (In)	*Crithidia sp* (E)	High v Low	-	-0.013	(51)
Trichoplusia ni (In)	Autographa californica multiple nucleopolyhedrovirus AcMNPV (Int)	Control v Low	+	0.319	(15)
		High v Control	+	-0.058	
Bombus terrestris (In)	*Crithidia bombi*	High v Control	-	0.219	(80)
		Control v Low	-	-0.868	
Mus sp (V)	*Listeria monocytogenes* (Int)	High v Control	-	-3.133	(81)
Cavia porcellus (V)	*Listeria monocytogenes* (Int)	High v Control	-	-22.580*	(81)
		Control v Low	-	-5.431	
Anopheles stephensi (In)	*Plasmodium yoelii yoelii* (Int)	Control v Low	+	1.992	(82)
Potamopyrgus antipodarum (In)	*Microphallus sp* (E)	Control v Low	+	1.474	(83)

		High v Control	+	0.634	
Daphnia magna (In)	*Glugoides intestinalis* (Int)	Control v Low	-	1.245	(20)
Drosophila melanogaster (In)	*Lactococcus lactis* (E)	Control v Low	=	0.229	(52)
	Pseudomonas entomophila (E)	Control v Low	-	2.500	

Supplementary Table 3: Outlier analysis for overall effect size.

Meta-analysis	Outliers	Overall Effect size (Hedge's g)	Standard error	K	T-value	P-value
Quality	Included	1.086	1.349	33	0.805	0.427
	Removed	0.187	0.575	31	0.325	0.748
Quantity	Included	-1.353	1.470	19	-0.929	0.307
	Removed	0.406	0.546	17	0.743	0.468

Supplementary Table 4: Outlier analysis for moderator variables.

Meta-analysis	Moderator	Outliers	F – Value	P – Value
Quality	Treatment type	Included	0.429	0.616
		Removed	0.632	0.544
	Vertebrate vs Invertebrate	Included	0.044	0.957
		Removed	0.053	0.948
	Host Type (Broad Grouping)	Included	0.317	0.813
		Removed	0.276	0.842
	Pathogen Type (Broad Grouping)	Included	0.782	0.514
		Removed	0.456	0.716
Quantity	Treatment type	Included	2.060	0.164
		Removed	0.249	0.783
	Vertebrate vs Invertebrate	Included	2.646	0.102
		Removed	0.199	0.174
	Host Type (Broad Grouping)	Included	0.233	0.796
		Removed	0.918	0.426
	Pathogen Type (Broad Grouping)	Included	0.233	0.796
		Removed	0.042	0.959

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Chapter 3

A meta-analysis of experimental work on the virulence of coinfections

A meta-analysis of experimental work on the virulence of coinfections

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Abstract

Coinfections of multiple parasites are widespread in nature, driving the need to understand the mechanisms that shape the harm they can cause hosts (virulence). Within-host competition between parasites and conflicting transmission strategies are hypothesized to increase the virulence of coinfections compared to single infections. However, the generality of coinfections impacting virulence remains unclear. Here we provide broad evidence that coinfection can increase the overall virulence of infection using a meta-analysis of 96 effect sizes and 84 different parasite genotypes and species from experimental studies. The magnitude of these effects was dependent on the phylogenetic divergence between coinfecting parasites, their mechanism of interaction, and whether they had different transmission modes. Where data was available, we investigated the relationship between the percentage change in the number of parasites between coinfection and single infection and parasite virulence. We did not observe a strong correlation between change in parasite abundance and virulence. These findings demonstrate the importance of studying multiple parasites within the ecological context of a host. Improving our understanding of how coinfections affect overall virulence will help us to predict novel coinfection outcomes, control clinically important coinfections and develop effective treatment strategies.

1. Introduction

A parasite rarely infects a host alone (Petney and Andrews 1998; Cox 2001; Pedersen and Fenton 2007; Rynkiewicz et al. 2015; Betts et al. 2016b; Hoarau et al. 2020). Most hosts are simultaneously infected by several parasite genotypes or species in coinfections (Cox 2001). Coinfections are highly prevalent in humans (Petney and Andrews 1998; Griffiths et al. 2011, 2015), with considerable overlap in particular between coinfections of human influenza virus (HIV) with tuberculosis (Lawn et al. 2006; Harries et al. 2010; Kwan and Ernst 2011) and malaria (Abu-Raddad et al. 2006). Similarly, a diverse parasite community can infect host species across the tree of life, from ticks (Moutailler et al. 2016) and voles (Telfer et al. 2010) to bacterial hosts (Betts et al. 2016a; Díaz-Muñoz 2017)). A similar diversity is found amongst the coinfecting parasites. Coinfections can occur between phylogenetically divergent species, such as helminth and malaria infections (Brooker et al. 2007; Mazigo et al. 2010; Knowles 2011), to more similar parasites (Lord et al. 1999), such as a mixture of Influenza A genotypes (Sharp et al. 1997). Despite the ecological (Tompkins et al. 2011), biomedical (Griffiths et al. 2011; Vaumourin et al. 2015) and agricultural ((Figueroa et al. 2017; Liu et al. 2020)) importance of coinfections, studies have largely focused on single infections (Pedersen and Fenton 2007; Telfer et al. 2010; Rigaud et al. 2010; Eswarappa et al. 2012).

Coinfecting parasites interact within hosts (Read and Taylor 2001; Pedersen and Fenton 2007; Buckling and Brockhurst 2008; Rynkiewicz et al. 2015), and the mechanisms of interaction can drive changes in host harm, or virulence (May and Nowak 1995; Frank 1996; Alizon et al. 2013). The direction in which virulence changes in coinfections, compared to single infections is not necessarily additive (Rigaud et al. 2010; Bordes and Morand 2011; Eswarappa et al. 2012; Alizon et al. 2013; Vaumourin et al. 2015). Coinfecting parasites may

have overlapping ecological niches encouraging within-host resource competition (Read and Taylor 2001; Mideo 2009), such as when the protozoan *Babesia microti* and the bacterium *Bartonells spp* compete for red blood cells within their field vole (*Miavtus agrestis*) host (Telfer et al. 2010). Theory suggests that competition can favour rapid within-host growth to achieve greater relative success and increase the overall virulence of the coinfection (Nowak and May 1994; Frank 1996; Ferguson and Read 2002). Virulence may similarly be increased by parasite facilitation in coinfections. For example, within mice *Plasmodium*-induced lysis of red blood cells causes iron to be released which in turn increases *Salmonella* growth, leading to an increased overall virulence (Kaye and Hook 1963; Kaye et al. 1965; Uneke 2008; Eswarappa et al. 2012).

Transmission mode may also impact how a parasite interacts with others in coinfections (Ewald 1983; Thomas et al. 2002; Walther and Ewald 2004; Haine et al. 2005; Graham et al. 2007; Jones et al. 2010; Rigaud et al. 2010; Antonovics et al. 2017). High virulence can negatively impact parasite transmission, particularly if a parasite requires a live host for transmission, leading to a trade-off between virulence and transmission (Anderson and May 1982; Koella and Antia 1995; Galvani 2003; Walther and Ewald 2004; Buckling and Brockhurst 2008). The trade-off varies depending upon transmission mode (Galvani 2003; Rigaud et al. 2010; Antonovics et al. 2017). Theory predicts that directly-transmitted parasites should be less virulent (Boldin and Kisdi 2012; Antonovics et al. 2017) as harming hosts may shorten the life span of their current host before they can transmit (Boldin and Kisdi 2012; Antonovics et al. 2017). Alternatively, it may be beneficial for indirectly-transmitted parasites to persist independently from the host for longer periods, and thus their virulence is predicted to be higher (Ewald 1983; Walther and Ewald 2004; Antonovics et al. 2017). Further complexity can arise in this trade-off between transmission and virulence

when parasites coinfect a host, particularly if the coinfecting parasites differ in their mode of transmission (Thomas et al. 2002; Haine et al. 2005; Jones et al. 2010; Rigaud et al. 2010). Different transmission modes may lead to conflict between coinfecting parasites with impacts on virulence. For example, the trematode *Microphallus papillorobustus* requires the host to be eaten by a bird (their definitive host) which is deleterious for the coinfecting nematode parasite, *Gammarinema gammari*, within an amphipod host (*Gammarus insensibilis*) leading to direct conflict between coinfecting parasite species (Thomas et al. 2002). Here the trematode virulence is reduced by the presence of the coinfecting nematode, as the nematode is able to sabotage the trematodes transmission strategy (Thomas et al. 2002).

Understanding the generality of the impact of coinfections on virulence is crucial for predicting coinfection outcomes and the development of effective treatments. Identifying broad patterns may be useful for predicting infection outcomes when novel coinfections arise (Hassell et al. 2021), such as between COVID-19 and Influenza A virus (Bai et al. 2021). Furthermore, the presence of coinfecting parasites can complicate treatments (Pedersen and Antonovics 2013; Ezenwa and Jolles 2015; Vaumourin et al. 2015; Clerc et al. 2019; Ezenwa et al. 2021) and few guidelines existing for the treatment of specific coinfections within humans (Griffiths et al. 2015). Thus, uncovering broad patterns may be applicable for treatment approaches potentially based on parasite-parasite interactions (Griffiths et al. 2015) across clinical and ecological settings.

We addressed the generality of the impact of coinfection on virulence with a formal meta-analysis. We searched the published literature for all available data sources focusing on experimental studies of human and wildlife diseases, to rigorously assess the relationship between coinfection and virulence. We use the term ‘parasite’ throughout to refer to micro

and macro parasites, as it is frequently used in ecological and evolutionary studies (Tompkins et al. 2011; Eswarappa et al. 2012; Alizon et al. 2013). We estimated the effect of coinfection on parasite virulence using the Hedges' *g* effect size (thus positive values indicate that coinfection increases virulence). We explored whether the phylogenetic divergence between coinfecting parasites, the mode of interaction between coinfecting parasites, and whether different transmission modes and infection sites influenced the virulence effect size. Finally, for the studies where data was available, we investigated the relationship between the percentage change in the number of parasites between coinfection and single infection and parasite virulence.

2. Methods

Literature search

We conducted a literature search on papers published up to and including 19th May 2021 on Web of Science (see Figure S1 for PRISMA flow diagram (Moher et al. 2009; Page et al. 2021)). We recorded the number of studies published per year with the term 'coinfection' as we wanted to track any trends in publishing over time and we also collected this data for the term 'virulence' for comparison. We performed forwards and backwards citation searches on the papers of interest. Papers were included in this study if they fit the following criteria:

1) The studies measured parasite virulence (harm caused to the host) *in-vivo*
2) The study collected a measure of parasite virulence from both parasite genotypes or species in single infection (virulence of parasite A and virulence of parasite B)
3) The study collected an overall measure of parasite virulence in coinfection (virulence of coinfection)

Criteria 2 and 3 enabled us to directly compare the virulence of each parasite in single infection and coinfection, and led to the exclusion of many studies including any studies on

151 humans. This was particularly important as the direct comparison allowed us to determine
152 that changes in virulence were the result of the addition of a coinfecting parasite, rather than
153 any other confounding variables. Furthermore, to meet these strict criteria, all the studies in
154 our analysis were experimental. We define virulence as the degree of harm caused to the host
155 from infection, and it was based on one of the following measures of mortality: days to death;
156 LT50; percentage survival; and scale of infection severity. We recorded the type of measure
157 of virulence to compare differences between the types of measurement. We also collected
158 data on parasite taxonomic grouping, transmission mode, site of infection and host type. We
159 noted the type of interaction between parasites (according to author definitions) which
160 included competition, cooperation, facilitation, coexistence (the presence of two parasites
161 which do not affect each other's fitness) and those where any interaction mechanism was
162 unknown. Finally, where available (in 16 studies), we recorded data on parasite load as a
163 proxy for fitness in single infections and coinfections. In total, we found 37 papers (see Table
164 1) that fulfilled all our inclusion criteria which incorporated a range of hosts (birds, fish,
165 insects, nematodes and zooplankton) and parasites (bacteria, fungi, viruses, nematodes,
166 microsporidia, protozoa and trematodes).
167
168 **Statistical analysis**
169 *Calculating effect sizes*
170 From the 37 studies in the analysis, we were able to calculate 96 effect sizes. From each
171 study, we calculated a minimum of two effect sizes using the following formula:
172
173 $$Effect\ size = Virulence\ of\ single\ infection\ (A\ or\ B) - Virulence\ of\ Coinfection\ (A + B)$$
174

For all the measures of virulence included in the analysis a higher numerical value (e.g. percentage survival) signified a lower virulence. Thus, we subtracted the mean virulence of the coinfection from the mean virulence of a single infection so that a positive effect size would represent a higher overall virulence of coinfections. For eight studies (Ben-Ami et al. 2008; Balmer et al. 2009; Bashey et al. 2011; Evison et al. 2015; Heinig and Thomas 2015; Roon et al. 2015; Ngba Essebe et al. 2017; Tang et al. 2020), there were more than two parasites included in the study, and we calculated more than two effect sizes (Table 1). For example, in (Evison et al. 2015), the virulence levels of three strains of fungal parasites (*Ascosphaera apis*) were measured in single infection and coinfection resulting in six effect sizes.

Our effect sizes were measured as Cohens' d which we then converted to values of Hedges' g (Hedges 1981) which is not biased by smaller effect sizes (Borenstein 2009) using the formula below:

$$Cohen's\ d = \frac{M_1 - M_2}{S_{pooled}}$$

Where:

$$S_{pooled} = \sqrt{\frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{(n_1 - 1) + (n_2 - 1)}}$$

We converted Cohen's d into Hedge's g using the following formula:

$$Hedge's\ g = J\ x\ Cohen's\ d$$

Where:

198 $$J = 1 - \frac{3}{4(n_1 + n_2 - 2) - 1}$$

199

200 Next, we calculated the variance of Hedge's g (V_g) using:

201

202 $$V_g = J^2 \; x \; Cohen's \; d$$

203

204 Positive values of Hedge's g represent infections where parasite virulence is higher in
205 coinfection than single infection. Negative values of Hedges' g represent infections where
206 parasite virulence in single infection is higher than in coinfection. Values of 0 represent equal
207 virulence in both infection scenarios.

208

209 *Analysing the data*

210 We first tested whether there was an overall effect of coinfection on parasite virulence by
211 fitting a meta-analytic mixed-effects model to the Hedges' g values (and the variance
212 associated with each value of Hedges' g) with 'dose' and 'measure of virulence' as random
213 effects on the intercept (Model 1). We included 'measure of virulence' as a random effect to
214 control for any variation caused by the different virulence measures across the studies.
215 Previous work has shown that small changes to dose have negligible effects on virulence
216 compared to relative parasite differences (Glynn and Bradley 1995; Glynn et al. 1995; Timms
217 et al. 2001). Nevertheless, we included 'dose' as a random effect. In some studies, the total
218 dose in the coinfection was kept the same as the total dose of each of the single infections,
219 whereas in others, the total doses were kept constant effectively doubling the overall dose in
220 coinfection. We did not use a multi-level approach to modelling the data because no specific
221 coinfection was repeated across the studies, and therefore there were no repeat measures.

222

On top of investigating the overall effect of coinfection on parasite virulence, we also carried out univariate moderator analyses using mixed-effects models (Assink and Wibbelink 2016)**.** These mixed-effects models included the following moderator variables as fixed effects: *i)* the mechanism of interaction (according to the authors definition) between coinfecting parasites (Model 2); *ii)* divergence between coinfecting parasites (Model 3); *iii)* type of host (vertebrate, invertebrate or protozoa) (Model 4); *iv)* type of parasite (micro or macro parasite) (Model 5); *v)* if the transmission method was the same or different between the two parasites (Model 6); *vi)* the transmission mechanism of the parasites (direct or indirect) (Model 7); *vii)* site of infection (same or different for the coinfecting parasites) (Model 8); and *viii)* if parasites were obligate killers (yes or no) (Model 9). Additionally, we tested for two interactions: *i)* for an interaction between divergence of coinfecting parasites and mechanism of interaction (Model 12); and *ii)* for an interaction between site of infection and mechanism of interaction (Model 13). We used an Analysis of Variance (ANOVA) to test whether the moderator variables significantly affected the effect sizes.

We also investigated the relationship between the percentage change in the number of parasites infecting the host and parasite virulence. We fit a meta-analytic mixed-effects model with 'percentage change in parasite abundance' as a fixed effect and 'dose' and 'measure of virulence' as random effects on the intercept (Model 10). We used the number of parasites in single infection and coinfection to calculate the percentage change in parasite abundance using the following formula:

$$\%\ change\ in\ parasite\ abundance = \frac{No.\ of\ parasites\ in\ coinfection - No.\ of\ parasites\ in\ single\ infection}{No.\ of\ parasites\ in\ single\ infection} \times 100$$

Data on the number of parasites in single infections and coinfections were available for 16 studies, allowing us to calculate 34 effect sizes for percentage change in parasite abundance.

To test for publication bias, we constructed funnel plots and performed a fail-safe *n* analysis. This analysis calculated the number of additional studies needed to reduce the significance level of the weighted average effect size (Rosenberg 2005). We did not find evidence of publication bias (see Figure S2) and found that one extreme value was driving the asymmetry of the funnel plots (see Figure S2A). Our Rosenberg's fail-safe *n* analysis showed an additional 16627 studies would be required to reduce the significance level of the meta-analysis.

All of the statistical analysis and data presentation was carried out in R version 3.6.2 (R Core Team 2019) and RStudio (RStudio Team 2021) using the packages 'metafor' (Viechtbauer 2010), 'dplyr' (Wickham et al. 2017), 'ggplot2' (Wickham 2009), 'multcomp' (Hothorn et al. 2008), 'cowplot' (Wilke 2020), 'meta' (Balduzzi et al. 2019), and 'stringr' (Wickham 2019) (all of our code is available as a supplementary file). Results were considered to be statistically significant when $p < 0.05$.

3. Results

Coinfections are more virulent than single infections

We found that coinfections are more virulent than single infections (Model 1: Overall effect size = 1.19, SE = 0.54, t = 2.21, p = 0.03, Figure 1, Table 2). Most virulence effect sizes were clustered around zero although there were several more extreme values, with most of these being positive (Figure 1). There was variation in the magnitude and direction of the difference between the pairs of effect sizes from each study (Figure 1), with half of the

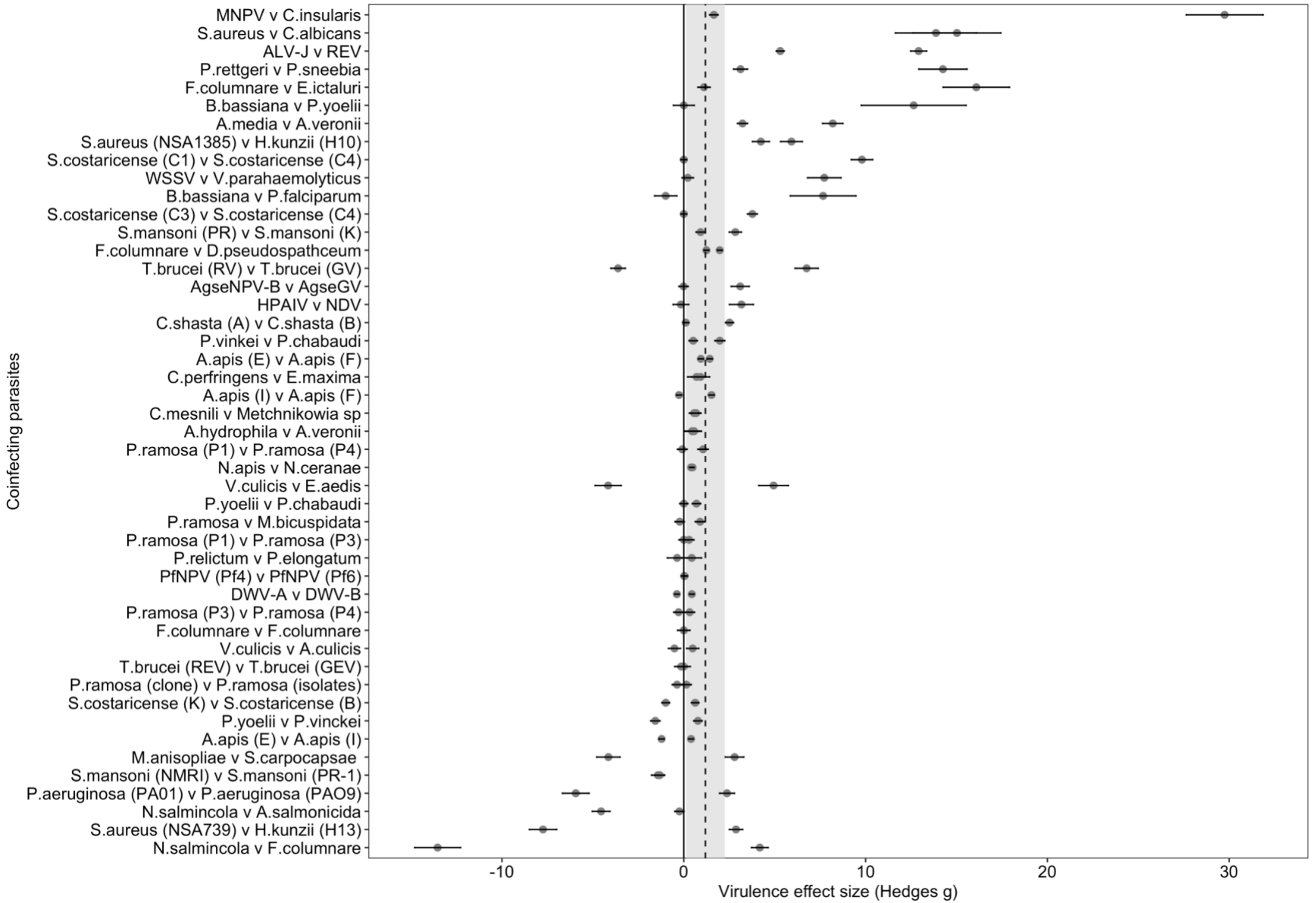


Figure 1: A forest plot of virulence effect sizes (Hedges' g) across coinfecting parasite species. The overall effect size, represented by the dotted line, is significantly different from zero. The grey area surrounding the dotted line represents the 95% confidence interval. Points represent means +/- one standard error. Positive effect sizes represent coinfections that are more virulent than single infections, negative effect sizes represent single infections that are more virulent than single infections and values of zero represent equal virulence of single and coinfections. There are two effect sizes per line as there are separate effect sizes for each parasite within a coinfection.

studies having effect sizes in the same direction (i.e., both effect sizes positive or both negative) and half having effect sizes in opposing directions. We also found the number of studies investigating coinfections has been increasing over time, particularly in recent years (Figure S5, (Griffiths et al. 2011)).

Virulence of coinfection increases as divergence between coinfecting parasites increases

Virulence of coinfections increased as the divergence between the coinfecting parasites increased (Model 3: $F_{3,90} = 76.85$, $p < 0.0001$, Figure 2, Table 2). Coinfecting parasites from different kingdoms were most virulent (mean = 1.64, SE = 0.43) followed by species (mean = 1.34, SE = 0.43) and strains (mean = 0.83, SE = 0.43). Coinfecting genotypes (mean = 0.59, SE = 0.43) were the least virulent, but this group also had the smallest sample size (n = 8). All groups were significantly different from each other (Figure 2, Table 2), but neither strain nor genotype significantly differed from zero. We also found that the likelihood of a mechanism of interaction between parasites was affected by the divergence between coinfecting parasites, as we found a significant interaction between the divergence between coinfecting parasites infection and the mechanism of interaction between parasites (Model 12: Overall effect = $F_{12,81} = 59.77$, $p < 0.0001$, Figure 3A, Table 2).

How and where parasites interact affects their virulence in coinfection

The virulence of coinfecting parasites differed depending on how the parasites interacted within the host and was a significant moderator variable (Model 2: $F_{4,89} = 72.79$, $p < 0.0001$, Table 2, Figure 3A&C). For parasites competing within the host, there was no difference in virulence between single infections and coinfections, with an effect size that was not significantly different from zero (mean = 0.52, SE = 0.59). Parasites that cooperated during

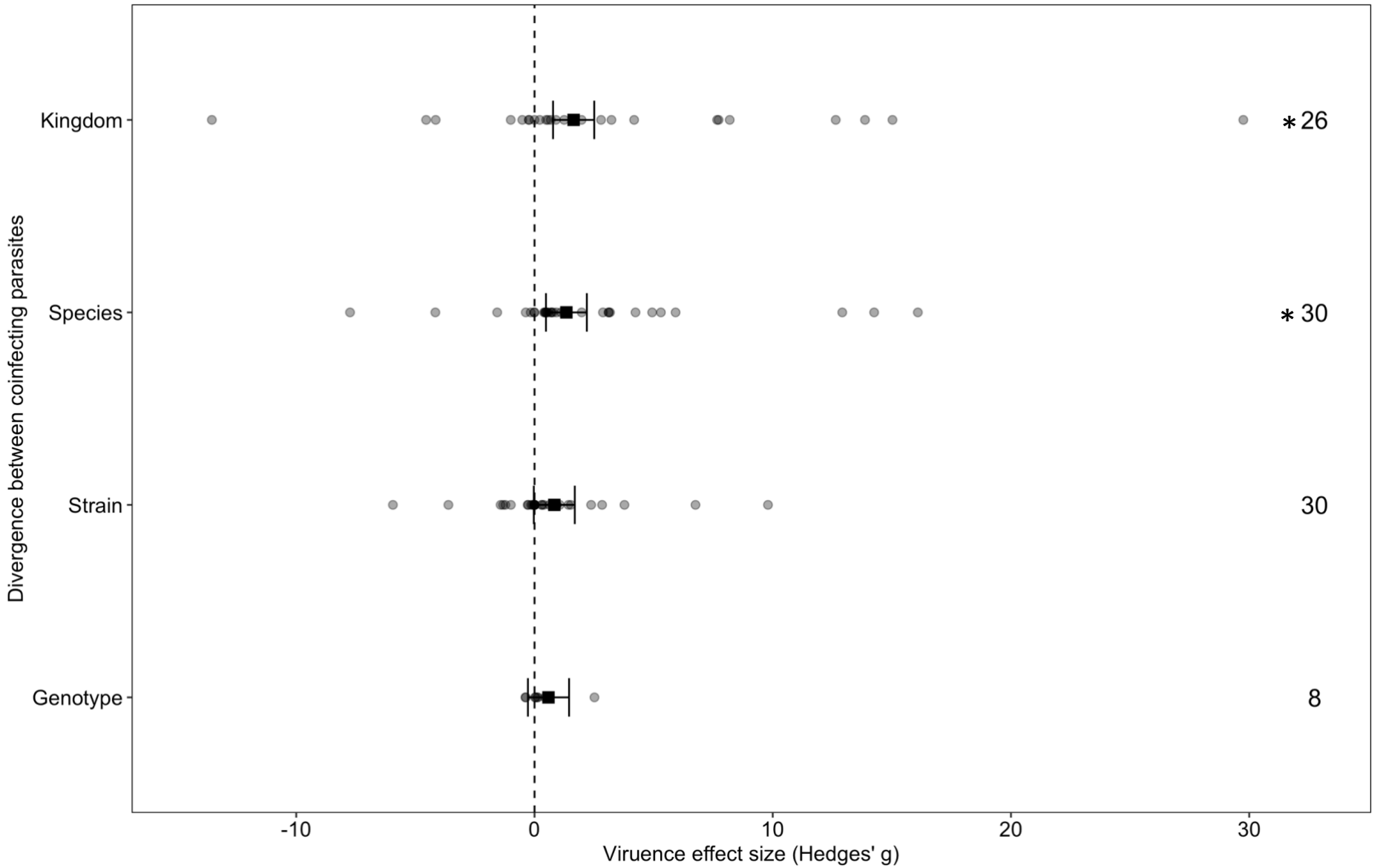


Figure 2: The virulence effect size (Hedges' g) of coinfecting parasites with different levels of phylogenetic divergence. All the means (square points) are significantly different from each other. Mean values and 95% confidence intervals are presented. Circular points represent all effect sizes within each group and numbers show the sample size of each grouping. * represent groups where mean values are significantly different from zero.

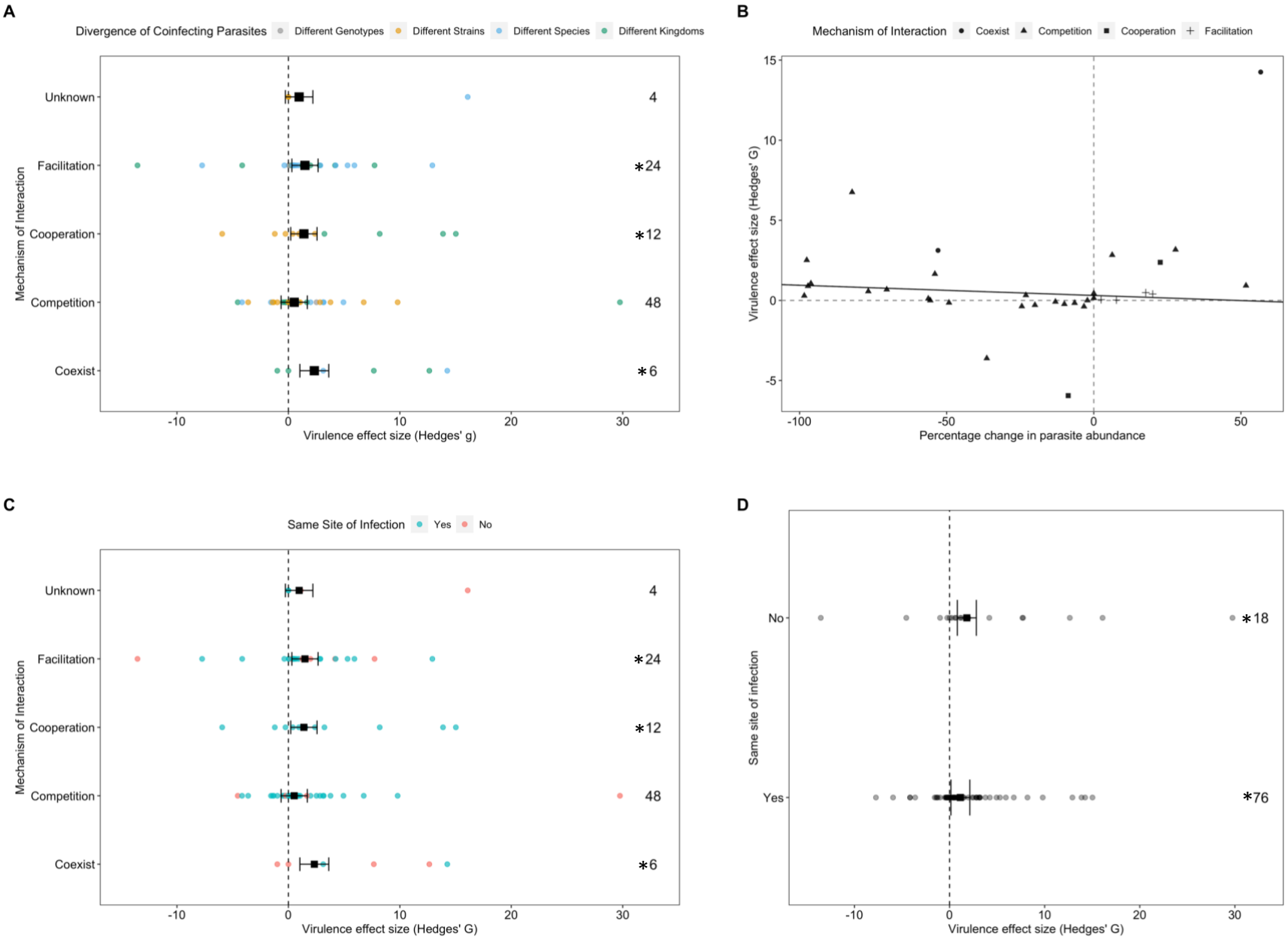


Figure 3: Mechanisms of within-host interactions and virulence. A) Virulence effect sizes across coinfecting parasites with different mechanisms of interaction. There is a significant interaction between 'Mechanism of interaction' and 'Divergence of coinfecting parasites' ($F_{12,81} = 59.77$, $p < 0.0001$). Colours differ according to the degree of divergence between parasites in coinfection. **B)** The relationship between the virulence effect size and percentage change in parasite load across studies. Regression line presented. Point shape varies by the mechanism of interaction. **C)** Virulence effect sizes across coinfecting parasites with different mechanisms of interaction. There is a significant interaction between 'Mechanism of interaction' and 'Site of infection' ($F_{8,85} = 45.77$, $p < 0.0001$). Colours depend upon whether the coinfecting parasites infect in the same or different sites within the host. **D)** Virulence effect sizes for coinfecting parasites with the same or different site of infection, points are significantly different from each other. In figures A, C and D mean values (square points) and 95% confidence intervals are presented,*represents a significant difference from zero and numbers represent the sample size of each grouping.

infection (mean = 1.39, SE = 0.59), coexisted (mean = 2.32, SE = 0.65) and facilitated each other (mean = 1.48, SE = 0.59) were more virulent in coinfections than single infections.

We found a marginal positive relationship between virulence effect size and percentage change in parasite abundance, which we considered to be a proxy for fitness (Model 10: Intercept = 0.35 (SE = 0.12), Gradient = 0.0004 (SE = 0.0002), Overall effect: $F_{1,33} = 7.01$, $p = 0.01$, Table 2). This slight positive gradient was driven by two extreme positive data points (see Figure S4). When we removed these points and re-ran the model, the overall gradient remained very small but significantly negative (Model 11: Gradient = -0.0064 (SE = 0.0011), t-value = -5.89, $p < 0.0001$, Figure 3B, Table 2). The gradient for this relationship (with and without extreme data points) is so close to zero that, although it is a statistically significant relationship, it is unlikely to be of any biological relevance. Aside from the overall relationship, coinfecting parasites that were competing were more likely to grow better in single infection with a high proportion of these points showing a negative percentage change in fitness (Figure 3B). Conversely, coinfecting parasites that facilitated each other were more likely to have a positive percentage change. It is notable that the two extreme positive values in Figure S4 were systems whereby parasites engaged in facilitation.

In addition, virulence was affected by whether coinfecting parasites infected the same or different sites within the host (Model 8: Overall effect: $F_{1,92} = 87.82$, $p < 0.0001$, Figure 3D, Table 2). Coinfections that occurred at different sites within the host (Mean = 1.81, SE = 0.50) were more virulent than those that occurred in the same site (Mean = 1.14, SE = 0.50). We also found that the likelihood of a mechanism of interaction between parasites was affected by site of infection, as we found a significant interaction between the site of parasite infection and the mechanism of interaction between parasites (Model 13: Overall effect =

$F_{8,85} = 45.76$, $p < 0.0001$, Figure 3C, Table 2). Coinfecting parasites that competed and cooperated were more likely to occur in the same site within the host (see Figure 3C).

Transmission modes of coinfecting parasites impacts virulence

Coinfections of parasites with different transmission modes were more virulent than parasites with the same mode (Model 6: Overall effect = $F_{1,92} = 67.70$, $p < 0.0001$, Table 2, Figure 4A). We also found that there was a difference in the virulence of coinfections compared to single infections for parasites with indirect or direct transmission (Model 7: Overall effect = $F_{1,92} = 59.09$, $p < 0.0001$, Table 2, Figure 4B). Coinfections with parasites that transmitted directly were more virulent (Mean = 1.29, SE = 0.58) than those which transmit indirectly (Mean = 0.85, SE = 0.58). When comparing parasites that were obligate killers to those that were not, the virulence of obligate killers was lower in coinfection (mean = 0.92, SE = 0.47) than non-obligate killers (mean = 1.24, SE = 0.47) (Table 2, Figure 4C).

Host type affects the virulence of coinfections

We found virulence of coinfections varied depending on the type of host (Model 4: Overall effect: $F_{2,91} = 116.90$, $p = <0.0001$, Table 2, Figure S3B). The virulence of parasites was significantly higher in coinfected vertebrates (mean = 1.90, SE = 0.60) than in invertebrates (mean = 0.84, SE = 0.60) or protozoa (mean = 0.70, SE = 0.60). Within vertebrates, hosts with the greatest effect sizes were birds, but there were only eight effect sizes in this group. We also found a significant difference in the virulence of coinfections compared to single infections depending on the type of parasite (Model 5: Overall effect: $F_{1,92} = 7.22$, $p = 0.01$, Table 2, Figure S3B). Coinfection with microparasites (mean = 1.21, SE = 0.52) had a higher virulence than coinfection with macroparasites (mean = 1.01, SE = 0.52) (Model 5, Table 2, Figure S3A). However, the difference in virulence between parasite types was only small and

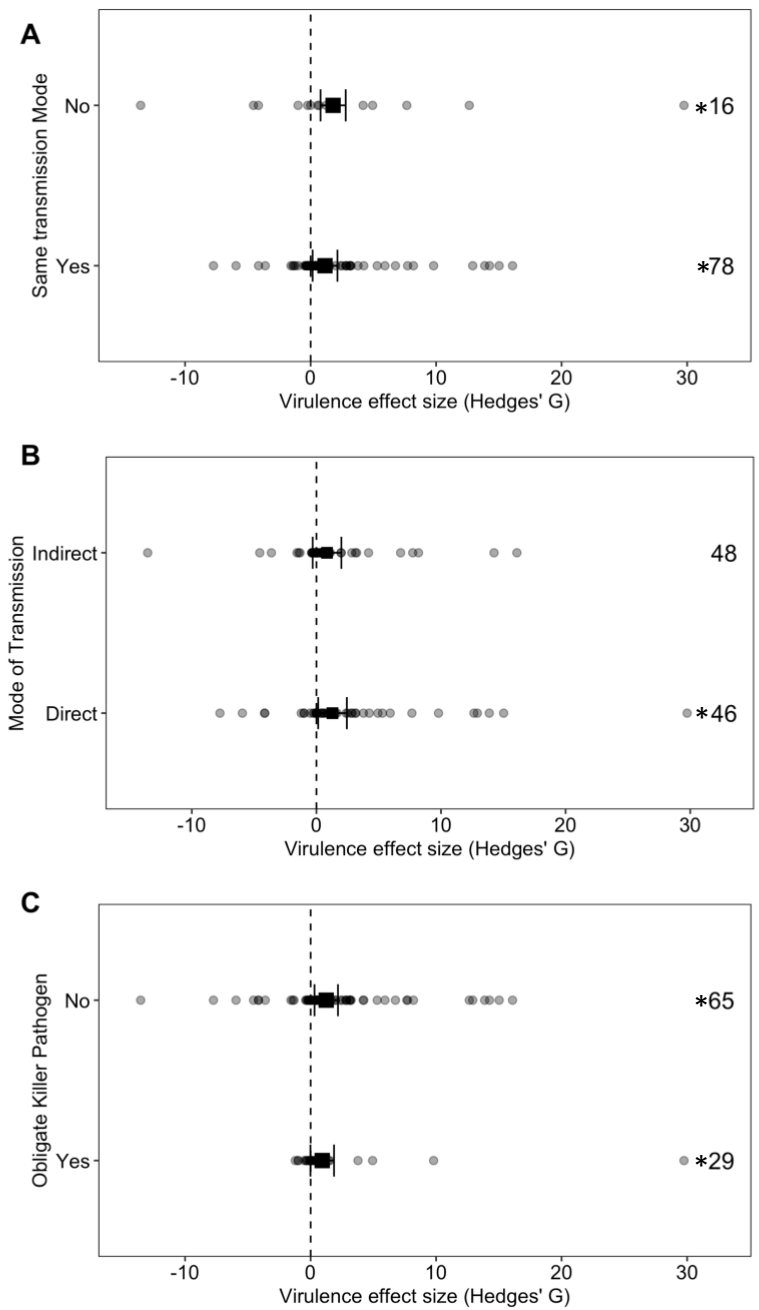


Figure 4: Transmission modes and Virulence. A) Virulence effect size for coinfecting parasites with same and different transmission modes. The points are significantly different from each other and from zero. B) Virulence effect size for coinfecting parasites with direct and indirect transmission modes. Points are significantly different from each other and the effect size for indirect transmission does not differ from zero. C) Displays the virulence effect size for parasites which are obligate killers compared to those which are not. The points are significantly different from each other and from zero. Mean values and 95% confidence intervals are presented. Numbers represent sample sizes. *represents groups where mean values are significantly different from zero.

346 may have been affected by the asymmetric number of effect sizes, with a lower number for
347 macroparasites (n = 15) than for microparasites (n = 79).
348
349 **4. Discussion**
350 Our meta-analysis revealed broad support for coinfections being more harmful to hosts than
351 single infections. When investigating this relationship further we found that the virulence of
352 coinfections was influenced by the divergence between coinfecting parasites, with highest
353 virulence between inter-kingdom and inter-specific coinfections but absent among
354 intraspecific coinfections. Virulence in coinfections also varied depending on how, and
355 where, parasites interacted within the host and their mode of transmission.
356
357 Our finding that when relatedness declines among coinfecting parasites, the overall virulence
358 is likely to increase, is in agreement with theoretical predictions proposed by Frank (Frank
359 1996). However, this theory is also dependent upon the mechanism of interaction between
360 coinfecting parasites, with Frank (1996) predicting that increased virulence is likely to occur
361 between less-related parasites because of increased competition to exploit hosts resources.
362 Similarly, we found that virulence varied depending upon the mechanism of interaction
363 between parasites, as has been experimentally demonstrated and predicted by theory (West
364 and Buckling 2003; Griffin et al. 2004; Buckling and Brockhurst 2008; Leggett et al. 2014),
365 and how parasites interacted varied across different levels of divergence. Coinfections of
366 cooperating and facilitatory parasites had a higher virulence in coinfection than in single
367 infection. Unexpectedly, we did not find that parasites which competed had higher virulence
368 in coinfection (Nowak and May 1994; Frank 1996; Ferguson and Read 2002). Furthermore,
369 we found the least divergent parasites were more likely to interact competitively (6 out of 8

370 coinfections with different genotypes, and 20 out of 30 coinfections with different strains
371 interacted competitively).
372
373 Although competition is predicted to lead to increased virulence (Frank 1996), we did not
374 find support of this. This may be explained by different types of competition which can arise
375 within a host (Read and Taylor 2001; Mideo 2009). For example, when parasites compete for
376 a limited supply of resources within the host (exploitation competition), it is advantageous for
377 parasites to utilise as many resources as possible which in turn is likely to lead to increased
378 harm to the host, and thus a higher virulence (Frank 1996; Read and Taylor 2001; Mideo
379 2009). Alternatively, if parasites evolve strategies to reduce the success of competitors
380 (interference competition) increased virulence may not be the predicted outcome (Massey et
381 al. 2004; Gardner et al. 2004; Mideo 2009). Interference competition has been found in many
382 species of bacteria, which produce a diverse range of antimicrobial compounds known as
383 bacteriocins capable of killing their competitors (Riley and Wertz 2002; Gardner et al. 2004).
384
385 Competition between more closely related parasites may be more likely to occur due to
386 similar life cycles or sites of infection (Pedersen and Fenton 2007; Griffiths et al. 2014).
387 We found that the type of interaction between coinfecting parasites varied depending upon
388 the site of infection. When coinfecting parasites were found within the same site within the
389 host, they were most likely to compete or cooperate with each other. Alternatively, for
390 parasites that coexisted, there was variation in the site of infection, with some parasites
391 infecting the same site and others infecting different sites (although this was a small sample).
392 A host could be considered an ecosystem to a parasite (Pedersen and Fenton 2007;
393 Rynkiewicz et al. 2015), within which there are compartmentalised sites of infection which
394 may have local host immune responses (Rynkiewicz et al. 2015). Interactions may be more

likely when parasites infect the same infection site (Rynkiewicz et al. 2015) or when two sites are linked by a common immune response (Griffiths et al. 2014). Likewise, coinfection within the same host site is likely to affect the types of interactions between parasites. For example, if cooperation is mediated by the production of public goods, being in close proximity may enable coinfecting parasites to directly benefit from the public goods (Harrison et al. 2006; West et al. 2006; Rezzoagli et al. 2019).

We found no correlation between percentage change in parasite abundance, our proxy for fitness, and the virulence effect size. Meaning that an increase in the number of parasites in coinfection, relative to single infections, didn't necessarily lead to a higher relative virulence in coinfections. A higher parasite load is often assumed to correlated with higher virulence (Read 1994; Alizon et al. 2009). However, our study and others (Alizon et al. 2009; Handel and Rohani 2015) highlight that this assumption is not always valid, or at least is should be put into context of the type of interaction between coinfecting parasites, as has been demonstrated in HIV (Fraser et al. 2007). Future work could investigate whether parasite abundance correlates with increased between-host transmissibility, as theory predicts (Hawley et al. 2013; Williams et al. 2014), across host-parasite systems.

In our study the majority of coinfecting parasites utilised the same mode of transmission as each other, and when this was the case there was no difference between virulence in single infections and coinfections. Where coinfecting parasites had different modes of transmission, the overall virulence in coinfection was higher than in single infections. Higher virulence in coinfections could arise because of conflict between the two parasites over their transmission strategies or due to trade-offs between transmission and virulence (Jones et al. 2010; Rigaud et al. 2010). Where there are extreme differences in transmission conflict has been shown to

affect interactions between parasites and their behaviour within the host (Thomas et al. 2002; Haine et al. 2005). Within the studies included in our analysis, Louhi *et al.* (2015) coinfected the rainbow trout (*Oncorhynchus mykiss*) with the trematode *Diplostomum pseudospathaceum* and the bacterium *Flavobacterium columnare*, which differed in their mode of transmission. Here, the trematode had a complex life cycle and required a mollusc intermediate host whereas the bacterium was indirectly-transmitted as an opportunistic waterbourne parasite (Louhi et al. 2015). Coinfection with the trematode and bacterium had a higher virulence than either of the parasites in single infection. The authors hypothesised that conflict may occur between transmission strategies as *F.columnare* may benefit from high virulence and saprotrophic transmission (Kunttu et al. 2009), but host death would result in transmission failure for *D. pseudospathaceum* (Louhi et al. 2015). Furthermore, we found that where parasites were obligate host killers, coinfections were less virulent than single infections [e.g. (Lohr et al. 2010)]. This counterintuitive finding is likely due to the conflict experienced between coinfecting parasites within the host (Thomas et al. 2002), meaning that obligate killers were less virulent in coinfection than in a single infection because of a within-host conflict.

Furthermore, coinfections involving parasites with direct modes of transmission were more virulent than those with indirect transmission modes. Directly-transmitted parasites experience conflicting selection pressures between transmitting between new hosts and shortening the life span of their current host, and so we predicted their virulence would be lower (Boldin and Kisdi 2012; Antonovics et al. 2017). Whereas, indirectly transmitted parasites may be able to persist for longer periods outside of the host in the environment, and thus we expected the virulence of these parasites to be higher (Ewald 1983; Walther and Ewald 2004; Antonovics et al. 2017). Our unexpected finding may be a result of the studies

included in our analysis, all of which were experimental. Thus, these parasites which experience homogeneous laboratory conditions may not experience the trade-offs that occur in naturally-persisting environmental strains (Walther and Ewald 2004; Rafaluk-Mohr 2019). More research is required to disentangle how between-host dynamics are influenced by the within-host interactions of parasites (Coombs et al. 2007; Mideo et al. 2008; Handel and Rohani 2015).

Our meta-analysis largely takes a parasite-centric view of coinfections, but we did find a small difference between the virulence effect size of vertebrate and invertebrate hosts. For vertebrate hosts we found that coinfections were more virulent than single infections, but for invertebrate hosts there was no difference. This discrepancy may be explained by the differing immune systems of vertebrate and invertebrate hosts with the latter lacking acquired immunity (Briggs et al. 1995). Furthermore, launching an immune response is costly (Sheldon and Verhulst 1996; Lochmiller and Deerenberg 2000) and can negatively impact other host abilities such as investment in reproduction (Vaumourin et al. 2015). Allocation strategies may vary between vertebrate and invertebrate hosts, resulting in different investments in immune responses which may explain why we observe a difference in virulence effect size between vertebrate and invertebrate hosts.

Further experimental work is required to deepen our understanding of coinfection and parasite virulence (Pedersen and Fenton 2007; Telfer et al. 2010; Rigaud et al. 2010; Eswarappa et al. 2012). The studies we included in our analysis are experimental on common coinfections, such as *Candida albicans* and *Staphylococcus aureus* (Sheehan et al. 2020) which enabled us to find broad trends across genotypes and species. However, the virulence of coinfections is likely to vary depending upon how frequently parasites interact in nature. If

coinfections between genotypes or species are rare, then selection is likely to favour a more prudent exploitation of the host and thus intermediate levels of virulence (Anderson and May 1982; Frank 1996; Levin 1996; Eswarappa et al. 2012). Anthropogenic-induced environmental change is affecting host-parasite interactions, for example temperature changes driving shifts in host and parasite ranges (Parmesan et al. 1999; Hance et al. 2007; Cahill et al. 2013; Longdon et al. 2015). These changes to the structure of ecological communities suggest that novel host-parasite interactions are likely to arise (Hance et al. 2007; Longdon et al. 2015; Hassell et al. 2021), highlighting the importance of understanding how novel host-parasite coinfections affect coinfection outcomes. Thus, when predicting coinfection virulence, it may be useful for future studies to consider both the mechanism of interaction between coinfecting parasites (Read and Taylor 2001; Pedersen and Fenton 2007; Buckling and Brockhurst 2008; Rynkiewicz et al. 2015) and the frequency with which parasites likely to co-occur within a host in nature (Anderson and May 1982; Frank 1996; Levin 1996; Eswarappa et al. 2012).

Our study, along with others (Pedersen and Fenton 2007; Graham 2008; Griffiths et al. 2014; Rynkiewicz et al. 2015; Betts et al. 2016b; Hassell et al. 2021), highlights the ecological importance and improved understanding that can arise from studying infections made up of multiple parasites. With coinfections particularly affecting the world's poorest human populations (Steinmann et al. 2010; Griffiths et al. 2011) and in this time of a global pandemic (Bai et al. 2021), now more than ever improving our knowledge of the degree of harm caused by coinfections is of vital importance. This knowledge will aid in the effective prediction and control of coinfections outcomes (Read and Taylor 2001; Vaumourin et al. 2015) and in the development of treatment strategies (Ezenwa and Jolles 2015; Vaumourin et al. 2015; Ezenwa et al. 2021).

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Author Contributions

V.L.P. and K.C.K. conceived and designed the study. V.L.P. collected the data and carried out the statistical analysis, with guidance from K.C.K. Both authors contributed to writing the manuscript.

Data Archiving

The data and code from this study will be made available in the Dryad digital repository.

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Conflict of Interest

We have no competing interests.

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Table 1: Summary of the literature on the effect of coinfection compared to single infection on parasite virulence across host-parasite systems.

Paper	Parasite		Host	Type of Transmission (same or different)	Type of transmission (direct or indirect)		Site of infection (same or different)	Mechanism of interaction	Divergence between parasites	Number of effect sizes per study
	Parasite A	Parasite B			Parasite A	Parasite B				
Hurst et al 2019	*Ceratonova shasta*	*Ceratonova shasta*	Chinook salmon (*Oncorhynchus tshawytscha*)	Same	Direct	Direct	Same	Competition	Different Genotypes	2
Clay et al 2018	*Pasteuria ramosa*	*Metschnikowia bicuspidata*	Daphia (*Daphnia dentifera*)	Same	Indirect	Indirect	Same	Competition	Different kingdom	2
Gleichsner et al 2017	*Schistosoma mansoni* (NMRI)	*Schistosoma mansoni* (PR-1)	Freshwater snail (*Biomphalaria glabrata*)	Same	Indirect	Indirect	Same	Competition	Different Strains	2
Ben-Ami & Routtu 2013	*Pasteuria ramosa* clones	*Pasteuria ramosa* isolates	Daphia (*Daphnia magna*)	Same	Indirect	Indirect	Same	Competition	Different Genotypes	2
Kinnula et al 2017	*Flavobacterium columnare*	*Flavobacterium columnare*	Zebrafish (*Danio rerio*)	Same	Indirect	Indirect	Same	Unknown	Different Strains	2
Fellous and Koella 2010	*Vavraia culicis*	*Ascogregarina culicis*	Mosquito (*Aedes aegypti*)	Same	Indirect	Direct	Same	Competition	Different kingdom	2
Harrison et al 2006	*Pseudomonas aeruginosa* (PaO1)	*Pseudomonas aeruginosa* (PAO9)	Wax moth (*Galleria mellonella*)	Same	Direct	Direct	Same	Cooperation	Different strains	2
Balmer et al 2009	*Trypanosoma brucei* (RV)	*Trypanosoma brucei* (GV)	Mouse (*Mus musculus*)	Same	Indirect	Indirect	Same	Competition	Different strains	4
	Trypanosoma brucei (REV)	*Trypanosoma brucei* (GEV)		Same	Indirect	Indirect	Same	Competition	Different strains	

Ben-Ami et al 2008	*Pasteuria ramosa* (P1)	*Pasteuria ramosa* (P3)	Daphia (*Daphnia magna*)	Same	Indirect	Indirect	Same	Competition	Different strains	6
	Pasteuria ramosa (P1)	*Pasteuria ramosa* (P4)		Same	Indirect	Indirect	Same	Competition	Different strains	
	Pasteuria ramosa (P3)	*Pasteuria ramosa* (P4)		Same	Indirect	Indirect	Same	Competition	Different strains	
Hodgson et al 2004	PfNPV (Pf4)	PfNPV (Pf6)	Moth (*Panolis flammea*)	Same	Direct	Direct	Same	Facilitation	Different genotypes	2
Lohr et al 2010	*Caullerya mesnili*	*Metschnikowia sp*	Daphnia (*Daphnia galeata*)	Different	Indirect	Indirect	Different	Competition	Different kingdom	2
Louhi et al 2015	*Flavobacterium columnare*	*Diplostomum pseudospathaceum*	Rainbow trout (*Oncorhynchus mykiss*)	Different	Indirect	Direct	Different	Facilitation	Different kingdom	2
Chandrarathna et al 2018	*Aeromonas hydrophila*	*Aeromonas veronii*	Zebrafish (*Danio rerio*)	Same	Indirect	Indirect	Same	Facilitation	Different species	2
Palinauskas et al., 2018	*Plasmodium relictum*	*Plasmodium elongatum*	Domestic canary (*Serinus canaria domestica*)	Same	Indirect	Indirect	Same	Facilitation	Different species	2
Milbrath et al 2015	*Nosema apis*	*Nosema ceranae*	Honey bee (*Apis mellifera*)	Same	Direct	Direct	Same	Facilitation	Different species	2
Tang et al 2020	*Plasmodium yoelii*	*Plasmodium vinckei*	Mosquito (*Anopheles stephensi*)	Same	Indirect	Indirect	Same	Competition	Different species	6
	Plasmodium yoelii	*Plasmodium chabaudi*		Same	Indirect	Indirect	Same	Competition	Different species	
	Plasmodium vinckei	*Plasmodium chabaudi*		Same	Indirect	Indirect	Same	Competition	Different species	
Dong et al 2015	*Flavobacterium columnare*	*Edwardsiella ictaluri*	Striped catfish (*Pangasianodon hypophthalmus*)	Same	Indirect	Indirect	Different	Unknown	Different species	2

Zhang et al 2016	White spot syndrome virus (WSSV)	*Vibrio parahaemolyticus*	Penaeid shrimp (*Litopenaeus vannamei*)	Same	Indirect	Indirect	Different	Facilitation	Different kingdom	2
Wennmann et al 2015	Agrotis segetum nucleopolyhedrovirus B (AgseNPV-B)	Agrotis segetum granulovirus (AgseGV)	Cutworm larvae (*Agrotis segetum*)	Same	Direct	Direct	Same	Competition	Different species	2
Costa-Hurtado et al 2015	Highly pathogenic avian influenza virus (HPAIV)	Newcastle disease virus (NDV)	Leghorn chickens (*Gallus gallus domesticus*)	Same	Direct	Direct	Same	Competition	Different species	2
Galac & Lazzaro 2011	*Providencia rettgeri*	*Providencia sneebia*	Fruit fly (Drosophila melanogaster)	Same	Indirect	Indirect	Same	Coexist	Different species	2
Evison et al 2015	*Ascosphaera apis* (E)	*Ascosphaera apis* (F)	Honey bee (*Apis mellifera*)	Same	Direct	Direct	Same	Cooperation	Different strains	6
	Ascosphaera apis (E)	*Ascosphaera apis* (I)		Same	Direct	Direct	Same	Cooperation	Different strains	
	Ascosphaera apis (I)	*Ascosphaera apis* (F)		Same	Direct	Direct	Same	Cooperation	Different strains	
Davies et al 2002	*Schistosoma mansoni (PR)*	*Schistosoma mansoni* (K)	Freshwater snail (*Biomphalaria glabrata*)	Same	Indirect	Indirect	Same	Competition	Different strains	2
Ponnusamy et al 2016	*Aeromonas hydrophila* (NF1)	*Aeromonas hydrophila* (NF2)	Mouse (*Mus musculus*)	Same	Direct	Direct	Same	Facilitation	Different strains	2
Mosser et al 2015	*Aeromonas media*	*Aeromonas veronii*	Nematode (*Caenorhabditis elegans*)	Same	Indirect	Indirect	Same	Cooperation	Different kingdom	2
Sheeehan et al 2020	*Staphylococcus aureus*	*Candida albicans*	Wax moth larvae	Same	Direct	Direct	Same	Cooperation	Different kingdom	2

			(*Galleria mellonella*)							
Dong et al 2014	Avian leukosis virus (ALV-J)	Reticuloendotheliosis virus (REV)	Chicken (*Gallus gallus domesticus*)	Same	Direct	Direct	Same	Facilitation	Different species	2
Roon et al 2015	*Nanophyetus salmincola*	*Flavobacterium columnare*	Chinook salmon (*Oncorhynchus tshawytscha*)	Different	Indirect	Indirect	Different	Facilitation	Different kingdom	4
	Nanophyetus salmincola	*Aeromonas salmonicida*		Different	Indirect	Indirect	Different	Competition	Different kingdom	
Heinig & Thomas 2015	*Beauveria bassiana*	*Plasmodium yoelii*	Mosquito (*Anopheles stephensi*)	Different	Direct	Direct	Different	Coexist	Different kingdom	4
	Beauveria bassiana	Plasmodium falciparum		Different	Direct	Direct	Different	Coexist	Different kingdom	
Ngba Essebe et al 2017	*Staphylococcus aureus* (NSA739)	*Helcococcus kunzii* (H10)	Nematode (*Caenorhabditis elegans*)	Same	Direct	Direct	Same	Facilitation	Different species	4
	Staphylococcus aureus (NSA739)	*Helcococcus kunzii* (H13)		Same	Direct	Direct	Same	Facilitation	Different species	
Escribano et al 2001	Multiple nucleopolyhedrovirus (MNPV)	*Chelonus insularis*	Fall armyworm (*Spodoptera frugiperda*)	Different	Direct	Direct	Different	Competition	Different kingdom	2
Bashley et al 2011	*Steinerne costaricense* (C1)	*Steinerne costaricense* (C4)	Wax moth (*Galleria mellonella*)	Same	Direct	Direct	Same	Competition	Different strains	4
	Steinerne costaricense (C3)	*Steinerne costaricense* (C4)		Same	Direct	Direct	Same	Competition	Different strains	
Bashley et al 2016	*Steinerne costaricense (K)*	*Steinerne costaricense (B)*	Wax moth (*Galleria mellonella*)	Same	Direct	Direct	Same	Competition	Different strains	2

Liu et al 2020	*Clostridium perfringens (S13)*	*Eimeria maxima*	Chicken (*Gallus gallus domesticus*)	Same	Direct	Direct	Same	Facilitation	Different species	2
Ruiz-Vega et al 2020	Metarhizium anisopliae	Steinernema carpocapsae	Scarab beetle larvae (*Phyllophaga vetula*)	Same	Direct	Direct	Same	Facilitation	Different kingdom	2
Zilio & Koella 2020	*Vavraia culicis*	*Edhazardia aedis*	Mosquito larvae (Aedes aegypti)	Different	Direct	Direct	Same	Competition	Different species	2
Norton et al 2020	Deformed wing virus (DWV)	Deformed wing virus (DWV)	Honey bee (*Apis mellifera*)	Same	Indirect	Indirect	Same	Competition	Different genotypes	2

Table 2: Output for all the models in the meta-analysis.

Model	Levels of Moderator Variable	Mean	SE	t value	P value	CIs		Moderator Analysis		N	Random Effects		Heterogeneity measure (Q)
						Lower	Upper	F value	P value		Measure	Estimate	
1 Overall effect	None	1.1875	0.5363	2.2145	0.0292	0.1226	2.2525	NA	NA	94	Dose Measure of virulence	0.0339 1.0385	4658.2019
2 Mechanism of Interaction	Coexist	2.3249	0.6521	3.5651	0.0006	1.0291	3.6206	$F_{4,89}$= 72.7889	< 0.0001	94	Dose	0.1795	4472.1709
	Competition	0.5189	0.5926	0.8757	0.3835	-0.6585	1.6964						
	Cooperation	1.3877	0.5945	2.3342	0.0218	0.2064	2.5691				Measure of Virulence	0.9949	
	Facilitation	1.4836	0.5916	2.5079	0.0140	0.3082	2.6590						
	Unknown	0.9615	0.6218	1.5464	0.1256	-0.2739	2.1969						
3 Divergence between coinfecting parasites	Different Kingdoms	1.6426	0.4355	3.7718	0.0003	0.7774	2.5077	$F_{3,90}$ = 76.85	< 0.0001	94	Dose	0	4430.6670
	Different Species	1.3388	0.4316	3.1022	0.0026	0.4814	2.1961				Measure of virulence	0.7001	
	Different Strains	0.8308	0.4332	1.9181	0.0583	-0.0297	1.6914						
	Different Genotypes	0.5871	0.4351	1.3494	0.1806	-0.2773	1.4515						
4 Type of host – Vert or Invert	Vertebrate	1.9024	0.5965	3.1893	0.0020	0.7176	3.0873	$F_{2,91}$ = 116.8990	< 0.0001	94	Dose	0.0121	4416.2941
	Invertebrate	0.8383	0.5951	1.4087	0.1623	-0.3438	2.0203				Measure of virulence	1.3452	
	Protozoa	0.7012	0.5999	1.1689	0.2455	-0.4904	1.8928						
5 Type of Parasite – Macro or Micro	Macro	1.0129	0.5284	1.9169	0.0584	-0.0366	2.0624	$F_{1,92}$ = 7.2226	0.0085	94	Dose	0.0289	4653.0384
	Micro	1.2085	0.5245	2.3042	0.0235	0.1668	2.2502				Measure of virulence	0.9983	

6 Transmission Mode – Same or Different	Same	1.1469	0.4994	2.2964	0.0239	0.1550	2.1389	$F_{1,92}$= 67.6954	< 0.0001	94	Dose	0.0006	4591.7434
	Different	1.7915	0.5049	3.5486	0.0006	0.7888	2.7943				Measure of Virulence	0.9526	
7 Transmission Mode – Direct or Indirect	Direct	1.2893	0.5791	2.2263	0.0284	0.1391	2.4395	$F_{1,92}$= 59.0895	< 0.0001	94	Dose	0.1165	4640.0270
	Indirect	0.8493	0.5806	1.4627	0.1470	-0.3039	2.0025				Measure of Virulence	1.0637	
8 Infection site – Same or Different	Same	1.1374	0.4996	2.2764	0.0251	0.1450	2.1297	$F_{1,92}$ = 87.8239	< 0.0001	94	Dose	0	4576.7928
	Different	1.8179	0.5042	3.6056	0.0005	0.8165	2.8193				Measure of Virulence	0.9546	
9 Obligate Killers	Yes	0.9253	0.4732	1.9557	0.0535	-0.0144	1.8650	$F_{1,92}$ =32.0123	< 0.0001	94	Dose	0.0030	4601.9267
	No	1.2439	0.4711	2.6406	0.0097	0.3083	2.1794				Measure of Virulence	0.8376	
10 Virulence and Fitness (with extreme values)	Intercept	0.3455	0.1172	2.9471	0.0058	0.1070	0.5841	$F_{1,33}$= 7.0054	0.0124	34	Dose	0.0008	818.7043
	Gradient	0.0004	0.0002	2.6468	0.0124	0.0001	0.0008				Measure of Virulence	0.0229	
11 Virulence and Fitness (without extreme values)	Intercept	0.3121	0.1409	2.2144	0.0343	0.0246	0.5995	$F_{1,31}$= 34.7675	< 0.0001	32	Dose	0.0367	735.1099
	Gradient	-0.0064	0.0011	-5.8964	<.0001	-0.0086	-0.0042				Measure of Virulence	0.0000	
12 Interaction between divergence of coinfecting parasites and	Competition: Different genotype	-0.0801	0.8118	-0.0987	0.9216	-1.6954	1.5351	$F_{12,81}$ = 59.7718	< 0.0001	94	Dose	0.6067	4039.2827
	Competition: Different strains	0.8154	0.8093	1.0076	0.3167	-0.7948	2.4257						
	Competition:	0.6178	0.5859	1.0544	0.2947	-0.5472	1.7827						

mechanism of interaction	Different species												
	Competition: Different kingdom	-0.0028	0.8154	-0.0035	0.9973	-1.6252	1.6195						
	Coexist: Different species	-0.4205	0.9056	-0.4644	0.6436	-2.2223	1.3813						
	Coexist: Different kingdom	-0.4205	0.9056	-0.4644	0.6436	-2.2223	1.3813						
	Cooperation: Different strains	1.2769	0.8096	1.5771	0.1187	-0.3341	2.8878						
	Cooperation: Different kingdom	5.2503	0.8440	6.2209	<0.0001	3.5710	6.9295						
	Facilitation: Different genotype	0.8841	0.8154	1.0843	0.2814	-0.7382	2.5065						
	Facilitation: Different species	0.8841	0.8154	1.0843	0.2814	-0.7382	2.5065						
	Facilitation: Different kingdom	1.2046	0.8146	1.4787	0.1431	-0.4162	2.8254						
	Unknown: Different strains	1.5041	0.9136	1.6463	0.1036	-0.3138	3.3219						
	Unknown: Different species	1.5041	0.9136	1.6463	0.1036	-0.3138	3.3219						
13 Interaction between site of	Coexist: Same site	4.3250	0.6810	6.3511	<.0001	2.9710	5.6790	$F_{8,85}$= 45.7694	< 0.0001	94	Dose	0.1399	4347.5579

infection (same or different) and mechanism of interaction	Coexist: Different site	0.0229	0.7020	0.0326	0.9740	-1.3729	1.4187						
	Competition: Same site	0.5716	0.5729	0.9979	0.3212	-0.5674	1.7107						
	Competition: Different site	0.6178	0.5859	1.0544	0.2947	-0.5472	1.7827						
	Facilitation: Same site	1.4312	0.5716	2.5037	0.0142	0.2946	2.5678						
	Facilitation: Different site	1.7735	0.5799	3.0584	0.0030	0.6206	2.9265				Measure of Virulence	0.9802	
	Cooperation: Same site	1.3934	0.5745	2.4256	0.0174	0.2512	2.5356						
	Cooperation: Different site	2.0413	0.7051	2.8952	0.0048	0.6395	3.4432						
	Unknown: Same site	0.7739	0.6190	1.2503	0.2146	-0.4568	2.0047						
	Unknown: Different site	1.4218	0.6619	2.1481	0.0346	0.1058	2.7378						

Supplementary Material

Chapter 3

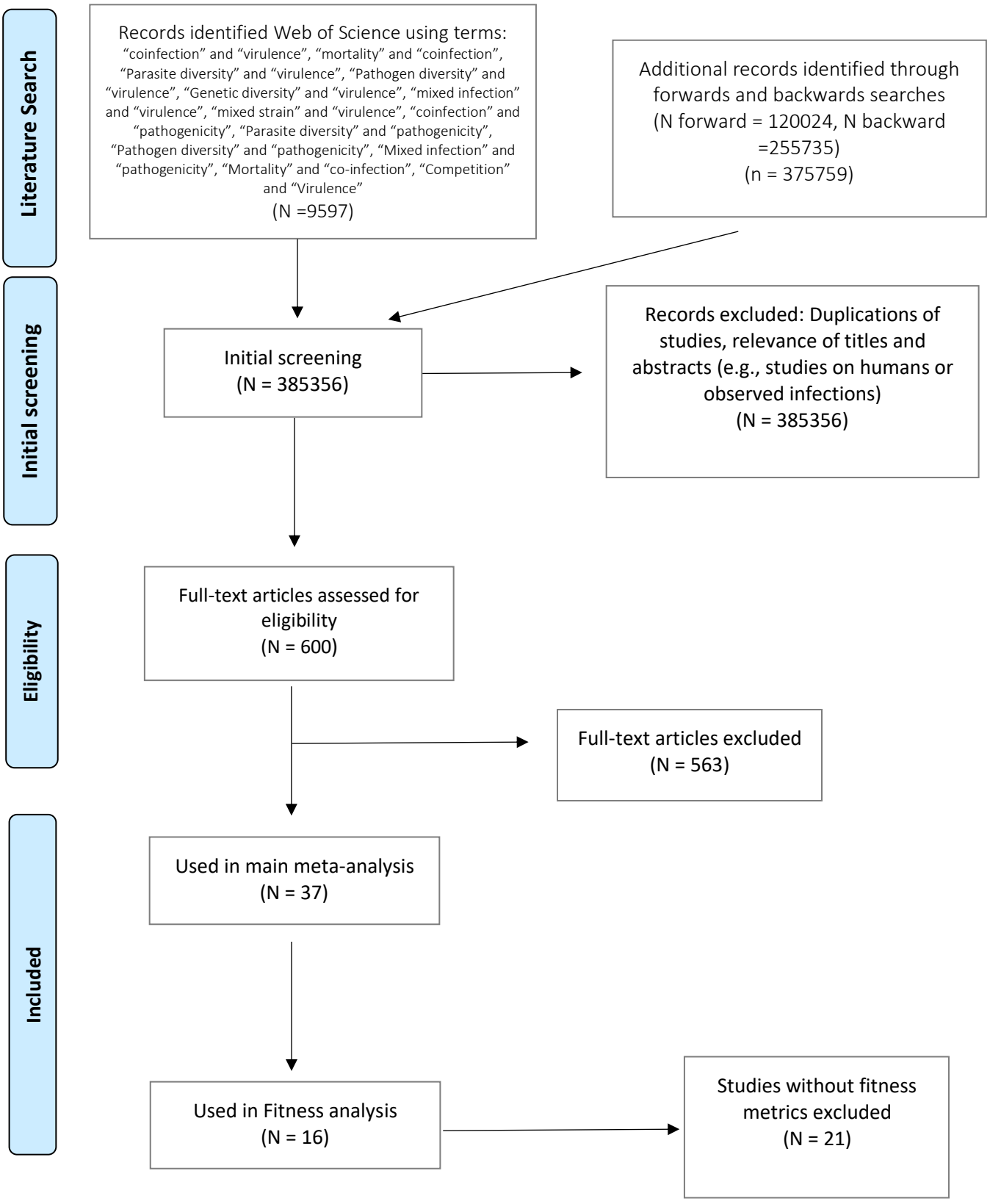


Figure S1: PRISMA flow chart detailing data collection (Moher *et al.*, 2009).

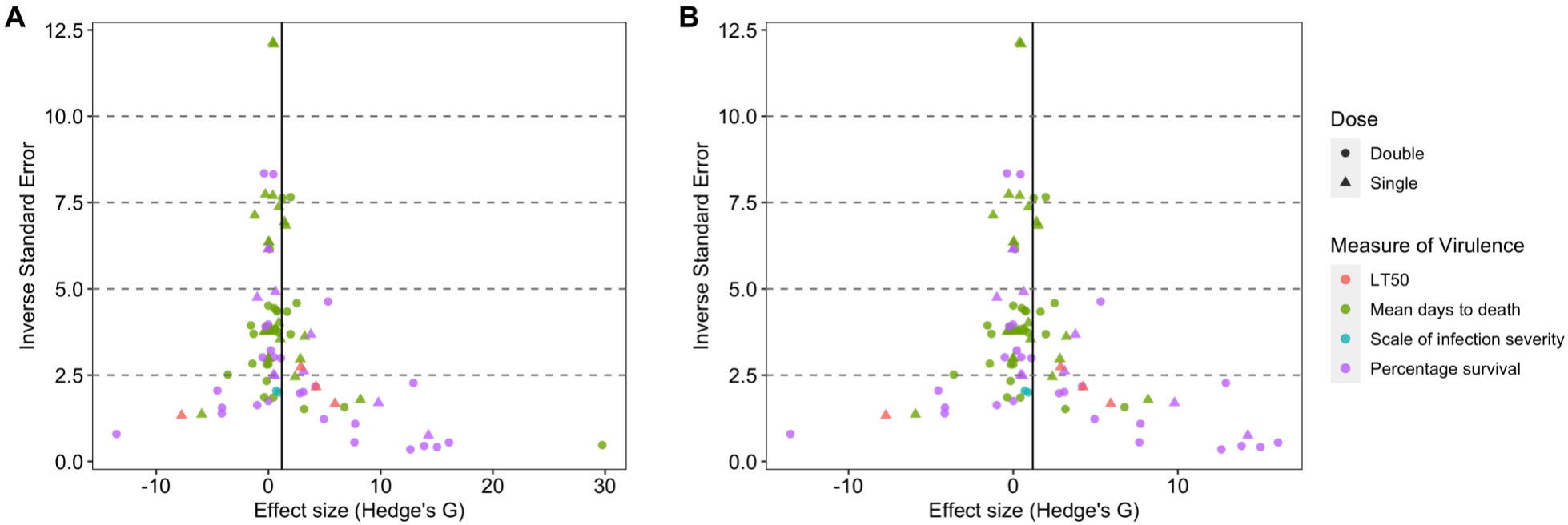


Figure S2: Funnel plots for virulence effect size (Hedge's g) with (A) and without (B) one outlier effect size. Shape (dose) and colour (measure of virulence) represent the two random effects included in our analysis.

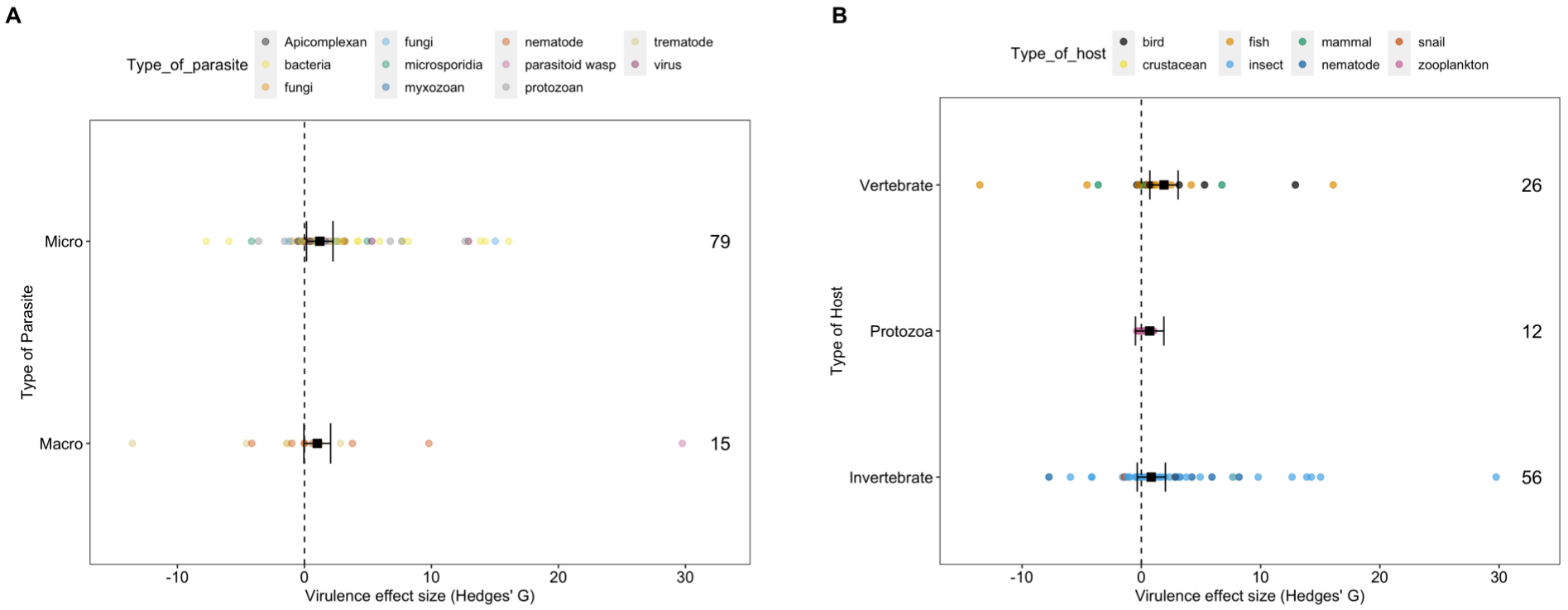


Figure S3: Virulence effect size (Hedges' g) for different types of A) host and b) parasites. Mean values and 95% confidence intervals are presented. Colours represent the type of host or parasite within each of the groups. Sample sizes are shown. * represents a significant difference from zero.

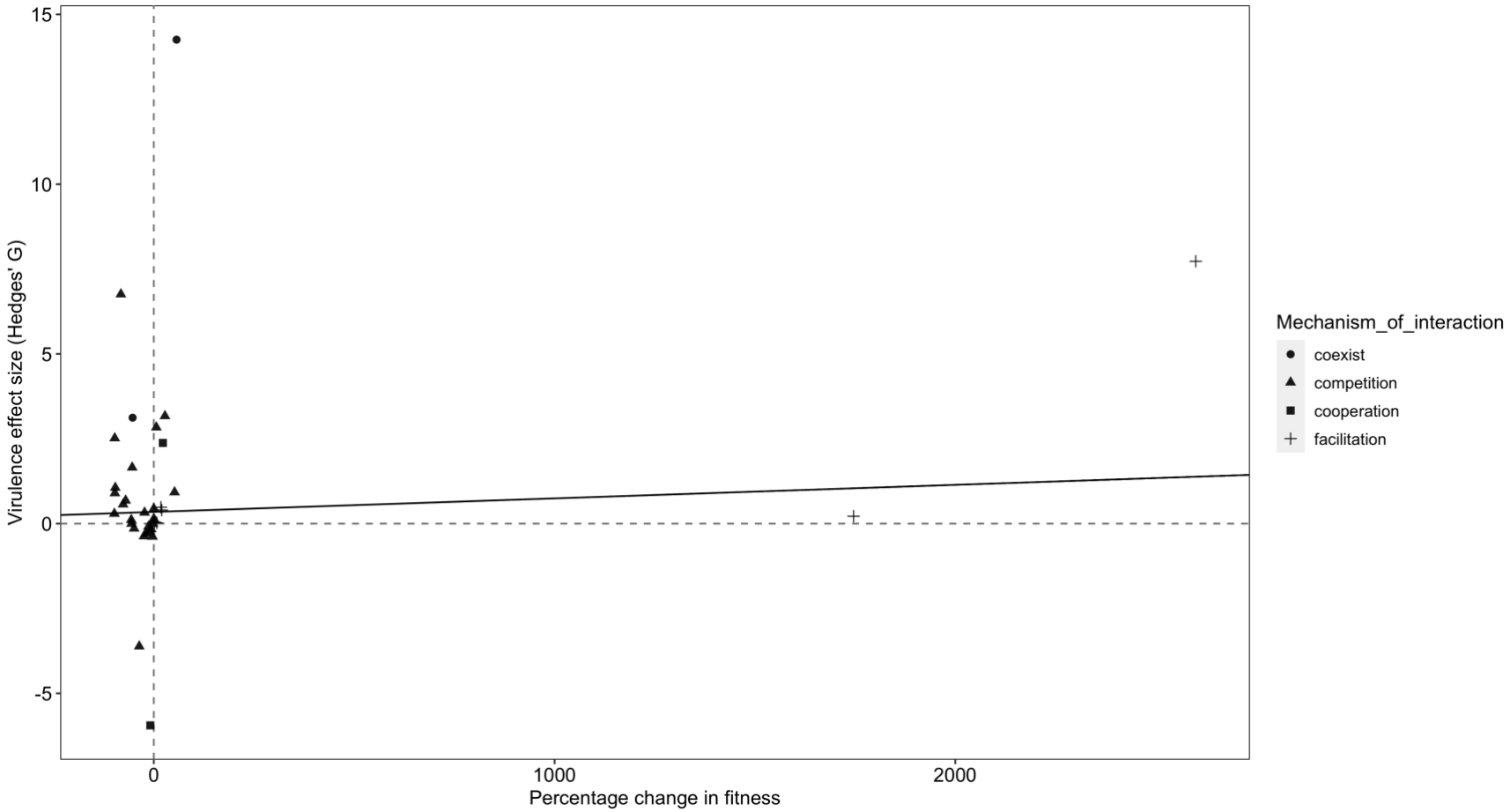


Figure S4: The relationship between virulence effect size and percentage change in fitness with outliers included. Shape represents the mechanism of interaction between the coinfecting parasites.

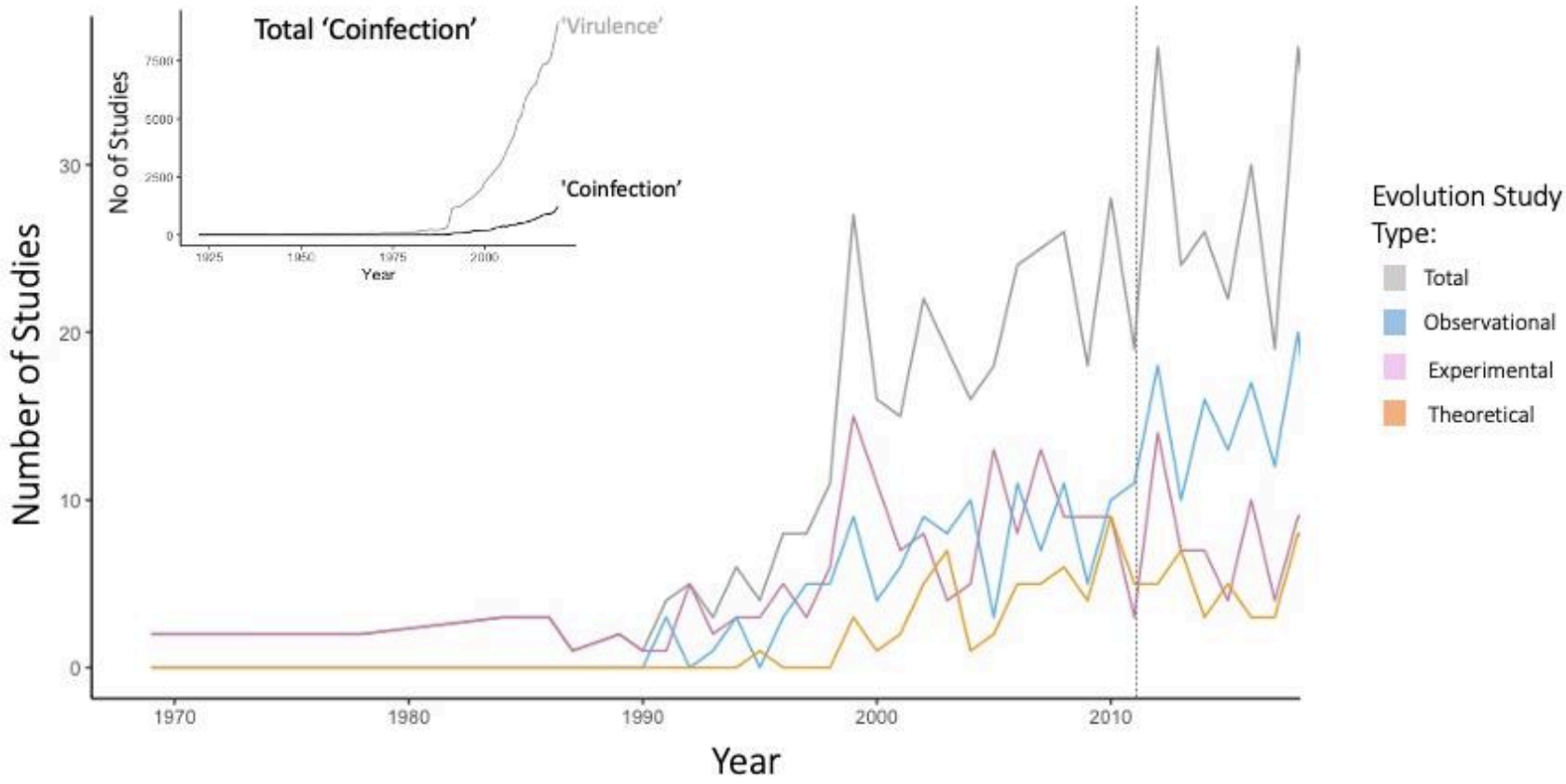


Figure S5: The number of coinfection studies over time. The large graph represents those within the field of evolutionary ecology and the smaller graph shows the total number. The dotted line represents the end of Griffiths et al 2011 sampling. Data was collected from Web of Science.

Chapter 4

Within-and between-host dynamics of producer and non-producer pathogens

Within- and between-host dynamics of producer and non-producer pathogens

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Abstract

For infections to be maintained in a population, pathogens must compete to colonise hosts and transmit between them. Within the host, much research has been conducted into pathogen interactions, yet less is known about whether these interactions can affect between-host transmission. In this study, we use an experimental approach to investigate within-and-between host dynamics using the pathogen *Pseudomonas aeruginosa* and the host *Caenorhabditis elegans.* Within-host interactions often involve the production of goods, that are beneficial to all the pathogens in the local environment but susceptible to exploitation by non-producers. Here we exposed the nematode host to a 'producer' and two 'non-producer' strains (specifically for siderophore production and quorum sensing) in single infections and coinfections to investigate within-host colonisation. Subsequently, we introduced infected nematodes to pathogen-naive populations, to allow natural pathogen transmission between hosts. We find that producer pathogens are consistently better at colonising hosts and transmitting between them than non-producers during coinfection and single infection. Non-producers were poor at colonising hosts and between-host transmission, even when coinfected with producers. Understanding pathogen dynamics across these multiple levels will ultimately help us to predict and control the spread of infections.

1. Introduction

To be successful, pathogens must be able to compete to colonise a host and transmit between new hosts (Anderson and May 1986). Theory has highlighted the trade-offs that can arise between these two levels as a result of counterbalancing selective pressures within and between hosts (Anderson and May 1982; Koella and Antia 1995; Walther and Ewald 2004). Within the host, pathogens must balance replication and transmission to new hosts (Reece et al. 2009), in an analogous trade-off between reproduction and growth within multicellular organisms (Bell 1980; Clutton-Brock 1984; Greischar et al. 2016). Studying pathogens at these two levels has mainly been theoretical (Anderson and May 1986; Coombs et al. 2007; Mideo et al. 2008; Handel and Rohani 2015), leaving an outstanding data gap in disease ecology (Coombs et al. 2007; Mideo et al. 2008; Handel and Rohani 2015). Understanding this interplay between different levels of infectious disease is crucial for understanding the fitness and transmissibility of pathogens, which could help contribute to developing effective intervention strategies (Hellriegel 2001; Handel and Rohani 2015) for clinically important pathogens, such as *Plasmodium sp* (Greischar et al. 2016) to those having major effects on ecosystems, such as the fungi *Batrachochytrium dendrobatidis* (Pedersen et al. 2007; Voyles et al. 2009; Kilpatrick et al. 2010; Wilber et al. 2017).

A pathogen rarely infects a host alone (Petney and Andrews 1998; Pedersen and Fenton 2007; Rynkiewicz et al. 2015; Betts et al. 2016). The presence of coinfecting species or strains may alter the within-host dynamics of an individual pathogen (Mideo 2009). For example, in the host there may be competition for resources (Pedersen and Fenton 2007; Mideo 2009), including key nutrients such as iron (Ratledge and Dover 2000; Nairz et al. 2010; Kramer et al. 2019). These within-host interactions may in turn alter their between-host dynamics, as the presence of competitors may mean host exploitation is optimal (Frank 1996;

Alizon et al. 2013) with reduced selection for transmission in order to secure a larger proportion of resources within the host (McKenzie and Bossert 1998; Greischar et al. 2016). Furthermore, coinfection will likely alter the evolutionary trajectory of pathogens, as adaptations that are beneficial in the presence of competitors may be costly on their own (Mideo 2009). For example, in the endoparasite *Pasteuria ramose* strains that were found to perform well in mixed infections within the host *Daphnia magna* had lower infectivity when infecting alone (Ben-Ami et al. 2008).

Within-host interactions are of vital importance for understanding infection outcomes (Foster 2005; West et al. 2007; Leggett et al. 2014; Rezzoagli et al. 2020). It has been well established that pathogens can interact cooperatively to successfully to colonise a host, for example via forming biofilms (Griffin et al. 2004; Kreft 2004; Foster 2005; Diggle et al. 2007b; West et al. 2007). These interactions frequently involve the production and secretion of public goods into the local environment (West et al. 2006, 2007; Leggett et al. 2014). Public goods benefit all the cells in the local environment, but they are costly to produce and thus are susceptible to exploitation by pathogens which may utilise but not produce public goods (non-producers) (Velicer 2003; West et al. 2007). For example, the production of compounds known as siderophores, that are used to bind to and uptake iron (Buckling et al. 2007; Kramer et al. 2019), benefitting all bacteria in the local area as iron is often limited within a host (Ratledge and Dover 2000; Nairz et al. 2010). The release of products such as siderophores is regulated by communication between pathogens using small autoinducer molecules in a process known as quorum sensing (Keller and Surette 2006; Williams et al. 2007; West et al. 2007; Rumbaugh et al. 2009). Non-producers for both siderophore production and quorum sensing (QS) have been shown to arise in infections (De Vos et al. 2001; Köhler et al. 2009; Jiricny et al. 2014; Andersen et al. 2015). Yet when populations are

sampled at random non-producers are rare (Köhler et al. 2009; Andersen et al. 2015), suggesting that non-producers could be poor at within-host colonisation or between-host transmission. Compared to our understanding of how pathogens interact within a host, relatively little is known about how within-host processes contribute to variation in pathogen transmission between hosts (Brown et al. 2009; Handel and Rohani 2015; VanderWaal and Ezenwa 2016; Stephenson et al. 2017).

In this study, we use the well-established model pathogen, *Pseudomonas aeruginosa* [e.g. (Griffin et al. 2004; Buckling et al. 2007; Diggle et al. 2007b)] to investigate the impact of two non-producer strains (siderophore and QS non-producers) on within- and between-host dynamics. Investigating pathogen transmission between hosts can be logistically challenging as it requires large population sizes (Handel and Rohani 2015). We thus used the model *Caenorhabditis elegans* as the host, enabling us to have large populations with natural between-host faecal-oral transmission (Kenney et al. 2005; Diaz and Restif 2014). We began by tracking the within-host dynamics of producers and non-producers, in single infections and coinfections, over time. We varied the order of exposure of the different types of pathogen to determine whether priority effects altered within-host interactions (Goodman and Ross 1974; de Roode et al. 2005; Jackson et al. 2006; Clay et al. 2018). We then introduced infected nematodes into uninfected populations and measured rates of between-host transmission. We investigated whether differences in pathogen strain transmissibility were due to variation in host preference for ingesting particular pathogen strains or in host shedding into the environment. Overall, our study demonstrates that producer pathogens are superior to non-producers at within-host colonisation and between-host transmission in this species interaction.

2. Materials and Methods

Bacterial Pathogen and Nematode Host

Caenorhabditis elegans is a nematode species whose natural diet is composed of microorganisms (Hope 1999). The gut of *C. elegans* can be colonised by a variety of microbes, including pathogens (Clark and Hodgkin 2014). We used two strains of *C. elegans* N2 and CB5584 (*mIs12 II*). CB5584 express fluorescence in their pharynx making them distinguishable from N2 under a fluorescent microscope. We selected CB5584 as it enabled us to identify nematodes from different populations, those exposed to pathogens (N2) and pathogen-naive populations (CB5584). Nematodes were maintained at 20^{o}C on a lawn of food (*Escherichia coli* (OP50)) on nematode growth medium (NGM) plates. To synchronise life cycles, nematodes were treated with bleach (NaClO and sodium hydroxide) which kills everything except unhatched eggs (Hope 1999). After bleaching, nematodes were synchronized overnight in M9 buffer and maintained for two days.

We exposed nematodes to the gram-negative pathogen, *Pseudomonas aeruginosa*. This bacterium is an opportunistic pathogen of plants and animals, including humans (Tan et al. 1999). We used three strains of *P. aeruginosa* in our experiments with the same genetic background (PAO1 strain background). The 'producer' strain was: PAO1 WT::GFP (WT labelled with GFP, which we herein refer to as PaO1) and two 'non-producer' strains were: PAO1 Δpvd::mCherry (siderophore (pyoverdin) non-producer strain labelled with mCherry, which we herein refer to as ΔPVD); and PAO1 ΔLASR::mCherry (quorum sensing non-producer labelled with mCherry, which we herein refer to as ΔLASR) (Rezzoagli et al. 2019).

For our investigation of nematode preference, we also used two strains of *Pseudomonas fluorescens:* a producer (CHA0) and a non-producer (CHA019) of *gacS* defensive toxins

151 (Jousset et al. 2009). *C.elegans* have previously been shown to preferentially graze on the
152 non-producer, CHA019 (Jousset et al. 2009), and so we used it as a positive control. All
153 bacteria were stored at -80^{o}C in a 1:1 ratio of sample to 50% glycerol solution in cryotubes.
154 Strains of *Pseudomonas* bacteria were grown overnight at 37^{0}C in Lysogeny broth (LB)
155 shaking at 200 r.p.m. OP50 was grown under the same conditions at 30^{o}C.
156
157 ***Within-host colonisation and dynamics***
158 Nematodes at the L4 stage were transferred onto a lawn of either PaO1, ΔLASR or ΔPVD for
159 24 hours. For the first stage of the experiment, a sample of the nematodes were removed from
160 the single pathogen treatment after 24 hours and washed according to the droplet method
161 (Ford et al. 2016) (Figure S1). For the second stage, there was a two-pathogen treatment,
162 where the pathogen strain was switched after 12 hours, with an additional washing stage
163 between transfers (See Figure S1). There were four different treatments according to the
164 order of primary and secondary pathogen exposure: *i)* PaO1 to △LASR; *ii)* PaO1 to △PVD;
165 *iii)* △LASR to PaO1; and *iv)* △PVD to PaO1. Approximately five nematodes were used to
166 calculate the number of colony-forming units (CFUs). CFUs were a metric of pathogen load
167 and estimated by taking 4-5 clean nematodes in 90µl of M9 in a 1ml Eppendorf tube with
168 microbeads which was placed in a bead-beater for one minute at 2800rpm. After crushing,
169 serial dilutions were plated onto KB and grown overnight at 30^{o}C. The number of colonies
170 was counted. Treatments for both stages of the experiment consisted of five biological
171 replicates, and the whole experiment was replicated four times.
172
173 With a two-pathogen treatment, within-host pathogen load was measured at the secondary
174 exposure stage (24 hours). To investigate within-host pathogen load at the primary exposure
175 stage (12 hours) we conducted an additional assay with an added stage of CFU collection

after the primary exposure. We added a time control of nematode food OP50 to this assay (Figure S2). After 12 hours, CFUs were collected from 4-5 clean nematodes, and the remaining nematodes were washed and transferred to the secondary exposure stage (Figure S2). After 12 hours on the second plate, CFUs were collected from 4-5 clean nematodes. In the statistical analysis below, we refer to the time at which pathogens were introduced to the nematodes (either at 0 hours or after 12 hours) as the 'transfer'. This experiment was replicated three times.

Between-host dynamics

To investigate between-host dynamics, we used nematodes from the within-host assay (see Figure S1). After the nematodes' exposure in the first instance to a single pathogen (either non-producer or producer) and in the second stage exposure to a two-pathogen treatment, a sample of 10 clean nematodes were transferred to a pathogen-naive population of GFP labelled nematodes (see Figure S1, Part 2). After 24 hours, we took 4-5 pathogen-naive nematodes from each plate to calculate pathogen load by counting the CFUs within nematodes. We were able to distinguish, and selectively pick, the pathogen-naive nematodes using a fluorescent microscope.

To determine whether nematodes preferred to consume producers or non-producers, we conducted a preference assay. Bacterial cell density was measured at an absorbance of 600 nm (A_{600}) and then standardized to the same density using M9. We used these standardised cultures to make equidistant 20µL spots on a 90mm Kings Broth (KB) media (Ghoul et al. 2016) plate (see Figure S3A). Each KB plate contained one spot of PaO1 and a spot of either ΔPVD, ΔLASR, CHA0 or CHA019. We left the bacteria to dry and then incubated the plates overnight at 30^{o}C prior to the aversion assays taking place. We

introduced approximately 40 clean nematodes to the centre of to the prepared plates with equidistant PaO1 and an alternative pathogen (ΔLASR, ΔPVD, CHA019 or CHA0) (as in (Abada et al. 2009)). We recorded the approximate time of bacteria drying as 0 hours and left the plates for 6 hours (by this time nematodes are likely to have established their preference (Shtonda and Avery 2006; Ballestriero et al. 2016)). Treatments consisted of six biological replicates and the whole experiment was replicated five times. After 6 hours we recorded the number of nematodes on the producer, non-producer or neither colony.

To investigate how many pathogens were shed from the nematode host into the environment we exposed nematodes to either a producer (PaO1) or non-producer *P. aeruginosa* (ΔLASR or PVD) for 12 hours. These exposed nematodes were cleaned and transferred to an empty NGM plate (with a minimal lawn of OP50). After 12 hours, these nematodes were picked off the plate and the pathogen cells liberated using M9 buffer, the bacteria from the plate were then grown and counted for comparison (Figure S3B).

Statistical analysis

Within-host colonisation and dynamics

To compare pathogen loads within hosts exposed to either a non-producer or producer, we fit a linear model with 'CFU per nematode' as the response variable and 'pathogen' (three treatments: *i*) PaO1; *ii*) ΔLASR; and *iii*) ΔPVD) as the explanatory variable. We then carried out an ANOVA with this model and ran post-hoc Tukey tests. When nematodes were exposed to both a non-producer and producer (four 'pathogen' treatments, written in order of exposure: *i)* PaO1 to ΔLASR; *ii)* PaO1 to ΔPVD; *iii)* ΔLASR to PaO1; and *iv)* ΔPVD to PaO1), we fit a quasipoisson generalised linear model to compare pathogen loads (with either 'non-producer CFU per nematode' or 'producer CFU per nematode' as the response variable

and ‘pathogen’ as the explanatory variable). To assess the significance of ‘pathogen’, we carried out a likelihood ratio test with this model and a second model from which the ‘pathogen’ term had been dropped. We then ran post-hoc Tukey tests using the ‘glht()’ function from the multcomp package (Hothorn et al. 2008) to assess pairwise differences.

For the next stage of the experiment, in which we investigated the within-host dynamics for both the primary (0-12 hours) and secondary (12-24 hours) exposure stages, our analysis had three components. Firstly, we compared pathogen loads across each treatment (PaO1, ΔPVD and ΔLASR) and investigated whether there was an interaction between the pathogen treatments and the transfer between exposure stages. We fit a mixed-effects model with ‘CFU per nematode’ as the response variable; ‘pathogen’, ‘transfer’ and their interaction as fixed effects; and plate number as a random effect on the intercept. The random effect was needed to account for the repeated measurement of CFUs from each population (once at 12 hours and again at 24 hours). We carried out an ANOVA with this model to compare CFUs across pathogen treatments and time. Secondly, we calculated the difference between the pathogen treatments CFUs at 12 hours and 24 hours (difference = CFU at 24 hours – CFU at 12 hours) to determine whether the pathogen introduced at 12 hours influenced load of the primary pathogen. We then compared these differences using paired t-tests. Finally, we compared pathogen loads for the pathogens that was introduced at the secondary exposure stage (after 12 hours). To do this, we fit a linear model and carried out an ANOVA, with introduced pathogen CFUs as the response variable and ‘pathogen’ as an explanatory variable, to determine whether the colonisation of the introduced pathogen was affected by the pathogen introduced at the primary exposure stage.

Between-host dynamics

To investigate between-host dynamics within the pathogen-naive nematode populations (introduced to nematodes that had been singly infected with either a producer or a non-producer) we compared pathogen loads across treatments by fitting a linear model with 'CFU per nematode' as the response variable and 'pathogen' (three treatments: *i)* PaO1; *ii)* ΔLASR; and *iii)* ΔPVD) as the explanatory variable. We carried out an ANOVA to calculate F ratios for the explanatory variable 'pathogen' and ran post-hoc Tukey tests. To compare the pathogen loads from the pathogen-naive populations, where the introduced infected nematodes had been exposed to both producers and non-producers (four 'pathogen' treatments, written in order of exposure: *i)* PaO1 to ΔLASR; *ii)* PaO1 to ΔPVD; *iii)* ΔLASR to PaO1; and *iv)* ΔPVD to PaO1), we fit a quasipoisson generalised linear model (GLM). For the GLM the response variable was either the pathogen load of non-producers ('Non-producer CFU per nematode') or that of the producer ('Producer CFU per nematode') and 'pathogen' as the explanatory variable. To assess the significance of the explanatory variable 'pathogen', we carried out a likelihood ratio test with this model and a second model from which the 'pathogen' term had been dropped. We then ran post-hoc Tukey tests.

To investigate whether nematodes displayed a preference for either producers or non-producers, we calculated a choice index:

$$Choice\ Index = \frac{Number\ of\ Nematodes\ in\ PaO1\ Colony - Number\ of\ Nematodes\ in\ Alternative\ Colony}{Total\ number\ of\ Nematodes}$$

Where a value of one indicates complete preference for the producer, 0 indicates a lack of preference and -1 indicates complete preference for the alternative strain (all non-producers except for CHA0). We tested whether the choice indexes were significantly different from zero (indicating no preference) using one sample t-tests. Finally, to compare the number of

CFUs present on the lawn of a plate after an infected population of nematodes had been removed, we fit a quasipoisson generalised linear model with 'CFU per nematode' as the response variable and 'pathogen' as the explanatory variable. We conducted a likelihood ratio test with this model and a second model from which the 'pathogen' term had been dropped and ran post-hoc Tukey tests between the pathogen treatments.

All of the statistical analysis and data presentation was carried out in R version 3.6.2 (R Core Team 2019) using RStudio (RStudio Team 2021) and the packages 'dplyr' (Wickham et al. 2017), 'ggplot2' (Wickham 2009), 'multcomp' (Hothorn et al. 2008), 'cowplot' (Wilke 2020) , 'stargazer' (Hlavac 2018), 'broom' (Robinson et al. 2021), 'lme4' (Bates et al. 2015) and 'lmerTest' (Kuznetsova et al. 2017). Results were considered to be statistically significant when $p<0.05$.

3. Results

Within-host dynamics: Producers are better than non-producers at colonising nematode hosts

The producer, PaO1, was better able to colonise the nematode host than both non-producers, as the pathogen load (CFUs per nematode) was significantly higher for PaO1 than ΔLASR or ΔPVD (Post-hoc Tukey tests; ΔLASR – PaO1: Difference = -11200, $p < 0.001$; ΔPVD – PaO1: Difference = -8870, $p < 0.01$, see Figure 1, Table S1). There was no difference between the pathogen loads for nematodes exposed to ΔLASR or ΔPVD (Post-hoc Tukey test; ΔPVD – ΔLASR: Difference = -2336, $p = 0.19$, Figure 1, Table S1), but overall, there was a significant effect of 'pathogen' treatment ($F_{2,57} = 39.8$, $p < 0.001$, Table S1, Figure 1).

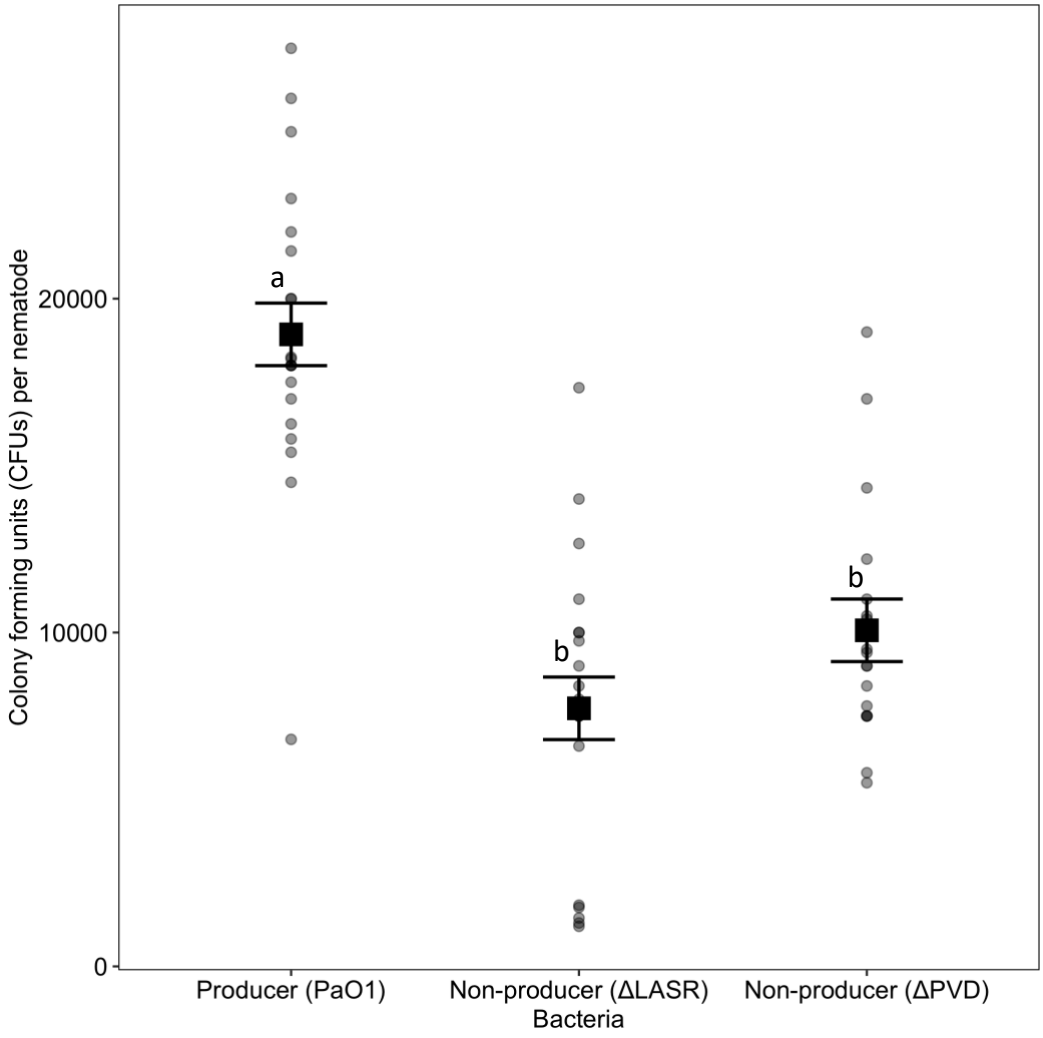


Figure 1: Within-host colonisation. Difference in the pathogen load, measured by the number of colony forming units (CFUs) per nematode, after exposure to one of the three strains of *P. aeruginosa* between pathogen treatments. Treatments with different letters are significantly different. Square points with error bars represent mean +/- 1 SE.

When nematodes were exposed to both producers and non-producers, producers colonised at a similar level across pathogen treatments within the nematode host (no effect of treatment: likelihood ratio: deviance = 2500, p = 0.365, Figure 2A, Table S2B). There was a significant effect of pathogen treatment for non-producer CFUs (deviance = 100414, $p < 0.001$, Figure 2A, Table S2A). The pathogen load of both ΔPVD treatments (PaO1 to ΔPVD and ΔPVD to PaO1) were significantly higher than both ΔLASR treatments (PaO1 to ΔLASR and ΔLASR to PaO1), regardless of the order of exposure, with ΔPVD to PaO1 having a significantly higher number of CFUs per nematode than all the other treatments (Figure 2A, Table S2A).

Between primary and secondary exposure stages (12 and 24 hours), there was a significant interaction of pathogen treatment and time for ΔLASR ($F_{1,16} = 6.40$, p = 0.02, Table S3A, Figure 2C), in which there was a significant decrease in pathogen load when the nematodes were transferred to PaO1 (Difference = -1776, SE = 602, t = -2.95, p = 0.01, Table S3B) but a small increase in pathogen load when transferred to food (Difference = 204, SE = 602). Similarly, for ΔPVD there was a greater decline in pathogen load when nematodes were transferred to PaO1 than when they were transferred to food (ΔPVD to PaO1: Difference = -3065, SE = 514, t = -5.96, $p < 0.001$; ΔPVD to food: Difference = -1536, SE = -3.04, t = -3.04, p = 0.0078, Figure 2B, Table S3B), but this interaction was marginally non-significant ($F_{1,16}$= 4.26, p = 0.0554, Table 3A).

For nematodes colonised by PaO1, between primary and secondary exposure stages there was a smaller effect of transferring the nematode to a second pathogen on pathogen load than there was for those nematodes colonised by ΔPVD or ΔLASR (Figure 2D), and there was no interaction between 'pathogen' and 'transfer' ($F_{1,16} = 2.70$, p = 0.08, Table S3A). There was no significant effect of transferring nematodes colonised with PaO1 to either ΔPVD or food

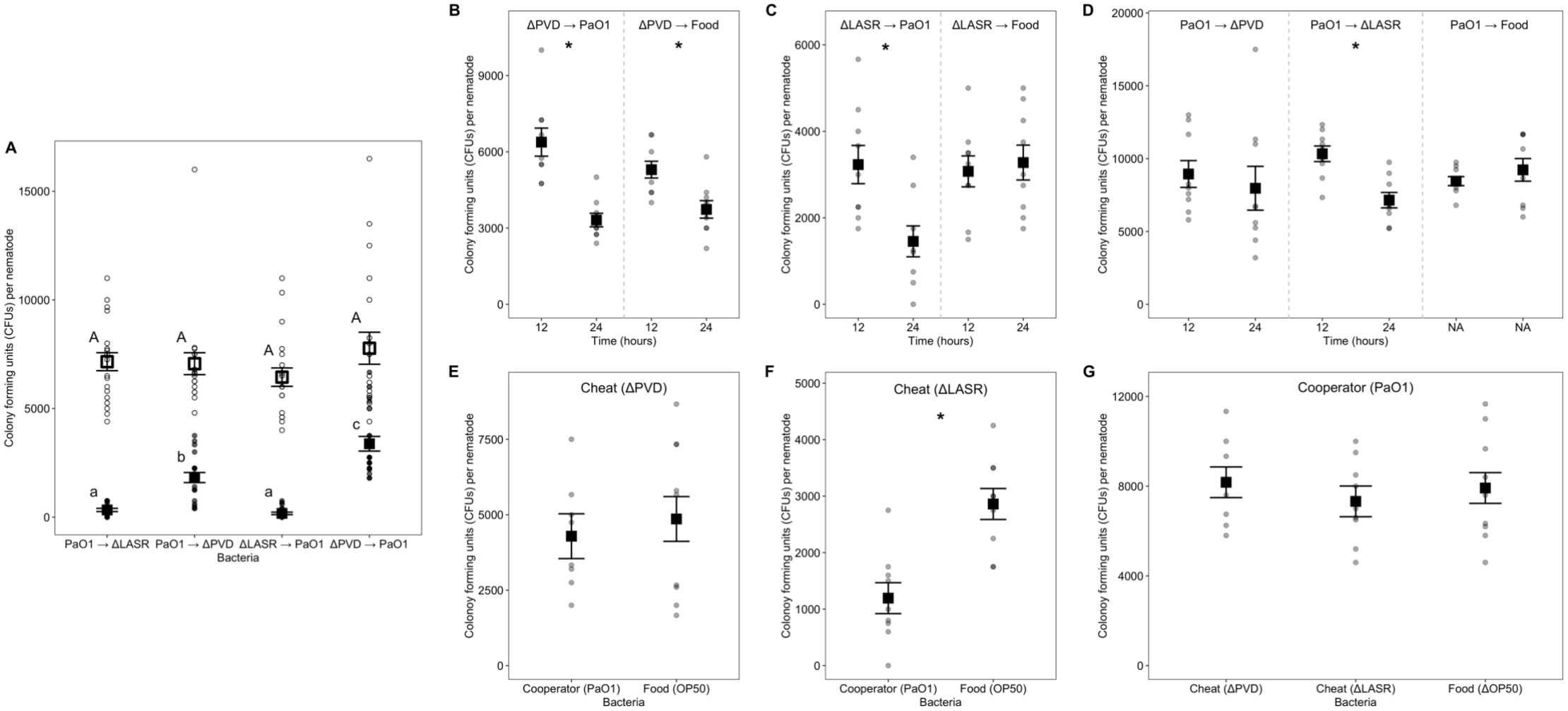


Figure 2: Within-host colonization and dynamics of producer and non-producer pathogens. A) Difference in the producer (white) and non-producer (black) pathogen loads, measured by the number of colony forming units (CFU) (per nematode), after the exposure to both pathogen treatments (PaO1, ΔLASR and ΔPVD). The x axis labels are given in order of exposure. **B-D)** Primary Exposure: CFU (per nematode) for the different pathogen treatments after 12 hours and 24 hours. The order of exposure to the different bacteria strains is shown at the top of the graphs. **E-G)** Secondary Exposure: Difference in the number of CFUs of each strain of bacteria when exposed to a control (Food (OP50)) vs in the presence of a producer (PaO1) or non-producer (ΔPVD or ΔLASR). Points with different letters are significantly different from each other in A. Significant differences in plots B-G are denoted by with a star (*). Square points with error bars represent mean +/- 1 SE.

but transferring these nematodes to ΔLASR caused a significant decrease in the load of PaO1 (Difference = -3183, SE =1302, t = -2.45, p = 0.02, Table S3B, Figure 2D). However, when a single outlier in the PaO1 to ΔPVD treatment is removed (see Figure 2D) there is a significant interaction between 'transfer' and 'pathogen' ($F_{1,16}$ = 4.24, p = 0.02, Table S3A). This interaction arises from the decrease in pathogen load of PaO1 when nematodes were transferred to either type of non-producer, but no change in pathogen load when the nematodes were transferred to food.

When directly comparing the pathogen load of nematodes colonised by non-producers transferred to producers to those transferred to food (OP50), we found no difference for ΔPVD ($F_{1,16}$= 0.296, p=0.594, Figure 2E, Table S4). Whereas, for ΔLASR, the pathogen load of nematodes transferred to PaO1 was significantly lower than those transferred to food ($F_{1,16}$ = 18.52, p = 0.0005, Figure 2F, Table S4). There was no change in PaO1 load when nematodes were transferred to either of the non-producers or food (ANOVA: $F_{1,16}$ = 0.408, p = 0.67, Figure 2G).

Between-host dynamics: Producers are better at between-host transmission than non-producers

When infected nematodes that were exposed to only a single type of pathogen were introduced to the pathogen-naive population, the producer (PaO1) was best able to spread to new hosts with a significantly higher pathogen load in the pathogen-naive worms than both non-producer pathogens ($F_{2,57}$ = 7.73, p = 0.001, Post-hoc Tukey tests; PaO1 to ΔLASR: Difference = -3440, p = 0.030; ΔPVD to PaO1: Difference = -5040, p = 0.0009, see Figure 3A, Table S5). Similarly, when nematodes had been infected by producers and non-producers, the pathogen load of PaO1 in the pathogen-naive populations of nematodes

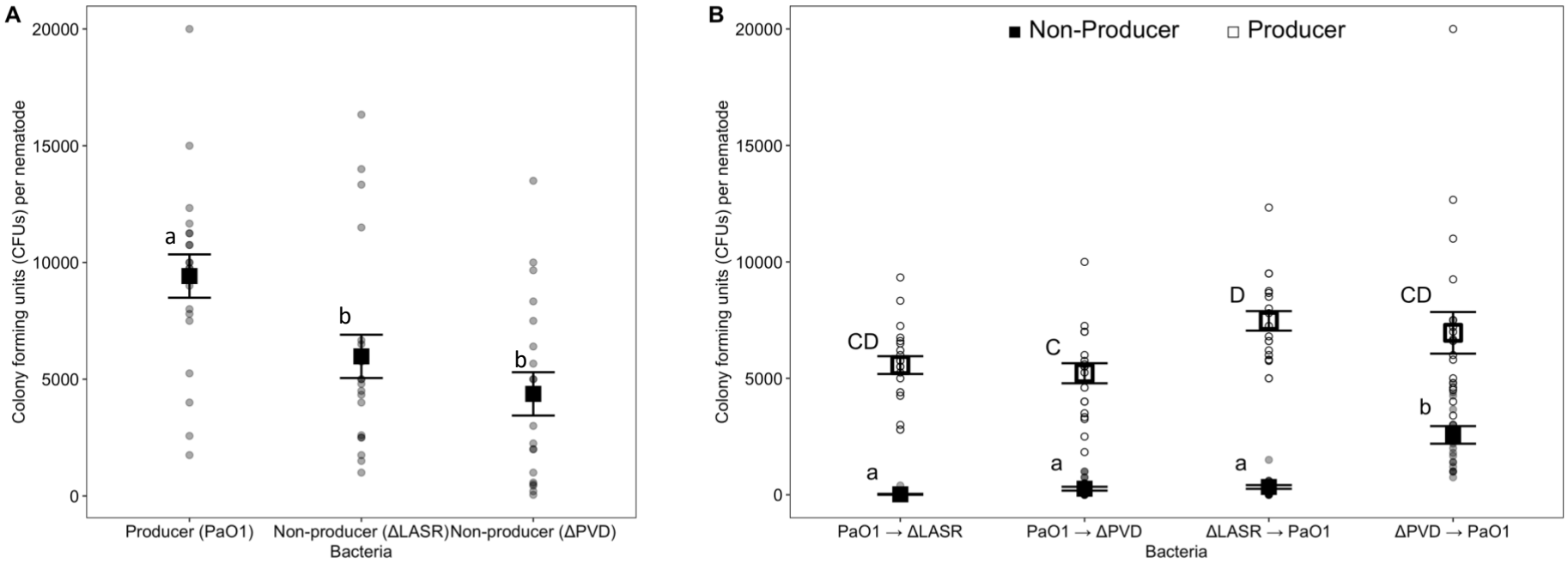


Figure 3: Between-host dynamics of producer and non-producer pathogens. A) Difference in the pathogen load (number of colony forming units (CFUs) per nematode) from the pathogen-naive population after the infected nematodes are introduced between the pathogen treatments. **B)** Difference in the number of producers (white) and non-producers (black) in the colony forming units (per nematode), from the pathogen-naive population after the infected nematodes are introduced into their population, between pathogen treatments. The x axis labels are given in order of exposure. Points with different letters are significantly different from each other. Square points with error bars represent mean +/- 1 SE.

remained consistently high (Figure 3B). Although pathogen loads were similar across treatments, there was a significant effect of 'pathogen' (Likelihood ratio test: 'producer' deviance = 11100, p = 0.01, Table S6B) likely driven by the statistically significant difference between the loads of the PaO1 to ΔPVD and ΔLASR to PaO1 treatments (Tukey post-hoc test of difference: 'ΔLASR to PaO1' – 'PaO1 to ΔPVD' = 0.358, p = 0.026). The pathogen loads of nematodes exposed to both ΔPVD and ΔLASR were low and close to zero across treatments (Figure 3B), except for the ΔPVD to PaO1 treatment where there was a significantly higher number of CFUs per nematodes than all the other types of non-producer (Table S6). This higher number of CFUs per nematode for ΔPVD to PaO1 treatment seemed to drive a significant effect of 'pathogen' (Likelihood ratio test: 'non-producer' deviance = 94200, $p < 0.001$).

C. elegans did not display a preference for the non-producer or producer strains of *P. aeruginosa* (see Figure 4A, Table S7A). The choice index was not significantly different from zero for either PaO1 v ΔLASR (Mean choice index = 0.0468, t = 0.733, p = 0.45) or PaO1 v ΔPVD (Mean choice index = -0.0333, t = -0.683, p = 0.5). As a positive control, we found *C.elegans* showed a preference for the non-producer *P. fluorescens* strain CHA019 (Mean choice index = -0.405, t = -12.28, $p < 0.001$, Figure 4B) as in (Jousset et al. 2009). There was no preference for the producer *P. protegens* strain CHA0 (Mean choice index = 0.00641, t = 0.188, p = 0.85, Figure 4B).

Nematodes shed significantly more producers into the environment than either type of non-producer (overall effect of 'pathogen' Likelihood ratio test: Deviance = 64100, $p < 0.001$; Post-hoc Tukey tests; PaO1 – ΔLASR: Difference = -0.825, $p<0.001$; ΔPVD – PaO1: Difference = -0.284, p = 0.007, Figure 4C, Table S7B). There was also a significant

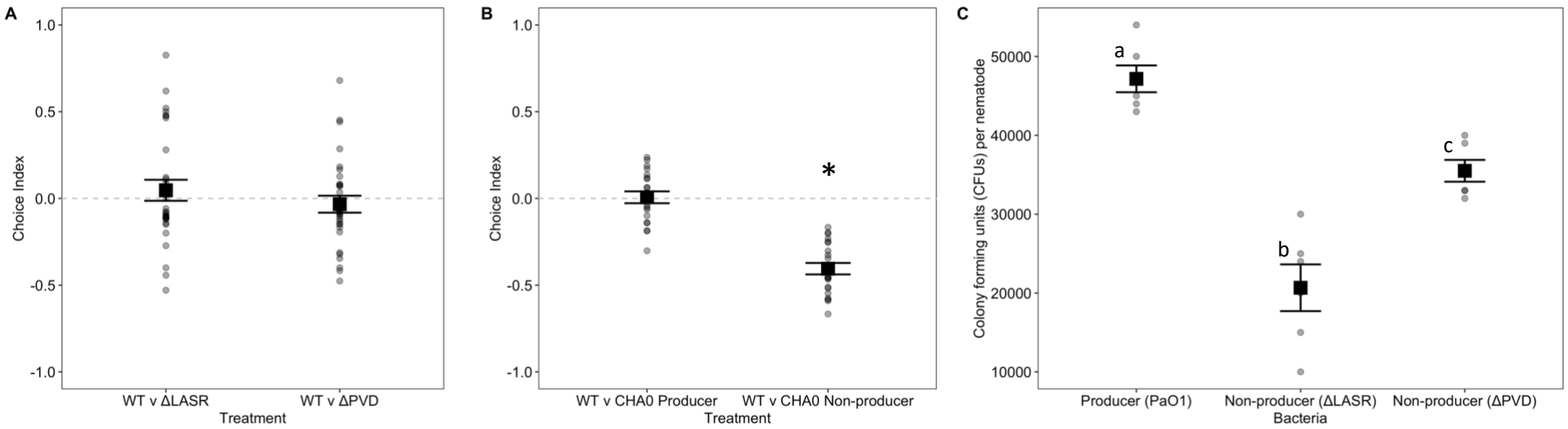


Figure 4: Host preference for picking-up pathogens and host shedding of pathogens in the environment. A&B) Choice Index of *C.elegans* for producer and non-producer strains of *P. aeruginosa*. Where 1 indicates complete preference for the producer strain (PaO1), 0 indicates a lack of preference for either and -1 indicates complete preference for the alternative strain (all non-producers (ΔPVD, ΔLASR and CHAO19) except CHA0). Figure A) shows the choice index for the two focal non-producer strains △LASR and △PVD in comparison to the cooperative PaO1 whereas B) shows the choice index for *P. fluorescens* in comparison to PaO1. * indicates significance. C) Difference the in the number of colony forming units present on a lawn after 12 hours of exposure to infected nematodes. Each of the three points are statistically significantly different from each other denoted by different letters. Square points with error bars represent mean +/- 1 SE.

375 difference between the number of CFUs present on a lawn between the two non-producer
376 strains, with more ΔPVD shed into the environment than ΔLASR (ΔPVD – ΔLASR = 0.541,
377 $p<0.001$, Figure 4C, table S7B).
378
379 **4. Discussion**
380 We revealed that producer pathogens are better at establishing infection within- and between-
381 nematode hosts than non-producers, during coinfection and single infection. There were
382 consistently more producers present in the nematodes when the hosts were directly exposed
383 to the pathogens as well as when the pathogens were spread naturally between hosts. Non-
384 producers were poor at both within-host colonisation and between host transmission, even
385 when coinfected with producers. Our results suggests that for producers, where there is a
386 higher pathogen load within the host than non-producers, there is an increase in their ability
387 to transmit between hosts. We provide empirical support that transmissibility of *P.aeruginosa*
388 depends upon on pathogen load in the host *C.elegans*. This link between pathogen load and
389 transmissibility is a common assumption in theoretical studies [see (Handel and Rohani
390 2015) and references therein], but with few empirical examples such as a guppy (*Poecilia*
391 *reticulata*) ectoparasite (*Gyrodactylus turnbulli*) system (Stephenson et al. 2017).
392
393 The counterbalancing selection pressures experienced by pathogens at the level of within-
394 host colonisation and between-host transmission can lead to trade-offs (Anderson and May
395 1982; Koella and Antia 1995; Walther and Ewald 2004). For example, within a host the
396 ability to reproduce rapidly may be advantageous to outcompete competitors, whereas
397 pathogens that reproduce more slowly may be able to persist for longer periods in the
398 external environment, which could be a beneficial trait for transmission but disadvantageous
399 for colonisation (Walther and Ewald 2004). Although, complexity can arise when hosts are

400 infected with more than one species, which is much more representative of what we observe
401 in nature (Petney and Andrews 1998; Pedersen and Fenton 2007; Betts et al. 2016). With
402 multiple species or strains interacting within a host there is greater competition for the host's
403 resources (Mideo 2009), which in turn may alter the pathogens strategy within the host,
404 tipping the balance towards the immediate benefit of host exploitation rather than the
405 potential future benefit of transmission (Mideo 2009; Alizon et al. 2013). In addition,
406 transmission mode may influence trade-offs between within-host colonisation and
407 transmission (Walther and Ewald 2004; Antonovics et al. 2017). Some pathogen species,
408 such as the malaria apicomplexan parasite (*Plasmodium sp*), require specialised forms to
409 infect new hosts leading to a trade-off in resource allocation for the production of these
410 different stages (Anderson and May 1981; Koella and Antia 1995; Greischar et al. 2016).
411 However, in our study, we do not find evidence for any trade-off between within-host
412 colonisation and transmissibility for the producer pathogens. Producers were more successful
413 at both within-and between-host transmission than non-producers when alone, and even in the
414 presence of co-infecting non-producers.
415
416 We predicted that in the absence of producers, non-producers would be poor at within host
417 colonisation as they lack the ability to either produce iron-scavenging siderophores (ΔPVD)
418 (Buckling et al. 2007) or induce the las quorum sensing pathway (ΔLASR) (Keller and
419 Surette 2006; Diggle et al. 2007b, a; West et al. 2007). We found that non-producers were
420 worse at within-host colonisation and between-host transmission than producers. ΔLASR was
421 consistently relatively poor at colonising and transmitting between hosts compared to ΔPVD,
422 an outcome likely due to the more severe fitness consequences experienced by the ΔLASR
423 non-producer than the ΔPVD non-producer. The ΔLASR non-producer is unable to
424 communicate with other pathogens via quorum sensing (Venturi 2006; Keller and Surette

2006), a crucial trait to the success of *P.aeruginosa* that controls behaviours such as biofilm development and the production of virulence factors (Diggle et al. 2007b; Williams et al. 2007). Nonetheless, the non-producer ΔPVD strain does not produce the primary siderophore pyoverdin (Buckling et al. 2007; Visca et al. 2007), which can negatively impact the fitness of the strain in an iron-limited host environment (Ratledge and Dover 2000; Nairz et al. 2010) in the absence of producers. However, this non-producer ΔPVD strain of *P.aerugionsa* can produce other siderophores and can take up iron from the environment in other ways (Buckling et al. 2007; Visca et al. 2007). In addition, nematode hosts did not preferentially consume pathogenic producers over non-producers, thus there was no difference in the intake of pathogen type, but more producers were shed into the local environment than non-producers, making them more successful at between-host transmission than non-producers.

Counter to our expectations, non-producers were also poor at within-host colonisation and between-host transmission in the presence of producers. This inability of non-producer strains to exploit producers has previously been found in *in-vivo* studies of a nematode host (Rezzoagli et al. 2019), as well as in larvae of the wax moth (*Galleria mellonella*) (Harrison et al. 2006) and the diamondback moth (*Plutella xylostella*) (Zhou et al. 2014). We expect that this is likely due to several factors. Firstly, within the nematode gut there is a relatively low pathogen density (Rezzoagli et al. 2019). Non-producers are most likely to benefit from producers when they exist at a low density (Ross-Gillespie et al. 2007; Rumbaugh et al. 2009) within a high density of producers (Ross-Gillespie et al. 2009) (negative frequency dependence). Secondly, the spatial heterogeneity within the host may not be conducive to cheating (Harrison et al. 2006; Leggett et al. 2014; Rezzoagli et al. 2019). The *in vitro* environment is more homogenous (Pedersen and Fenton 2007; Mideo 2009), allowing non-producers to mix with producers and potentially utilise the products released into the

environment (Griffin et al. 2004; Diggle et al. 2007b; Kümmerli et al. 2009). Whereas, the environment within the host is likely to be more structured, non-producers are more likely to be surrounded their non-producer relatives (Frank 1998), and thus will be less able to directly benefit from the public goods of the producers (Harrison et al. 2006; West et al. 2006; Rezzoagli et al. 2019). Finally, the nematode host itself may impact the non-producer's colonisation ability; for example limited iron availability within the host may remove any benefit of not producing the costly siderophores (Ratledge and Dover 2000; Nairz et al. 2010).

Understanding how social interactions affect within- and between-host dynamics requires further experiments (Mideo et al. 2008; Handel and Rohani 2015). Our study, among others (Diaz and Restif 2014), highlights the benefits of *C.elegans* as a model host for naturally studying between-host dynamics without major logistical challenges (Handel and Rohani 2015). A future avenue for exploration within this system could involve experimentally evolving non-producers de-novo within the nematode that can successfully cheat (Ghoul et al. 2014) to determine how within-host cheating impacts pathogen transmissibility. Cheats have been shown to evolve in natural populations, for example quorum sensing cheats are found in the lungs of cystic fibrosis patients (Diggle et al. 2007). It is predicted that successful within-host cheating could negatively impact the transmission dynamics of an infection, and it could be explored as a potential disease intervention strategy (Brown et al. 2009; Jiricny et al. 2014; Leggett et al. 2014).

Furthermore, when investigating disease dynamics across multiple levels, heterogeneity can arise from multiple sources that could be incorporated further. In particular, host heterogeneity has been demonstrated to impact within- and between-host infection dynamics (Hellriegel 2001; Ganusov et al. 2002; Handel and Rohani 2015), with some hosts

475 contributing disproportionately to the maintenance of an infection within a population
476 (Fenton et al. 2015; VanderWaal and Ezenwa 2016; Stephenson et al. 2017). Further studies
477 incorporating heterogenous host populations, would be highly informative and be much more
478 representative of wild populations (Ganusov et al. 2002; Fenton et al. 2015). In addition,
479 disease dynamics across levels can be hugely impacted by abiotic and biotic stressors in the
480 environment (Greischar et al. 2019). With anthropogenic activities leading to major global
481 ecological changes and environmental stressors on host-pathogen systems (Tylianakis et al.
482 2008; Cahill et al. 2013; Becker et al. 2015; Cable et al. 2017), it is vital we understand
483 parasite interactions within- and between-hosts in order for us to predict their evolutionary
484 responses to change (Cable et al. 2017; Greischar et al. 2019).
485
486 We show that within-host pathogen interactions affect between-host infection dynamics. We
487 provide empirical evidence linking processes occurring at different scales, in a field
488 dominated by theory (Coombs et al. 2007; Mideo et al. 2008; Handel and Rohani 2015),
489 investigating the role of pathogenic social traits importance of disease. Taking a multilevel
490 experimental approach to within- and between-host dynamics is an outstanding and major
491 challenge within evolutionary biology (Mideo et al. 2008). Addressing this challenge will
492 improve our ability to predict and control infection in less well understood systems, such as
493 disease-induced population extinctions (Pedersen et al. 2007; Smith et al. 2009; Wilber et al.
494 2017) and may also allow us to harness pathogen interactions for our own benefit (Smith and
495 Holt 1996; Mideo 2009; Leggett et al. 2014; Rezzoagli et al. 2020).
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Author contributions

V.L.P, K.C.K and A.S.G conceived and designed the study. V.L.P. performed the experiments and analysed the data, with guidance from K.C.K. All authors contributed to writing the manuscript.

Data Archiving

The data and code from this study will be made available from the Dryad digital repository.

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Conflict of Interest

We have no competing interests.

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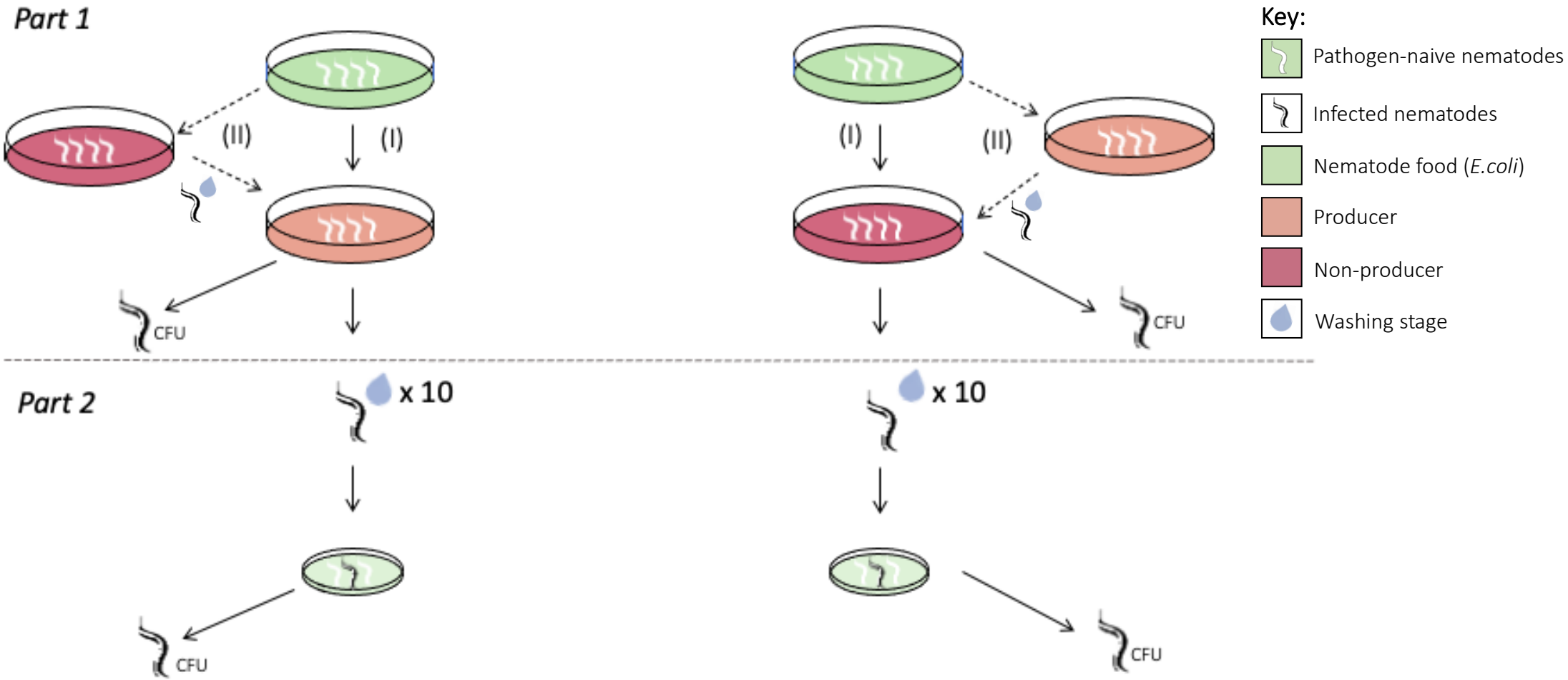
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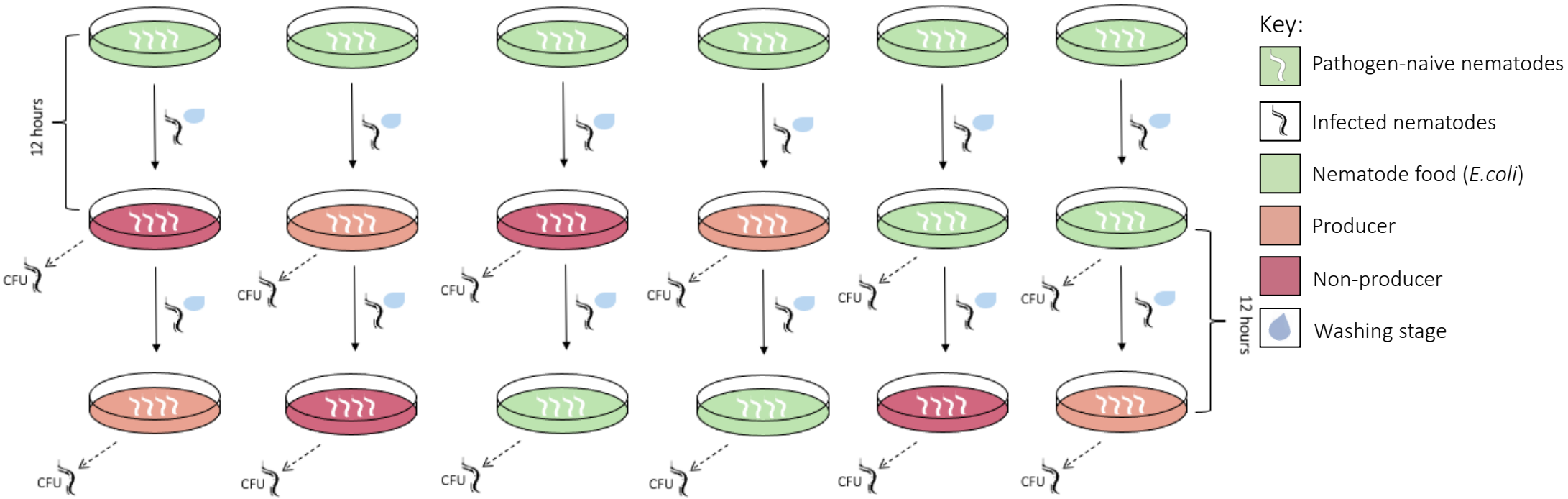
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Supplementary Material

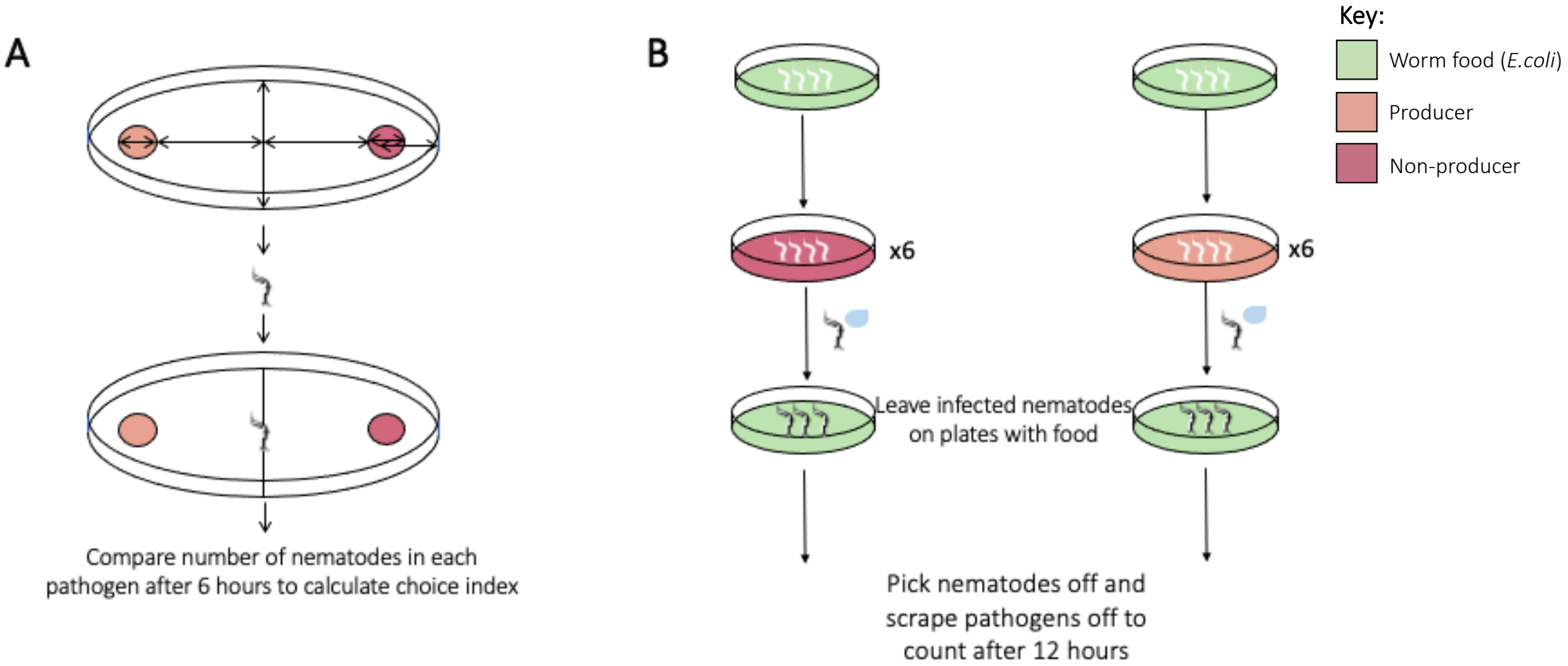
Chapter 4



Supplementary Figure 1: Investigating within- and between-host dynamics. Part 1: Within-host dynamics (I) Nematodes are grown up on food (OP50) until they reach fourth larval stage (L4) when they are exposed to either wild type producer (PaO1) or a non-producer (△LASR or △PVD) of *P.aeruginosa* for 24 hours. The nematodes are washed and the pathogen CFU's are calculated per worm. (II) Follows the same steps as (I) but with one added exposure stage and a transfer after 12 hours. Part 2: Between-host dynamics: ten washed nematodes are transferred to a population of GFP-labelled uninfected worms for 24 hours to transmit infection. Pathogen CFU's per GFP-labelled nematode are recorded.



Supplementary Figure 2: Experimental set up for investigating within-host pathogen loads at the primary (12 hours) and secondary (24 hours) exposure stages. Nematodes are washed between transfers.



Supplementary Figure 3: Experiments investigating host preference for picking-up pathogens and host shedding of pathogens in the environment. A) Preference assay. Equal density samples of wild type producer (pale) and non-producer (dark) *P. aeruginosa* strains are placed equidistant from the center of the plate. 40 nematodes are added to the center of the plate and left for 6 hours at 20^{o}C after which the number of nematodes in the bacteria are recorded to calculate a choice index. B) Host shedding of pathogens in the environment assay. Nematodes are exposed to a producer or non-producer for 24 hours, they are then washed and transferred to a new plate. After 12 hours the nematodes are picked off the plate and the CFUs grown from the plate.

Table S1: Within-host dynamics. Model output for single exposure and post-hoc test.

ANOVA output for transmission experiment stage 1 – non-producer only (exposed N2)

	df	Sum Sq	Mean Sq	F	P
Pathogen	2	1,396,773,445	698,386,723	39.797	1.52246987475705 x 10-11
Residuals	57	1,000,265,918	17,548,525		

Tukey post hoc test – non-producer only (exposed N2)

Comparison	Difference	CI Lower	CI Upper	Adjusted P
ΔLASR - PaO1	-11,201	-14,389	-8,014	4.74479344703127 x 10-11
ΔPVD - PaO1	-8,865	-12,053	-5,677	3.09603214176946x 10-8
ΔPVD - ΔLASR	2,337	-851	5,524	0.190944792332241

Table S2: Within-host dynamics A) Model output for two stage exposure and post hoc test output for non-producer CFUs (ΔPVD and ΔLASR) and for B) producer CFUs (PaO1).

A) Likelihood ratio test for quasipoisson glm for non-producer CFUs

Model	Residual df	Residual Deviance	df	Deviance	P
Intercept only	79	139,611			
Pathogen	76	39,197	3	100,414	3.24176664162092 x 10-44

Tukey post hoc test for non-producer CFUs

Comparison	Difference	Std Error	Z	P
"PaO1→ΔPVD" - "PaO1→ΔLASR"	1.706	0.296	5.770	2.37994173257761 x10 -08
"ΔLASR→PaO1" - "PaO1→ΔLASR"	-0.646	0.464	-1.394	0.475890964188774
"ΔPVD→PaO1" - "PaO1→ΔLASR"	2.323	0.285	8.152	1.66533453693773 x 10-15
"ΔLASR→PaO1" - "PaO1→ΔPVD"	-2.352	0.393	-5.982	6.63580213000614x 10-09
"ΔPVD→PaO1" - "PaO1→ΔPVD"	0.617	0.144	4.293	0.000158475002713931
"ΔPVD→PaO1" - "ΔLASR→PaO1"	2.970	0.385	7.708	4.18554080283684x10-14

B) Likelihood ratio test for quasipoisson glm for producer CFUs

Model	Residual df	Residual Deviance	df	Deviance	P
Intercept only	79	55,976			
Pathogen	76	53,469	3	2,507	0.3648

Tukey post hoc test for producer CFUs

Comparison	Difference	Std Error	Z	P
"PaO1→ΔPVD" - "PaO1→ΔLASR"	-0.013	0.105	-0.122	0.999
"ΔLASR→PaO1" - "PaO1→ΔLASR"	-0.105	0.108	-0.971	0.766
"ΔPVD→PaO1" - "PaO1→ΔLASR"	0.083	0.103	0.808	0.851
"ΔLASR→PaO1" - "PaO1→ΔPVD"	-0.092	0.108	-0.848	0.831
"ΔPVD→PaO1" - "PaO1→ΔPVD"	0.096	0.103	0.930	0.789
"ΔPVD→PaO1" - "ΔLASR→PaO1"	0.188	0.106	1.775	0.285

Table S3A: Investigating interactions between pathogen and stage of exposure in within-host dynamics for A) ΔPVD, B) ΔLASR, C) PaO1 and D) PaO1 with the outlier removed.

A) ANOVA output - N2 12 and 24hr interaction – Non-producer ΔPVD

Term	Sum Sq	Mean Sq	Numerator df	Denominator df	F	P
Pathogen	777,454	777,454	1	16	0.654	0.430710191400823
Transfer	48,186,736	48,186,736	1	16	40.506	9.38616540965786e-06
Pathogen:Transfer	5,075,008	5,075,008	1	16	4.266	0.0554736896615176

B) ANOVA output - N2 12 and 24hr interaction - Non-producer ΔLAS

Term	Sum Sq	Mean Sq	Numerator df	Denominator df	F	P
Pathogen	6,236,119	6,236,119	1	32	4.525	0.0412
Transfer	5,561,736	5,561,736	1	32	4.036	0.0531
Pathogen:Transfer	8,817,600	8,817,600	1	32	6.398	0.0165

C) ANOVA output - N2 12 and 24hr interaction - Producer PaO1

Term	Sum Sq	Mean Sq	Numerator df	Denominator df	F	P
Pathogen	1,443,591	721,795	2	48	0.110	0.8960
Transfer	17,226,858	17,226,858	1	48	2.628	0.1116
Pathogen:Transfer	35,389,290	17,694,645	2	48	2.699	0.0775

D) ANOVA output - N2 12 and 24hr interaction - Producer PaO1 (outlier removed)

Term	Sum Sq	Mean Sq	Numerator df	Denominator df	F	P
Pathogen	8,044,702	4,022,351	2	46	0.879	0.4219
Transfer	32,474,175	32,474,175	1	46	7.100	0.0106
Pathogen:Transfer	38,804,516	19,402,258	2	46	4.242	0.0204

Table S3B: Differences between pathogen treatments when investigating the interactions between bacteria and stage of exposure in within-host dynamics for A) ΔPVD, B) ΔLASR and C) PaO1.

A) Primary (12hr) and secondary (24 hr) exposure differences – Non-producer ΔPVD

	Difference	Std Error	t	P
ΔPVD→PaO1	-3,065	514	-5.961	1.99478141444201e-05
ΔPVD→OP50	-1,563	514	-3.040	0.0078

B) Primary (12hr) and secondary (24 hr) exposure differences – Non-producer ΔLAS

	Difference	Std Error	t	P
ΔLAS→PaO1	-1,776	602	-2.952	0.0094
ΔLAS→OP50	204	602	0.339	0.7393

C) Primary (12hr) and secondary (24 hr) exposure differences - Producer PaO1

	Difference	Std Error	t	P
PaO1→ΔPVD	-980	1,302	-0.753	0.4590
PaO1→ΔLAS	-3,183	1,302	-2.445	0.0222
PaO1→OP50	774	1,302	0.595	0.5576

Table S4: Model output for comparing control CFUs when exposed to food to those exposed to a producer or non-producer treatment.

12 and 24 hr – secondary exposure stage – comparison of pathogen and food treatments

Second Pathogen	Term	df	Sum Sq	Mean Sq	F	P
ΔPVD	Pathogen	1	1,463,951	1,463,951	0.296	0.5941
	Residuals	16	79,201,173	4,950,073		
ΔLASR	Pathogen	1	12,500,000	12,500,000	18.525	0.0005
	Residuals	16	10,796,111	674,757		
PaO1	Pathogen	2	3,439,588	1,719,794	0.408	0.6697
	Residuals	24	101,241,975	4,218,416		

Table S5: Between host dynamics. Model output for single exposure stage and post-hoc tests.

ANOVA output for transmission experiment stage 1 – Non-producer only (exposed N2 Naive)

	df	Sum Sq	Mean Sq	F	P
Pathogen	2	266,000,423	133,000,212	7.726	0.0011
Residuals	57	981,175,992	17,213,614		

Tukey post hoc test – Non-producer only (exposed N2 Naive)

Comparison	Difference	CI Lower	CI Upper	Adjusted P
PaO1 - ΔLASR	3,440	283	6,597	0.0297
ΔPVD - ΔLASR	-1,608	-4,765	1,550	0.4433
ΔPVD - PaO1	-5,048	-8,205	-1,891	0.0009

Table S6: Between host dynamics. Model output for two stage exposure and post-hoc tests for A) non-producers (ΔPVD and ΔLASR) and B) producers (PaO1).

A) Likelihood ratio test for quasipoisson glm non-producers (exposed N2 naive)

Model	Residual df	Residual Deviance	df	Deviance	P
Intercept only	79	133,436			
Pathogen	76	39,259	3	94,178	9.79059749265308e-34

Tukey post hoc test non-producers (exposed N2 naive)

Comparison	Difference	Std Error	Z	P
"PaO1→ΔPVD" - "PaO1→ΔLASR"	2.575	1.272	2.024	0.1543735260584
"ΔLASR→PaO1" - "PaO1→ΔLASR"	2.823	1.262	2.237	0.0957031056723139
"ΔPVD→PaO1" - "PaO1→ΔLASR"	4.857	1.231	3.946	0.000339384657785713
"ΔLASR→PaO1" - "PaO1→ΔPVD"	0.249	0.451	0.551	0.93841964903327
"ΔPVD→PaO1" - "PaO1→ΔPVD"	2.282	0.355	6.423	4.31572111381229e-10
"ΔPVD→PaO1" - "ΔLASR→PaO1"	2.033	0.318	6.398	5.01496844051985e-10

B) Likelihood ratio test for quasipoisson glm producers (exposed N2 naive)

Model	Residual df	Residual Deviance	df	Deviance	P
Intercept only	79	80,169			
Pathogen	76	69,056	3	11,113	0.0112

Tukey post hoc test producers (exposed N2 naive)

Comparison	Difference	Std Error	Z	P
"PaO1→ΔPVD" - "PaO1→ΔLASR"	-0.065	0.136	-0.476	0.964243999944474
"ΔLASR→PaO1" - "PaO1→ΔLASR"	0.293	0.125	2.343	0.0884608791614553
"ΔPVD→PaO1" - "PaO1→ΔLASR"	0.222	0.127	1.747	0.298710576573992
"ΔLASR→PaO1" - "PaO1→ΔPVD"	0.358	0.128	2.808	0.0258013574782604
"ΔPVD→PaO1" - "PaO1→ΔPVD"	0.287	0.130	2.216	0.118429799065706
"ΔPVD→PaO1" - "ΔLASR→PaO1"	-0.071	0.118	-0.604	0.930652962888251

Table S7: Investigating host preference and the shedding of pathogens into the environment. A) Output for choice assay t-test and B) Output for shedding into environment assay and associated post-hoc test.

A) Choice assay - Producer vs Non-producer t-tests - output table

Comparison	Difference	Std Error	t	P
PaO1 - ΔLASR	0.047	0.061	0.773	0.445980843262594
PaO1 - ΔPVD	-0.033	0.049	-0.683	0.499995772345799
PaO1 - CHA0 Producer	0.006	0.034	0.188	0.853084623174605
PaO1 - CHA0 Non-producer	-0.405	0.033	-12.276	9.0882955154233e-11

B) Likelihood ratio test for quasipoisson glm – Pathogens shed into the environment (Lawn CFUs)

Model	Residual df	Residual Deviance	df	Deviance	P
Intercept only	17	81,119			
Pathogen	15	17,029	2	64,090	1.30607652055112e-13

Tukey post hoc test - Pathogens shed into the environment (Lawn CFUs)

Comparison	Difference	Std Error	Z	P
ΔLASR - PaO1	-0.825	0.112	-7.372	2.42361686275672e-13
ΔPVD - PaO1	-0.284	0.094	-3.014	0.00699912031736816
ΔPVD - ΔLASR	0.541	0.117	4.608	9.06211326889839e-06

Chapter 5

Why don't all animals avoid inbreeding?

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Why don't all animals avoid inbreeding?

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Abstract

Individuals are expected to avoid mating with relatives as inbreeding can reduce offspring fitness, a phenomenon known as inbreeding depression. This has led to the widespread assumption that selection will favour individuals that avoid mating with relatives. However, the strength of inbreeding avoidance is variable across species and there are numerous cases where related mates are not avoided. Here we test if the frequency that related males and females encounter each other explains variation in inbreeding avoidance using phylogenetic meta-analysis of 41 different species from six classes across the animal kingdom. In species reported to mate randomly with respect to relatedness, individuals were either unlikely to encounter relatives, or inbreeding had negligible effects on offspring fitness. Mechanisms for avoiding inbreeding, including active mate choice, post-copulatory processes and sex biased dispersal, were only found in species with inbreeding depression. These results help explain why some species seem to care more about inbreeding than others: inbreeding avoidance through mate choice only evolves when there is both a risk of inbreeding depression and related sexual partners frequently encounter each other.

1) Introduction

Mating with relatives (inbreeding) can result in offspring with reduced survival and fertility, a phenomenon known as inbreeding depression (1). Inbreeding depression has been observed to occur in wild animals with severe consequences (2,3). This has led to the prediction that selection will favour individuals that base mate choice decisions on relatedness (2,4–7) . However, studies of mate choice have failed to provide consistent support for this prediction, with a recent meta-analysis highlighting the lack of evidence for inbreeding avoidance across species (8). While many species have been found to strongly prefer unrelated mates, for example long-tailed tits, *Aegithalos caudatus* (9), in other species, such as the yellow-bellied toads, *Bombina variegata* (10) and common terns, *Sterna hirundo* (11), mate choice decisions are not influenced by relatedness. Some species even show a preference for mating with relatives, such as the ground tit *Parus humilis* (12), the cichlid fish *Pelvicachromis taeniatus* (13,14) and White's skink *Liopholis whitii* (15). Why do some species actively avoid mating with relatives while others do not, and does this pose a problem for our understanding of inbreeding depression and mate choice?

A potential answer to why species vary in the extent to which they choose unrelated mates may be found by considering how inbreeding is avoided (4,5,16–18). Individuals can reduce the chance of mating with relatives via a number of different mechanisms, including sex biased dispersal, kin recognition and extra-pair copulations (4,5,19). Some mechanisms, such as sex bias dispersal, are expected to reduce average relatedness in the population, resulting in a low risk of inbreeding and weakening selection for mate avoidance (17). In olive baboons (*Papio Anubis*), for example, relatedness between males and females is low because of sex

biased dispersal (20), a phenomenon that is widespread across mammals and birds (18). In other species, sex biased dispersal is absent and avoiding inbreeding may require other mechanisms, such as use of genetic cues for distinguishing between related and unrelated individuals (9,21).

The mechanism of inbreeding avoidance determines the strength of selection for actively discriminating against related mates through two key processes. First, there must be a chance of encountering a related sexual partner (i.e. the risk of inbreeding is high) (22). If related mates do not interact, then selection for active mate avoidance will be weak or absent, even if there is inbreeding depression (16). Second, choosing unrelated mates is only predicted to occur when there is a risk of inbreeding depression (23). The severity of inbreeding depression can be highly variable both within (2) and across species with there being strong reductions in fitness sometimes, such as in collared flycatchers, *Ficedula albicollis* (24) while in other species inbreeding can even be beneficial (13,25,26). It is only when these two conditions are combined - related individuals interact and there is inbreeding depression - that there will be selection for mate avoidance. However, the risks of mating with relatives are often overlooked in studies of inbreeding avoidance and are rarely matched with data on inbreeding depression.

To understand variation in inbreeding avoidance across species, we conducted a phylogenetic meta-analysis collecting data on relatedness between mates, average male-female relatedness within populations, inbreeding depression and mechanisms of inbreeding avoidance that do not require active mate choice (mating behaviour and sex-biased dispersal). Data was extracted from 40 studies on 41 species from across the animal kingdom. The presence and

absence of inbreeding depression was recorded based on reports from authors, as well as calculating an effect size of the relationship between offspring fitness and the level of inbreeding, which was possible for a subset of species (16 species). Using this data, we addressed the following questions: Is variation in relatedness between mates explained by the frequency that relatives encounter each other and inbreeding depression? Are all mechanisms of inbreeding avoidance equally effective? What mechanism of inbreeding avoidance do species use most frequently? Is random mating with respect to relatedness more likely to occur when other mechanisms of inbreeding avoidance reduce the frequency with which relatives interact?

2) Methods

Literature Search

We conducted a literature search on Web of Science using the following keyword search strings 'inbreeding avoidance', 'incest avoidance', 'inbreeding preference' and 'incest preference' up to and including 6th January 2021 (see Figure S1 for PRISMA flow chart (27) and Tables S24-S26 for a full list of studies and searches). We manually screened these results based on titles and abstracts. Studies on humans, plants and captive populations were excluded, which made up a large proportion of the search results (Figure S1) as they are often used to investigate inbreeding (2,28). Next, we performed full text screening of articles of interest (for details of the inclusion and exclusion see Table S24) and carried out backwards and forwards citation searches on the studies. Our final dataset comprised of 40 papers representing 41 different species from six different classes: Aves, Mammalia, Reptilia, Amphibia, Insecta and Actinopterygii.

Data Collection

Estimating Inbreeding avoidance

The standard measure of inbreeding is Wright's inbreeding coefficient (f) (29). The value of f represents the probability that two alleles in an individual are identical by descent (1,29). However, f does not indicate the level of inbreeding avoidance, as it does not consider levels of relatedness across the whole population. We therefore analysed the strength of inbreeding avoidance by examining the degree of relatedness between pairs in relation to the average relatedness between males and females in the study population (Table 1). To do this, we collected two measures from each study population:

i) The average relatedness between mating pairs (*rPairs*)

ii) The average relatedness between males and females in the population (*rAverage*)

For two species (*Pan troglodytes* and *Marmota flaviventris*) there were estimates from two different populations with the same sample size for which averages of *rPairs* and *rAverage* were included in analyses. Consequently, all analyses included one estimate per species. Author reports of whether mate choice was random with respect to relatedness (presence and absence of inbreeding avoidance) and estimates of population size were also recorded for each study (Table 1).

Estimates of relatedness between mated pairs and opposite sex pairs are correlation coefficients bounded by -1 (lower than average relatedness) and 1 (clonal population) (30). These estimates were transformed using Fisher's Z (*Zr*) before analysis using the following formula:

Table 1: Details of data collection

Information required	**Data collected**
Does the species avoid inbreeding?	Authors statement on inbreeding avoidance (yes/no) If the author did not find any evidence for inbreeding avoidance, we classified the species as having no mechanism of inbreeding avoidance in our analysis
What method of inbreeding avoidance does a species employ?	Mechanism(s) of inbreeding avoidance recorded by the author: Active Mate Choice (yes/no)? Sex Biased Dispersal (yes/no)? Post-Mating Avoidance (Post-Copulatory and Extra-Pair Copulation) (yes/no)?
Does the species have inbreeding depression?	Authors statement on inbreeding depression (yes/no) If this was not available and no other studies investigated inbreeding in the species, risk of inbreeding depression was assumed to exist. Quantitative estimates of inbreeding depression were included in analysis if available (a mean value or using a correlation between inbreeding and fitness)
Relatedness between pairs (*rPairs*)	The average relatedness reported between breeding pairs in the study population
Average relatedness between males and females in the population *(rAverage)*	The average relatedness between males and females in the study population. We considered these pairs to be 'potential mates' We excluded studies where the relatedness between males and females in the population may be affected by other factors e.g., if all opposite sex pairs in a population are related
Information on species dispersal	If all individuals disperse If dispersal is sex biased If mating occurs pre- or post-dispersal
Does the species mate randomly?	Species were considered to mate randomly if there was no effect of relatedness on mate choice

141 $$Z_r = \frac{1}{2} log_e \left| \frac{1+r}{1-r} \right|$$

142

143 Throughout the analyses these are referred to as: 1) *ZrPairs*, transformed effect sizes of mean
144 relatedness between breeding pairs in the study population, and 2) *ZrAverage,* transformed
145 effect sizes of mean relatedness between all pairs in the study population which we consider
146 to be the pool of potential mates.

147

148 ***Classifying mechanisms of inbreeding avoidance***

149 We classified the mechanisms of inbreeding avoidance according to the author's evidence.
150 We classified the mechanisms into three groups: active mate choice, sex-biased dispersal and
151 post-mating avoidance (Table 1). A species was classified as having 'active mate choice'
152 when the author reported evidence of any type of mate choice based on relatedness. When the
153 author had reported evidence of female or male biased dispersal from the natal group prior to
154 mating, a species was classified as having sex-biased dispersal. Post-mating avoidance was
155 defined as any mechanism of inbreeding avoidance occurring after mating including extra-
156 pair copulations (in these cases extra-pair copulations were a mechanism of avoiding
157 inbreeding, although this is not always the case (31)) and post-copulatory inbreeding
158 avoidance. Species without any known mechanism of inbreeding avoidance were classified as
159 "none" (Table 1). We also recorded whether a species was a cooperative breeder, as they
160 frequently interact with relatives which could facilitate the recognition of relatives.

161

162 ***Estimating inbreeding depression***

163 Inbreeding depression was estimated in two ways. First, we calculated an effect size of
164 inbreeding depression as the Pearson's correlation coefficient (*r*) between fitness and levels of

inbreeding (*rDepression*). Studies presented data on inbreeding depression as means, standard deviations and the sample sizes of inbred versus outbred offspring that were converted to *r*. Estimates of *rDepression* were transformed to Fisher's Zr using the formula above (*ZrDepression)*. Values were extracted from the studies in our main analysis, except for three cases (32–34) where different studies were cited (35–37). Values were either extracted from the main text of the study or from figures using 'Webplotdigitizer'(38). Inbreeding depression was estimated using a variety of different fitness measures (3,39,40) which we categorised into three groups; reproductive success ($N_{Species} = 5$), mortality ($N_{Species} = 7$) and body mass ($N_{Species} = 4$). We examined the sensitivity of our results to the type of fitness measure used by analysing differences in *ZrDepression* between these categories (see SI).

Inbreeding depression is often difficult to quantify in natural populations (3,26). For example, in some species the occurrence of inbreeding is too rare to calculate any associated costs (e.g. (9)). As a result, it was only possible to get estimates of *ZrDepression* for 16 species. In order to examine inbreeding depression in a greater number of species, a second method based on authors statements on the presence or absence of inbreeding depression was used to classify species (referred to as "reported inbreeding depression", Table 1). Where authors did not report whether a species suffered from inbreeding depression or not ('not mentioned') we assumed species suffer deleterious consequences of mating with related individuals, as this is the most likely outcome (41) (for analyses and discussion of this assumption see SI). For details of classifications for all species see Table S1. We examined the robustness of reported inbreeding depression by examining the relationship between reported measures of inbreeding depression and *ZrDepression* (see SI). There was good correspondence between the methods, and we therefore present analyses using reported inbreeding depression due to the larger

sample size (41 species versus 16 species). Analyses using *ZrDepression* are presented in the supplementary information (Tables S14-20 and S22).

Statistical Analysis

General statistical techniques

All statistical analysis were conducted in R (42). Data were analysed using Bayesian Phylogenetic Multi-level Meta-regressions (BPMM) with Markov chain Monte Carlo (MCMC) estimation implemented in the 'MCMCglmm' package (43). Data were from a taxonomically diverse range of species. Phylogenetic non-independence between species was accounted for by creating a phylogenetic tree (see Figure 1A) using the R package 'rotl' (44) that accesses information from the open tree of life (45). A phylogenetic variance-covariance matrix was included in all models as a random effect. Data points were weighted by the inverse sampling variance (1/(n–3)) associated with each of the effect sizes *ZrPairs*, *ZrAverage* and *ZrDepression* using the "mev" term in MCMCglmm.

Specific analyses

We ran seven models in total. First, intercept-only BPMMs were used to quantify heterogeneity, I^2 (46,47), in *ZrPairs*, *ZrAverage* and *ZrDepression* (Models 1.1, 1.4 & 1.7). We calculated phylogenetic heritability (H^2) and total heterogeneity (I^2_{Total}) as specified in (46) using modified code from (48)) which was as follows:

$$H^2 = \frac{\sigma^2_{phylogeny}}{\sigma^2_{phylogeny} + \sigma^2_{residual}}$$

$$I^2_{Total} = \frac{\sigma^2_{phylogeny} + \sigma^2_{residual}}{\sigma^2_{phylogeny} + \sigma^2_{residual} + \sigma^2_{m}}$$

Where σ_m^2 is the measurement error variance. Second, we tested if there was a general effect of inbreeding avoidance across species using a BPMM with *ZrPairs* as the response variable, and *ZrAverage* as a fixed effect (Model 2). If there is no evidence for inbreeding avoidance across species then there will be a 1:1 relationship between *ZrPairs* and *ZrAverage,* indicating no deviation in relatedness between mated pairs from between random pairs of males and females from the population. This was tested by examining if the 95% credible interval (CI) of the slope of *ZrAverage* encompassed one. A slope of *ZrAverage* statistically significantly less than 1 is expected if there is inbreeding avoidance, indicating lower relatedness between mated pairs than the average relatedness between random pairs from the population. Third, the impact of inbreeding depression on mate choice for unrelated mates was tested using a BPMM with *ZrPairs* as the response variable, and *ZrAverage*, reported inbreeding depression (categorical variable with two levels: yes/no) and their interaction term as the fixed effects (Model 3). Fitting the interaction between inbreeding depression and *ZrAverage* enabled us to test if there was inbreeding avoidance (relationship between *ZrPairs* and *ZrAverage* significantly less than one) for species with and without inbreeding depression.

Fourth, we tested if the probability of evolving at least one mechanism for avoiding inbreeding was dependent on inbreeding depression using a BPMM (binomial error distribution with logit link function). The response variable was the number pairs with a mechanism of inbreeding avoidance versus number pairs without. In practice, authors reported that species either had or did not have a mechanism of inbreeding avoidance, but using number of pairs as a binomial response rather than a binary response of presence absence enabled differences in sample size across studies to be modelled. Reported inbreeding depression was fitted as a fixed effect. This model (Model 4) and the following

three (Models 5-8) were restricted to only species with reported evidence of inbreeding depression ($N_{species}$ = 34). Model 5 investigated whether different mechanisms of inbreeding avoidance were equally effective at reducing relatedness between pairs, and more effective than the species without a known mechanism for inbreeding avoidance. *ZrPairs* was the response variable and the mechanism of inbreeding avoidance (4-level categorical variable; active mate choice, sex bias dispersal, post-mating avoidance and no avoidance), *ZrAverage* and their interaction were fitted as fixed effects. Model 6 investigated whether some mechanisms of inbreeding avoidance work by reducing the probability of individuals encountering related mates. *ZrAverage* was the response variable, and the mechanism of inbreeding avoidance was a fixed effect. Model 7 tested whether choosing mates randomly with respect to relatedness was more likely to occur when other mechanisms of inbreeding avoidance reduced average relatedness in populations. Random mating was a binomial response variable (logit link function) of the number of pairs with and without random mating and the mechanism of inbreeding avoidance was included as a fixed effect. Model 8 addressed whether there was an effect of cooperative breeding on inbreeding avoidance. *ZrPairs* was the response variable with cooperative breeding (2-level categorical variable: yes, no), *ZrAverage* and their interaction term as fixed effects. A social insect was excluded from this analysis as it did not fit into the category of cooperative or non-cooperative breeder leaving 33 species.

Default priors were used for fixed effects (independent normal priors with zero mean and large variance (10^{10})) and inverse gamma priors were used (V = 1, nu = 0.002) were used for random effects. Gaussian models were run for 2,000,000 iterations with a burn-in of 1,000,000 and a thinning level of 1000 and binomial models were run for 6,000,000 iterations

with a burn-in of 1,000,000 and a thinning level of 5000. We checked for convergence of the models by running them three times, and visually checking their traces. Convergence was quantitively assessed using Gelman and Rubin's convergence diagnostic (see Table S21 & S23). We selected the results of one of the three models at random for estimating model parameters. Results were considered to be statistically significant when the 95% CI's for differences did not overlap specified values (e.g. slope of 1) and when the pMCMC (percentage of iterations above or below a test value correcting for the finite sample size of posterior samples) values were <0.05. The percentage of variation explained by fixed effects ($R^2_{marginal}$) was calculated for all BPMMs following (49) using code modified from (49).

Publication bias tests

Evidence for publication bias was examined in three ways for *ZrPairs* and *ZrDepression*. First, asymmetry in funnel plots was examined using meta-analytic residuals from intercept only models plotted against precision estimates (square root of inverse sampling variance). Second, an Egger's regression test for funnel plot asymmetry was performed by fitting models with the effect size as the response variable weighted by their inverse sampling variances, the standard error of effect size as a fixed effect and the same random effects used in meta-analytical models. For *ZrPairs* we also included *ZrAverage* as we expect publication bias to influence the residual variation around the relationship between *ZrPairs* and *ZrAverage*: high values of *ZrPairs* relative to *ZrAverage* are used to conclude there is inbreeding avoidance. There was no evidence of publication bias for *ZrPairs* or *ZrDepression* (see Models PB 1-PB 3; Figures S2-3, Tables S10 & S12). Third, a test for time-lag bias was conducted by fitting the same models as specified for the Egger's regressions, but the 'year of publication' was

included as a fixed effect instead of the effect size standard error (as in (50)). There was no evidence of time-lag bias (CIs of year spanned 0 in all models: Tables S11 & S13).

For all data, code, model runs, results of analyses (Tables S1-S20) and methods and results of the verification analyses see supplementary information. All details of the R packages used and their versions can be found in the supplementary code (session_info object).

3) Results

Animals generally avoid inbreeding

We found that species generally avoid inbreeding (Figure 1). Average relatedness between pairs was close to zero and significantly lower than expected from population averages (slope of Zraverage versus 1: posterior mean (PM) = -0.8, CI = -1.25 to -0.42, pMCMC <0.0001. Table S2 & S3). There were exceptions, such as the social ant *Hypoponera opacior* (51) that had relatively high rates of inbreeding, but overall relatedness between pairs was consistently low (Figure 1). Consequently, there was relatively little variation in estimates of relatedness between pairs (I^2_{Total} = 21%, CI = 5% to 42%. Table S2), particularly compared to variation in average relatedness in populations and inbreeding depression (ZrAverage: I^2_{Total} = 37%, CI = 19% to 59%. ZrDepression: I^2_{Total} = 97%, CI = 94% to 99%. Table S2).

The strength of inbreeding avoidance depends on the risk of inbreeding depression

In most species where inbreeding depression has been reported, mated pairs were less related than the population average (22 species out of 34 are below the 1:1 line in Figure 1B & S6). In fact, across all species where inbreeding depression has been found, relatedness between mates was low (Figure 2, Table S4; Model 3: BPMM: PM = 0.21, CI = -0.22 to 0.69,

pMCMC = 0.18, Tested against 1: Difference = -0.79, CI = -1.22 to -0.31, pMCMC = 0.001). Even in species where the average relatedness in the population was high, such as cooperatively breeding species, we found no evidence for increased levels of inbreeding (Table S9; Model 8: BPMM: Difference between cooperative and non-cooperative breeders = -0.02, CI = -1.41 to 1.35, pMCMC = 0.49, Cooperative breeders difference from 1 = -0.91, CI = -2.24 to 0.42, pMCMC = 0.081; non-cooperative species difference from 1 = -0.88, CI = -1.42 to -0.3, pMCMC = 0.003).

In contrast, in species where inbreeding depression has not been reported, mates were as related, or even more closely related, than to the population average (5 species out of 7 are above the 1:1 in Figure 1B): Relatedness between pairs was positively correlated with average relatedness in the population and did not statistically significantly differ from one (Figure 1B & 2, Table S4. BPMM Model 3: PM = 0.55, CI = -0.88 to 2.03, pMCMC = 0.25, Difference of slope from 1; PM = -0.45, CI = -1.88 to 1.03, pMCMC = 0.29). This indicates that for species where there is no evidence of inbreeding depression, individuals do not avoid mating with relatives. However, it is important to note that there are a limited number of species for which there is no evidence of inbreeding depression (n = 7). Species with and without evidence of inbreeding depression were widely distributed across the phylogeny showing inbreeding avoidance and inbreeding depression are relatively labile over evolutionary time (Figure 1). It is also possible that inbreeding depression has been reduced in some species as a result of purging deleterious alleles (28,52).

Mechanisms of inbreeding avoidance only evolve when there is a risk of inbreeding depression

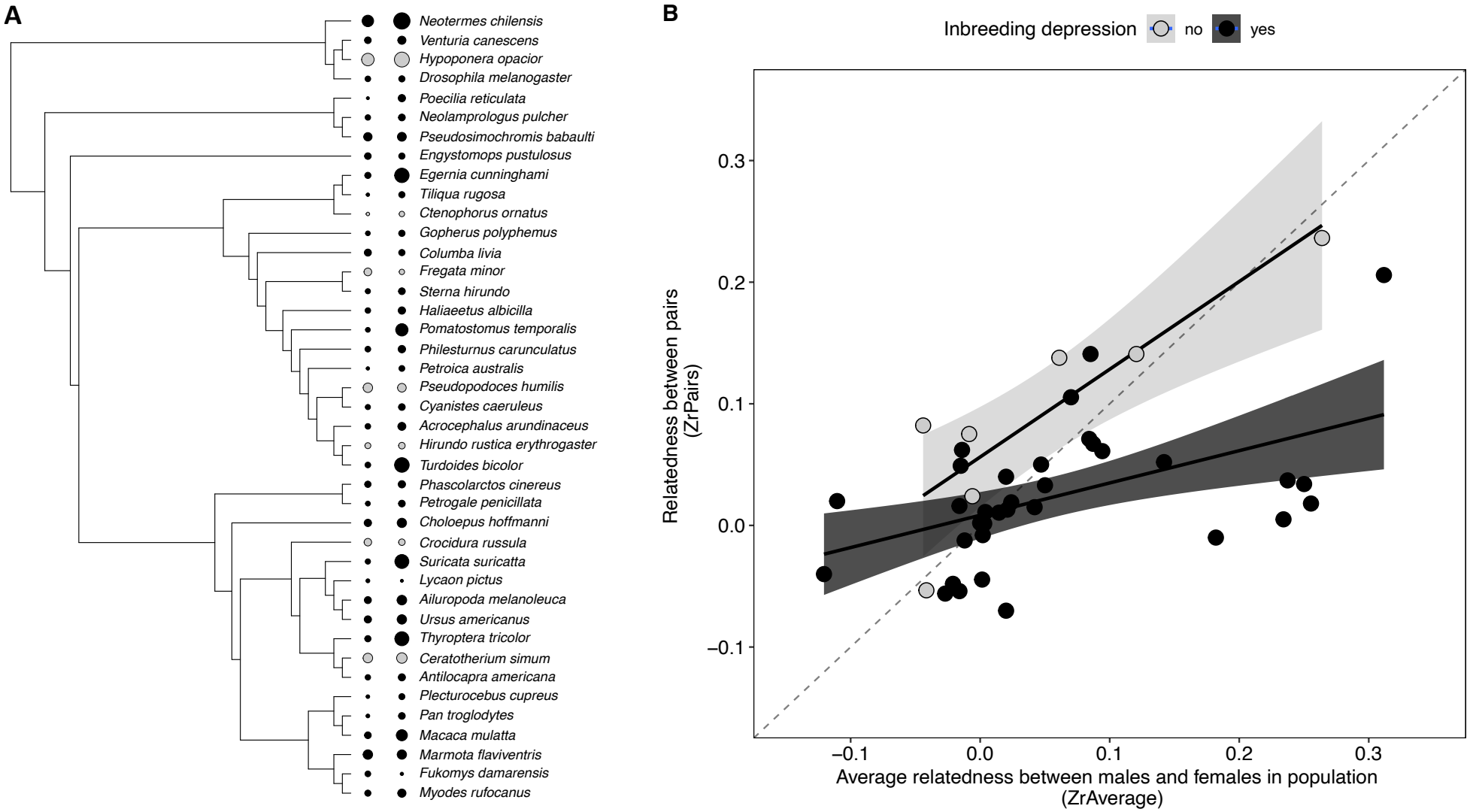


Figure 1: Variation in inbreeding avoidance across species. A) Phylogenetic tree of species included in the analysis with *rPairs* (left) and *rAverage* (right) represented by the size of points and inbreeding depression by colour (black = reported inbreeding depression, grey = no reported inbreeding depression). B) The relationship between average relatedness between males and females in the population and between mates for species with (black) and without (grey) reported inbreeding depression. The dashed line represents 1:1 relationship and the solid lines and shaded areas are regression lines with 95% confidence intervals.

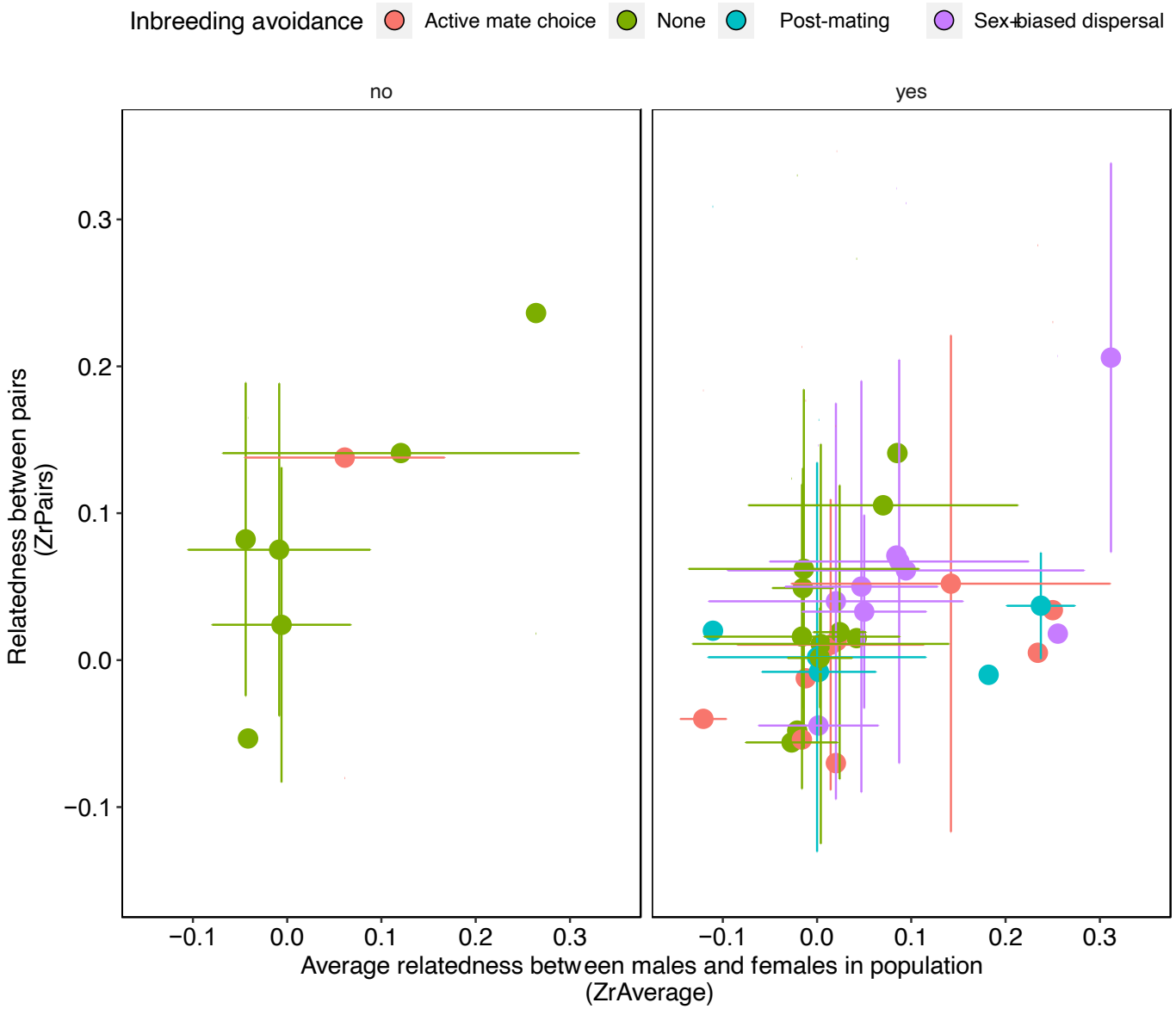


Figure 2: Inbreeding avoidance depends on how frequently relatives are encountered and the presence of inbreeding depression. In species with inbreeding depression, relatedness between mates is consistently low including in populations where average relatedness between potential partners is high whereas for species without inbreeding depression related partners frequently encounter each other and there is no active avoidance of related mates. A) shows species without reported inbreeding depression and B) shows species with inbreeding depression. Points represent mean values ± standard error. The dashed line represents the 1:1 relationship. Different colors represent the mechanism of inbreeding avoidance; no mechanism (green), active mate choice (red), sex-bias dispersal (purple) and post mating avoidance (blue).

330 In species reported to suffer from inbreeding depression ($N_{Species}$=34), avoidance of
331 inbreeding through mate choice (26% of species) was as common as sex biased dispersal
332 (26% of species), and both mechanisms were more common than post-mating avoidance
333 (15% of species). These mechanisms of inbreeding avoidance were only observed in species
334 reported to suffer from inbreeding depression (Figure 2, Table S5; Model 4: BPMM:
335 Difference in posterior means = -0.91, CI = -1 to 0, pMCMC = 0.002). In contrast,
336 mechanisms of inbreeding avoidance were never reported in species without inbreeding
337 depression ($N_{species}$=7). One study on the ground tit *Parus humilis,* observed active mate
338 choice based on relatedness for a preferentially mating with relatives (12). The evolution of
339 all types of inbreeding avoidance mechanisms therefore appears to rely exclusively on the
340 presence of inbreeding depression, although the restricted sample sizes highlight the need
341 further data collection particularly in species without inbreeding depression.
342
343 **Random mating does not necessarily increase the risk of inbreeding**
344 Different mechanisms of inbreeding avoidance were equally effective at reducing relatedness
345 between pairs among species with inbreeding depression. Species where mate choice was
346 random with respect to relatedness, were equally successful at avoiding inbreeding as species
347 where mating with relatives was actively avoided (Figure 2 & S8, Table S6; Model 5;
348 BPMM: Differences between active mate choice & sex biased dispersal PM = -0.28, CI = -1.9
349 to 1.33, pMCMC = 0.353; active mate choice & post mating avoidance PM = 0.12, CI = -1.59
350 to 1.49, pMCMC = 0.43; sex biased dispersal & post mating avoidance PM = - 0.4, CI = -
351 1.82 to 1.11, pMCMC = 0.29). Contrary to expectation, we did not find that species with sex
352 biased dispersal had a lower risk of encountering relatives (Table S7; Model 6, Figure S9):
353 species with different mechanisms of inbreeding avoidance did not significantly differ in their

354 average relatedness amongst potential mates (BPMM: Difference between sex biased
355 dispersal & active mate choice PM = 0.11, CI = -0.04 to 0.24, pMCMC = 0.068, Difference
356 between sex biased dispersal & post mating avoidance PM = -0.04, CI = -0.19 to 0.11,
357 pMCMC = 0.26, Difference between sex biased dispersal & no avoidance PM = 0.08, CI = -
358 0.05 to 0.2, pMCMC = 0.26). However, we did find that species with sex bias dispersal and
359 post-mating avoidance were more likely to choose their mates at random with respect to
360 relatedness (Figure 3, Table S8; Model 7; sex biased dispersal PM = 0.58, CI = 0 to 1,
361 pMCMC = 0.42, post mating avoidance PM = 0.34, CI = 0 to 1, pMCMC = 0.42, active mate
362 choice PM = 0.01, CI = 0 to 0, pMCMC = 0.01, no avoidance PM = 0.89, CI = 0 to 1,
363 pMCMC = 0.11).
364
365 In some species with inbreeding depression, mechanisms of inbreeding avoidance were not
366 reported ($N_{species}$ = 10), a phenomenon often referred to as an 'inbreeding paradox'(53,54).
367 Relatedness between partners in these species, although higher, was not statistically
368 significantly different from species where mechanisms of inbreeding avoidance were known
369 (Figure S8, Table S6, Model 5, Mean relatedness between breeding pairs ± SE: None = 0.033
370 ± 0.014, active mate choice = -0.0069 ± 0.014, post mating avoidance = 0.0082 ± 0.009, sex
371 biased dispersal = 0.052 ± 0.024). Although data are limited and results not statistically
372 significant, species without known mechanisms of inbreeding avoidance were also at a lower
373 risk of encountering related mates in the population (Figure S9, Table S7, Model 6, Mean
374 relatedness between all pairs in the population ± SE: None = 0.019 ± 0.016, active mate
375 choice = 0.058 ± 0.041, post mating avoidance = 0.061 ± 0.044, sex biased dispersal = 0.107
376 ± 0.034). Such trends indicate the need for more data on species with varying degrees of
377 inbreeding depression, but suggest that species which are less likely to meet related mates

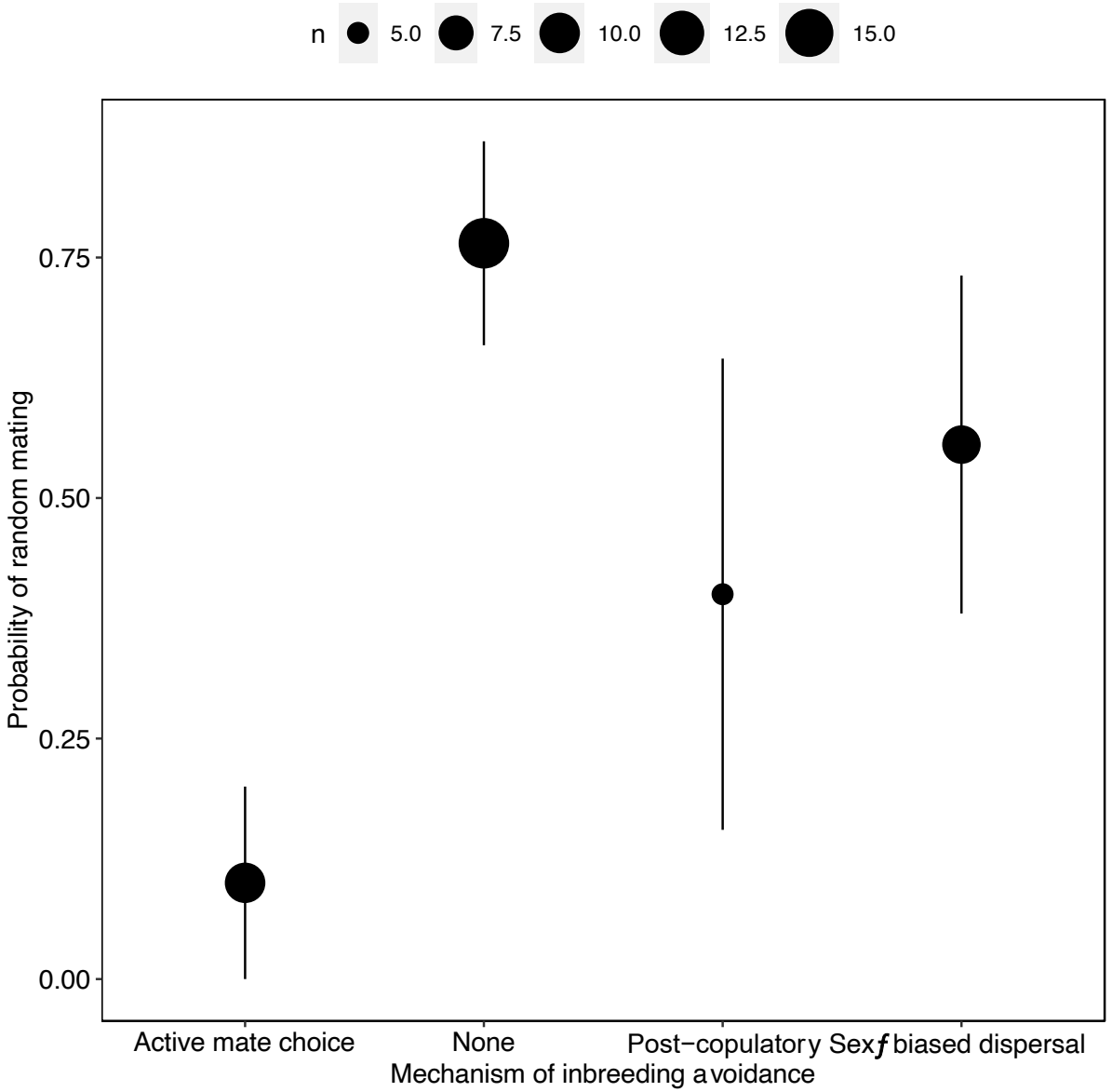


Figure 3: Probability of random mating with respect to relatedness in relation to different mechanisms of inbreeding avoidance (active mate choice ($N_{Species}$=10), none ($N_{Species}$=16), post mating avoidance ($N_{Species}$=5) and sex-biased dispersal ($N_{Species}$= 9**).** Points represent means with 95% confidence intervals and the size of the point represents $N_{Species}$.

lack ways of avoiding inbreeding, increasing the chances of inbreeding if relatives are encountered.

4) Discussion

Patterns of inbreeding avoidance across species show there is little justification for the notion that a lack of inbreeding avoidance in natural populations is surprising, or counter to predictions based on evolutionary theory (2,4,5,8). Animals choose mates based on relatedness when two conditions are fulfilled: when there is a risk related mates encounter each other and when there is inbreeding depression (Figure 2). If these conditions are not met, selection for mate choice based on kinship will be weak, even when inbreeding depression is extremely costly. In species without inbreeding depression, the relatedness between pairs tended to increase with relatedness to potential mates, suggesting inbreeding avoidance does not occur (Figure 2). In some species, there may even be a net benefit of mating with relatives (13,25,26).

Active mate choice did not offer any measurable advantage over other methods of avoiding inbreeding. Individuals can avoid mating with relatives by actively discriminating on the basis of relatedness (either by mate choice or post-mating avoidance) or by reducing the risk of encountering related mates in the population (17). In species where the likelihood of encountering related mates in the population was reduced (e.g., dispersal from the natal territory), mate choice was more likely to be random. The apparent lack of avoiding mating with relatives in some species may therefore be explained by there being a negligible risk of inbreeding. Alternatively, in some species, past inbreeding may have resulted in a selective

decrease in rare, deleterious and recessive alleles that cause inbreeding depression (28,52), a phenomenon known as purging (52), as has been shown in Chillingham cattle (*Bos taurus*) (55). Thus, potential purging could also explain a lack of inbreeding depression and avoidance in some species.

Multiple mechanisms of inbreeding avoidance were not reported in the same species, suggesting that each mechanism might be effective on its own. It should be noted, however, that this could be a by-product of the fact that few studies investigate multiple mechanisms of inbreeding avoidance. One exception is a study by Aguilera-Olivares et al (56) which investigated two different mechanisms of inbreeding avoidance in drywood termites (*Neotermes chilensis*) and found that sex-bias dispersal is the main mechanism of avoiding mating with kin and that there is little evidence of active mate avoidance. Such studies increase the chance of determining which mechanisms of inbreeding avoidance are more likely to evolve in particular species and why. This may help resolve our unexpected finding that sex-biased dispersal was not associated with an overall reduction in average relatedness. For example, other mechanisms may be in place when sex-biased dispersal fails to reduce the chances of inbreeding. In the cooperatively breeding noisy miner (*Manorina melanocephala*), where there is female biased dispersal, average relatedness in the population is high, yet relatedness between breeding pairs is low. The authors suggest that the noisy miners may be capable of detecting kin and actively avoid choosing to mate with relatives (57) given that noisy miners have been shown to recognise kin based on vocalizations in different contexts (58).

425 Selection for adopting a strategy based on reducing encounter rates between relatives is likely
426 to depend on constraints on dispersal. For example, although sex-biased dispersal can reduce
427 the chance of inbreeding (17,18), there are also various costs associated with dispersal, such
428 as energetic costs, increased exposure to predators and risk of failure in finding a suitable
429 breeding site (17,59,60). In addition, habitat fragmentation, as a result of anthropogenic and
430 climate induced factors, may limit the ability of species to effectively disperse, as is the case
431 for male koalas (61). Constraints on dispersing from the natal group are, therefore, likely to
432 generate selection for avoiding mating with relatives by active discrimination. This maybe the
433 case in cooperative breeders such as meerkats (*Suricata suricatta*), which live in extended
434 family groups. Subordinate meerkats are often directly related to other adults in the group and
435 must either "sit and wait" for a potential mating partner to turn up or face increased risks of
436 mortality from leaving the group (62,63).
437
438 Lack of inbreeding avoidance through mate choice in the animal kingdom is often presented
439 as surprising, even a challenge to our understanding of how species should respond to the
440 costs of inbreeding depression (2,4,5). More broadly our results show that the strength of
441 selection against deleterious actions is not simply a product of the cost incurred, but
442 frequency with which it happens. Evidence for the importance of risk on the strength of
443 selection for optimising behaviour also comes from: 1) kin discrimination, where helping kin
444 has a fitness advantage but actively recognising kin only occurs when related and unrelated
445 individuals are frequently encountered (64); 2) paternal care, where caring for unrelated
446 offspring is costly, but adjustment of care by males is not always needed because cuckoldry is
447 rare (65), and 3) sex ratio adjustment, where producing more female offspring is beneficial for
448 single foundresses, but this does not occur in species where multiple females typically lay

449 together (66). Similarly, reproducing with relatives can result in severe fitness costs, but
450 active mate choice is only selected for when related males and females interact (16).
451
452
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457
458 **Data accessibility**
459 The data and code in this study are available on Dryad digital repository
460 (doi:10.5061/dryad.6hdr7sr0t).
461
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467
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Supplementary Material

Chapter 5

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Supplementary analyses

Verification analyses Methods

To examine the robustness of our results to using reported inbreeding depression and differences in the methodological approaches studies used we ran seven supplementary models (Tables S14-20 and S22). First, we tested whether reported inbreeding depression corresponded to *ZrDepression.* A BPMM with *ZrDepression* as the response variable (Gaussian error distribution) and reported inbreeding depression (categorical variable with two levels: yes/no) was included as a fixed effect (Model S1). Second, we repeated the analysis of inbreeding avoidance using *ZrDepression* instead of reported inbreeding depression. A BPMM with *ZrPairs* as the response variable and *ZrDepression* and its interaction with *ZrAverage* fitted as the fixed effects (see Model S2, Table S22). Third, we tested whether the results of Model 2 in the main analysis could be repeated with the smaller sample of studies for which there was a measure of *ZrDepression* (n = 16). A BPMM with *ZrPairs* as the response variable and reported inbreeding depression with its interaction with *ZrAverage* as fixed effects (Model S3).

Fourth, the analysis of the probability of evolving an inbreeding avoidance mechanism was repeated using *ZrDepression* instead of reported inbreeding depression. A BPMM of the probability of evolving inbreeding avoidance as the response variable and *ZrDepression* as a fixed effect (Model S4). Fifth, we compared the three different categories (offspring mortality, offspring mass and reproductive success) of fitness measures used to calculate *ZrDepression,* to see if there was any difference in *ZrDepression* between these measures. A BPMM with *ZrDepression* as the response variable and fitness measure as the fixed effect (categorical variable with three levels) (Model S5).

Sixth, we examined estimates of *ZrPairs* across species with and without known levels of inbreeding depression to test the assumption that for species where inbreeding depression was 'not mentioned' by authors there were similar patterns of inbreeding avoidance to those known to have inbreeding depression. We ran a BPMM with *ZrPairs* as the response variable and the three-category author classification of inbreeding depression (categorical variable with three levels; yes/no/not mentioned) as the fixed effect (Model S6). Finally, we examined if estimates of inbreeding avoidance depended on how relatedness was measured (either via pedigree or genetic markers) in the main analysis with *ZrPairs* as the response variable and the two types of relatedness measure (categorical variable with two levels, Model S7).

Supplementary Results

1) **Do reports of inbreeding depression correspond to estimates of *ZrDepression?***

There was good correspondence between the authors classification of inbreeding depression and *ZrDepression* (Figure S4, Table S22 & S14: Model S1). Effect sizes for 'no' classifications were significantly positive (posterior mode (PM) = 0.65, 95% credible interval (CI) = 0.18 to 1.34, pMCMC = 0.013). This suggests that in some species inbreeding could potentially be beneficial. In contrast, effects sizes for the 'yes' classification were significantly negative (PM= -0.53, CI = -1 to -0.02, pMCMC = 0.017) and were significantly different from the 'no' category (PM = 1.19, CI = 0.72 to 1.59, pMCMC <0.0001).

2) **Are patterns of inbreeding avoidance similar when using *ZrDepression* and reported inbreeding depression?**

There was not a statistically significant interaction between *ZrDepression* and *ZrAverage* (PM: 0.42, CI = -3.29 to 4.08, pMCMC: 0.41, n = 16, Model S2, Figure S6, Table S15 & S22). This result does not align with the findings from Model 2 in our main analysis (Table 2). However, this difference is potentially because of the difference in sample size (n=16 compared to n=41 in main analysis). Furthermore, *ZrDepression* is a continuous variable which tests whether mate avoidance quantitatively increases with inbreeding depression. The presence of even low levels of inbreeding depression may generate sufficient selection for the active avoidance of related mates. If this is the case, then any species with a *ZrDepression* value below zero will avoid related mates. We tested this hypothesis in model S3.

The gradients of *ZrAverage* for species (where ZrDepression>0, 'no') and those (where ZrDepression<0, 'yes') were not different from 1 ("*no":* Difference from 1: PM = -0.41, CI = -4.21 to 3.06, pMCMC = 0.41 , "*yes"* Difference from 1: PM = -0.47, CI = -3.56 to 2.85, pMCMC = 0.39, see Figure S7, Model S3, Table S16 & S22). It is worth noting that for the '*yes*' category, the lack of a difference from 1 is seemingly driven by few data points, especially when compared to the full data set (see Figure 2). Patterns of inbreeding avoidance were therefore somewhat qualitatively similar to those found using measures of reported inbreeding depression, but more quantitative data on *ZrDepression* is clearly needed, especially in species with higher levels of average relatedness in the population (*ZrAverage*).

3) Is the association between the probability of an inbreeding avoidance mechanism and inbreeding depression similar when using *ZrDepression* and reported inbreeding depression*?*

For Model S4, there was not a significant relationship between the probability of species evolving an inbreeding avoidance mechanism and *ZrDepression* (PM: 0.35, CI = 0 to 1,

pMCMC = 0.35, n = 16, Model S4, Table S17 & S22). However, there was a trend of *ZrDepression* being lower for species which displayed inbreeding avoidance (Mean *ZrDepression* ± SE: Species which displayed inbreeding avoidance= -0.29±0.26, Species without a reported mechanism of inbreeding avoidance = -0.11±0.22). The lack of a significant relationship is likely due to the sample of species displaying a mechanism of inbreeding avoidance being much smaller in this analysis than the main analysis ($N_{Species}$ = 7 compared to $N_{Species}$ = 24 in the main analysis).

4) Do estimates of *ZrDepression* differ between fitness measures?

ZrDepression was broadly similar across the different categories of fitness measures, although there was a tendency for estimates of inbreeding depression to be greater when mortality was measured as opposed to body mass (offspring mortality (n=7), offspring mass (n=4) and reproductive success (n=5); Model S5; PM of Difference between two categories: reproductive success + offspring mass = 0.30, CI = -0.71 to 1.23, pMCMC = 0.24, PM of difference between offspring mortality + offspring mass: -0.68, CI = -1.66 to 0.14, pMCMC =0.06 and PM of difference between between offspring mortality + reproductive success: -0.38, CI = -1.21 to 0.44, pMCMC = 0.17). In addition, the percentage of species reported to have inbreeding depression was similar across each fitness measure (species reported to have inbreeding depression: offspring mortality = 6 out of 7; offspring mass = 2 out of 4; and reproductive success 3 out of 5).

5) Testing the assumption that species where the presence of inbreeding depression is not mentioned have inbreeding depression.

We found that estimates of *ZrPairs* for the 'not mentioned' and 'yes' categories tended to be lower than for the 'no' category (Figure S1), providing support for the assumption that

species for which the presence of inbreeding depression is not mentioned are likely to have inbreeding depression. Despite *ZrPairs* being lower for "yes" and the "not mentioned" differences between categories were not significantly different (Difference between 'yes' vs 'not mentioned':PM = -0.01, CI = -0.11 to 0.09, pMCMC = 0.38), Model S6, Table S20 & S22). This is perhaps not surprising given the large variation in estimates of *ZrPairs* across species (Figure 1), which clearly highlights that more detailed data on inbreeding depression is needed, especially in species where inbreeding avoidance has already been measured.

6) Examining if estimates of inbreeding avoidance depend on how relatedness was measured

For the two different types of relatedness measurements across studies, pedigree (n = 6) and genetic markers (n = 34), we found no difference in estimates of inbreeding avoidance (Table S20 & S22, Model S7: Difference between slope of *ZrAverage* for genetic markers and Pedigree PM = -0.32, CI = -2.96 to 2.03, pMCMC = 0.40). This suggests that the type of technique used to measure relatedness is unlikely to influence our results. However, it is important to note the much smaller sample of studies using pedigree based measures (n = 6) may contribute to a lack of difference with genetic measures.

References

1. Olson L, Blumstein D, Pollinger J, Wayne R. No evidence of inbreeding avoidance despite demonstrated survival costs in a polygynous rodent. Molecular Ecology. 2012;21(3):562–71.

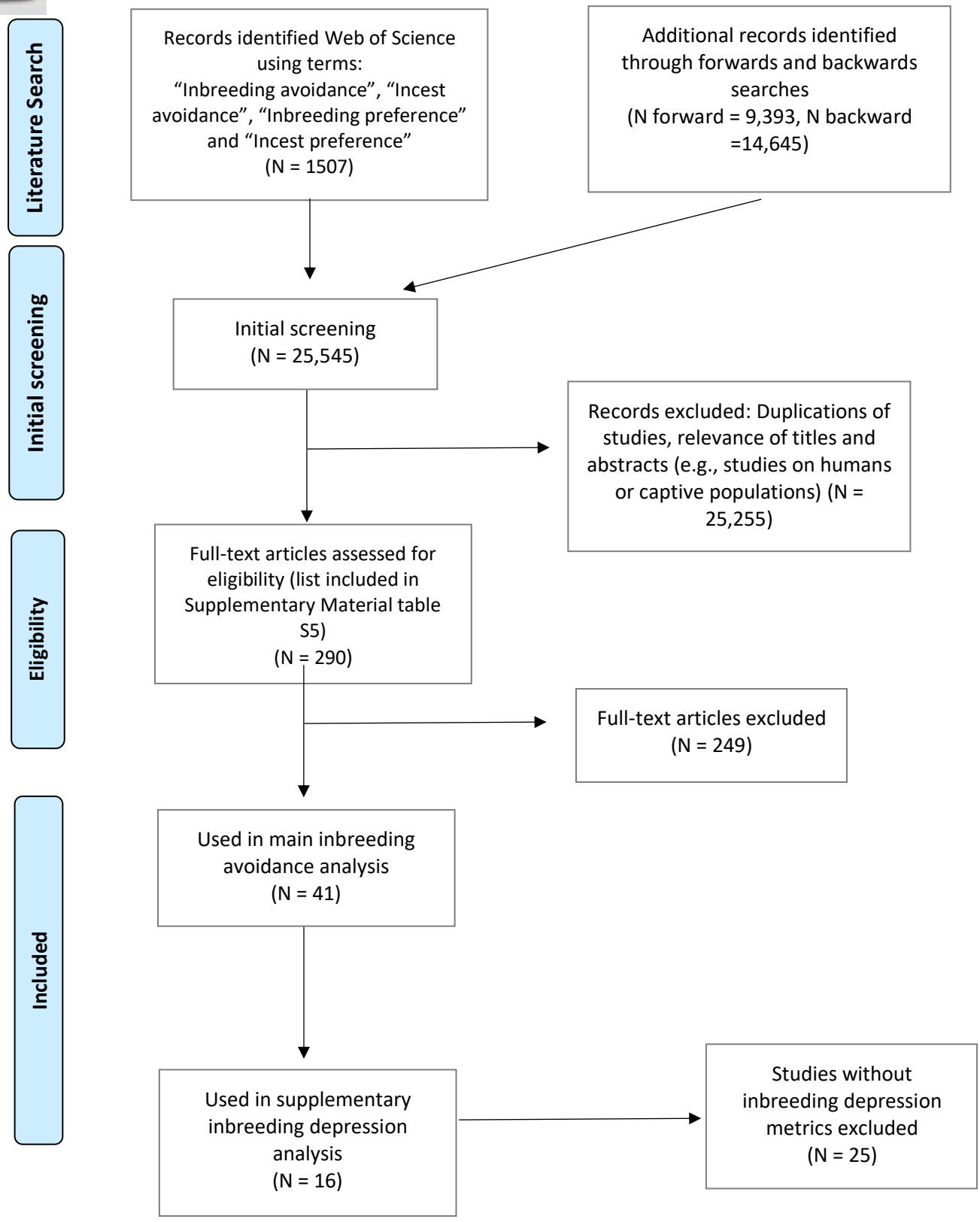
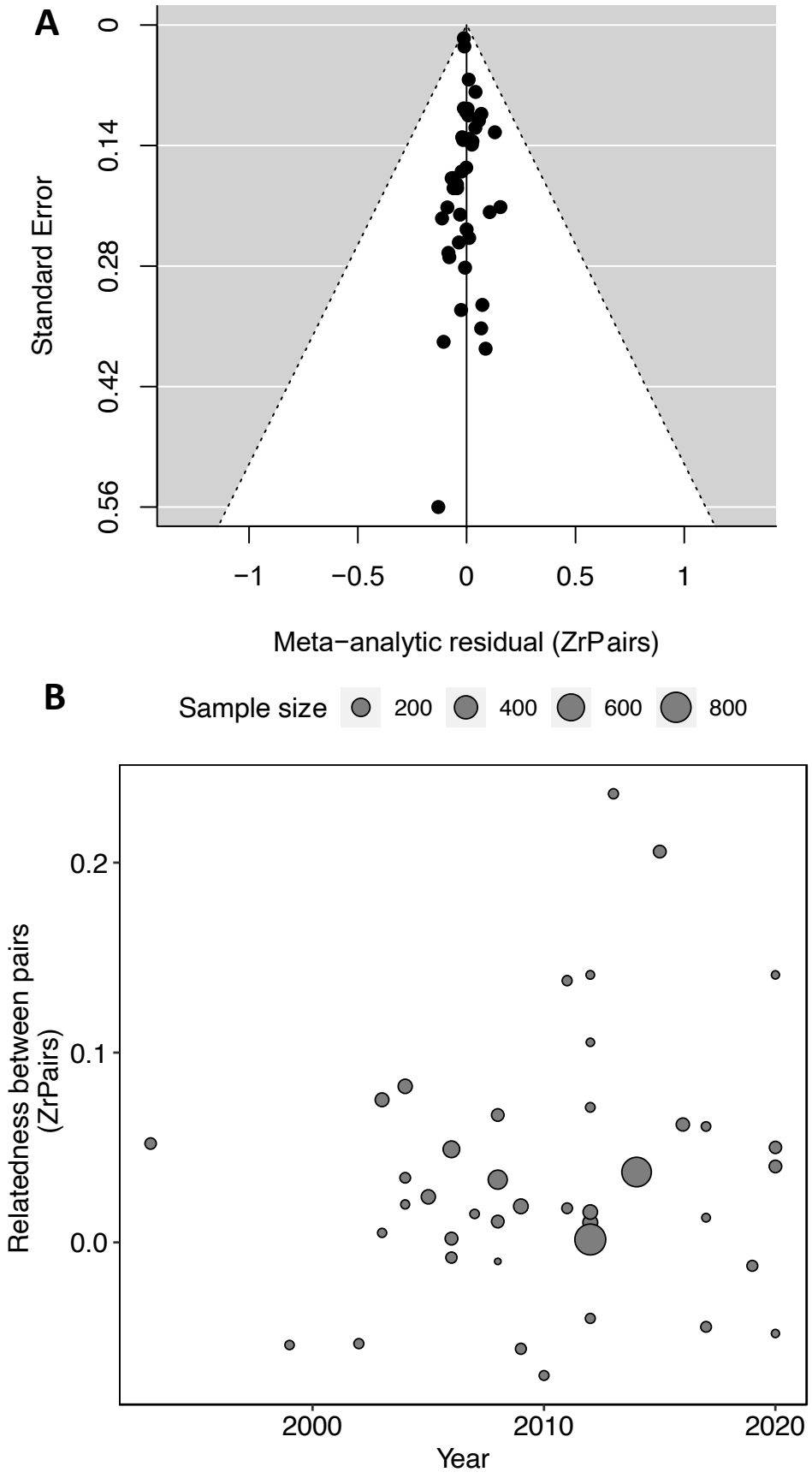


Figure S1: PRISMA flow chart detailing data collection (Moher *et al.*, 2009)

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). *P*referred *R*eporting *I*tems for *S*ystematic Reviews and *M*eta-*A*nalyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097

For more information, visit www.prisma-statement.org.

Figure S2: A) Funnel plot and B) time lag plot for relatedness between pairs (*ZrPairs*).



153 **Figure S3: A) Funnel plot and B) Time lag plot for inbreeding depression effect size**

154 **(*ZrDepression*).**

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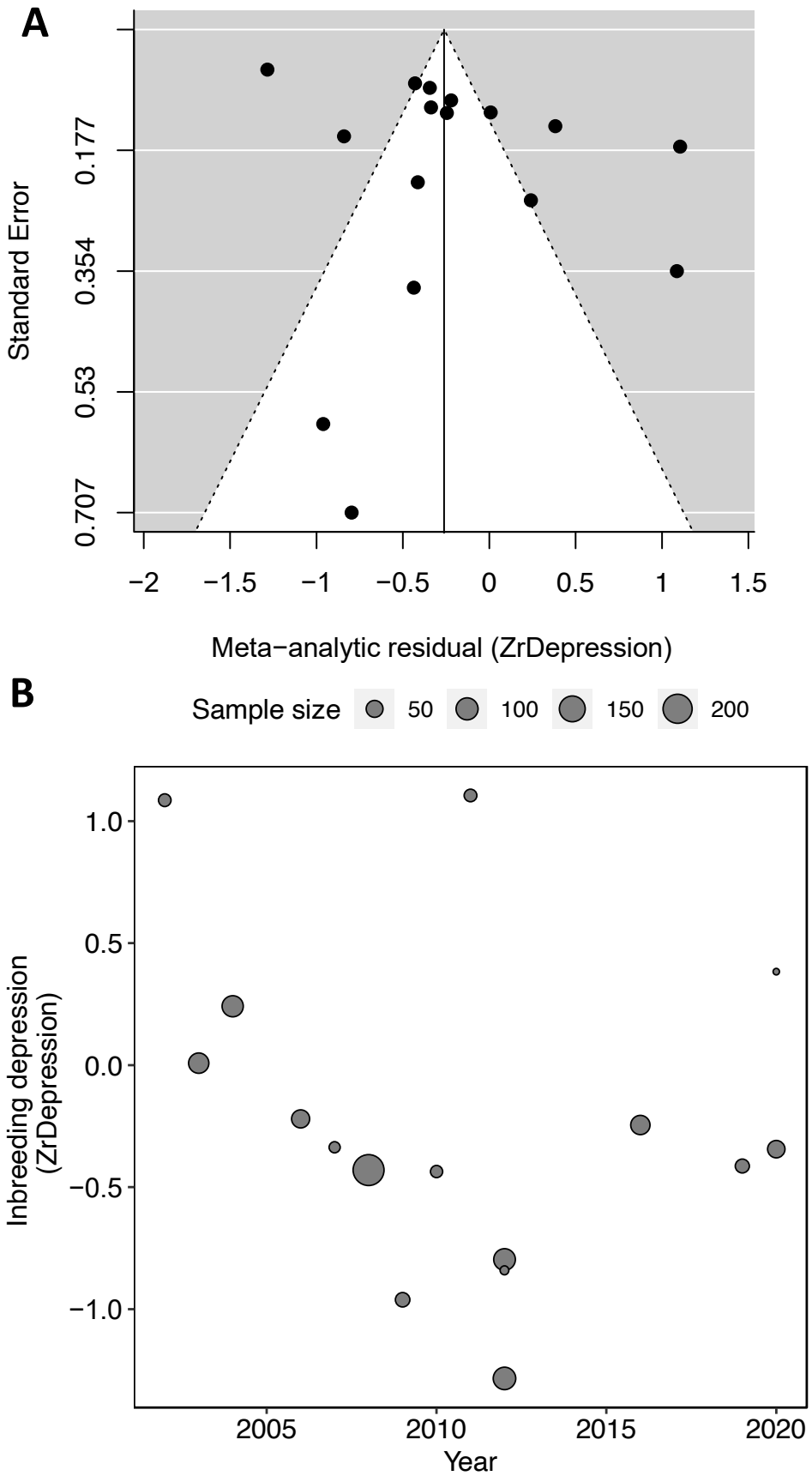


Figure S4: Author's classification of inbreeding depression (yes/no) compared to inbreeding depression effect size *(ZrDepression).* Points represent means +/- standard errors.

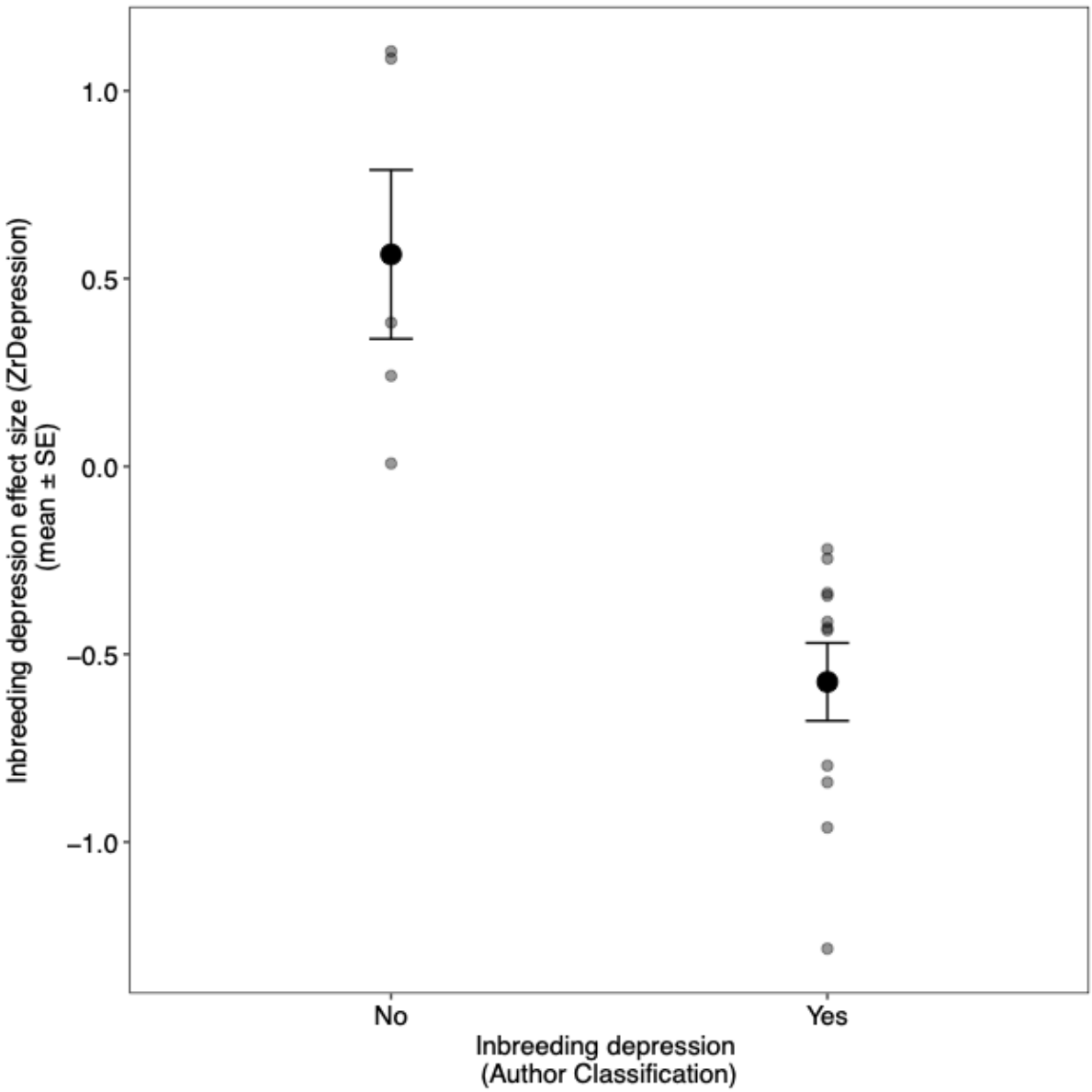


Figure S5: Author's classification of inbreeding depression (yes/no) compared to relatedness between pairs *(ZrPairs).* Points represent means +/- standard errors.

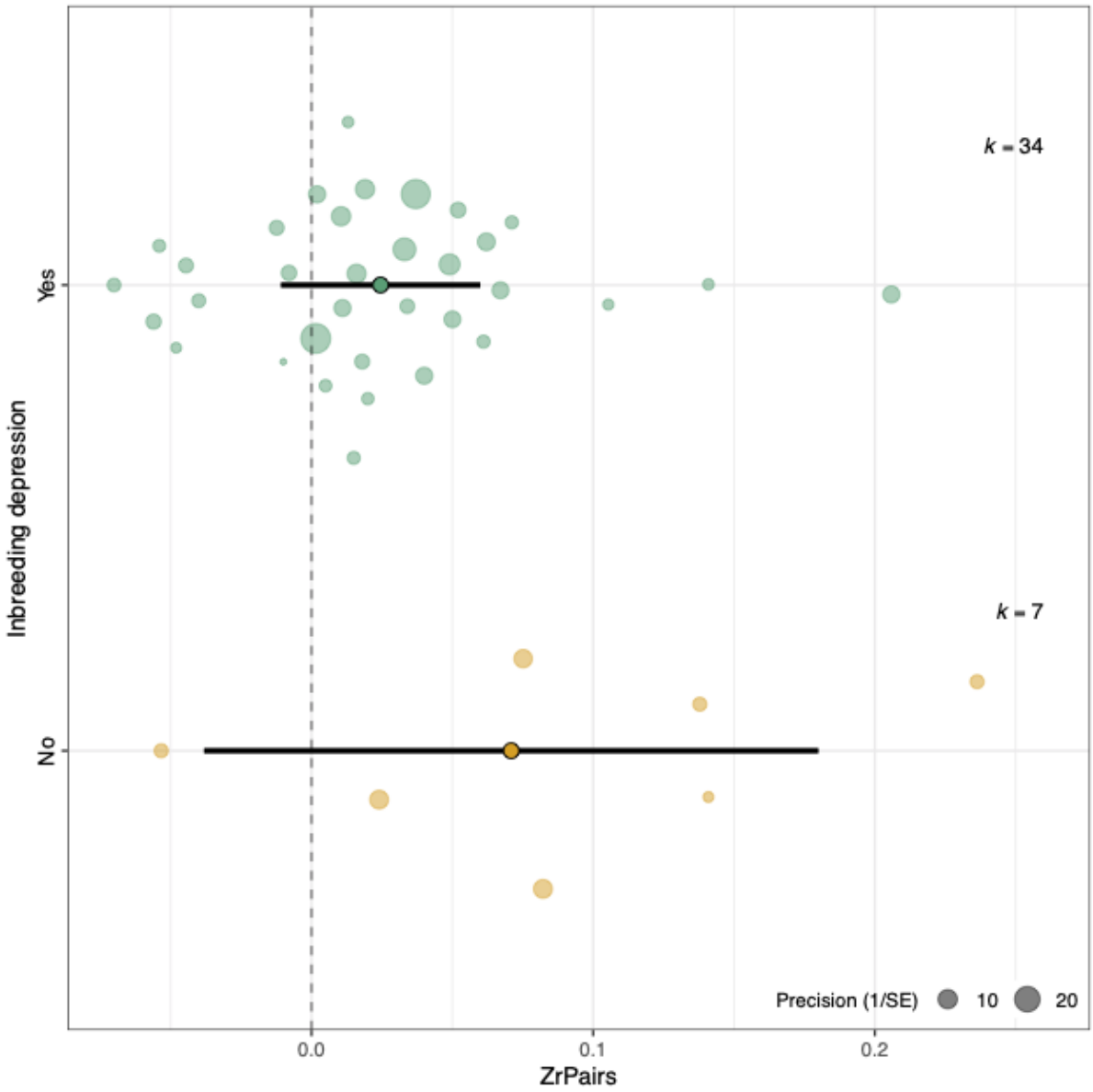


Figure S6: Relationship between Inbreeding depression effect size (*ZrDepression)* and relatedness between pairs (*ZrPairs)*. Open circles represent species without inbreeding depression, according to the authors definitions, and filled circles represents species with inbreeding depression.

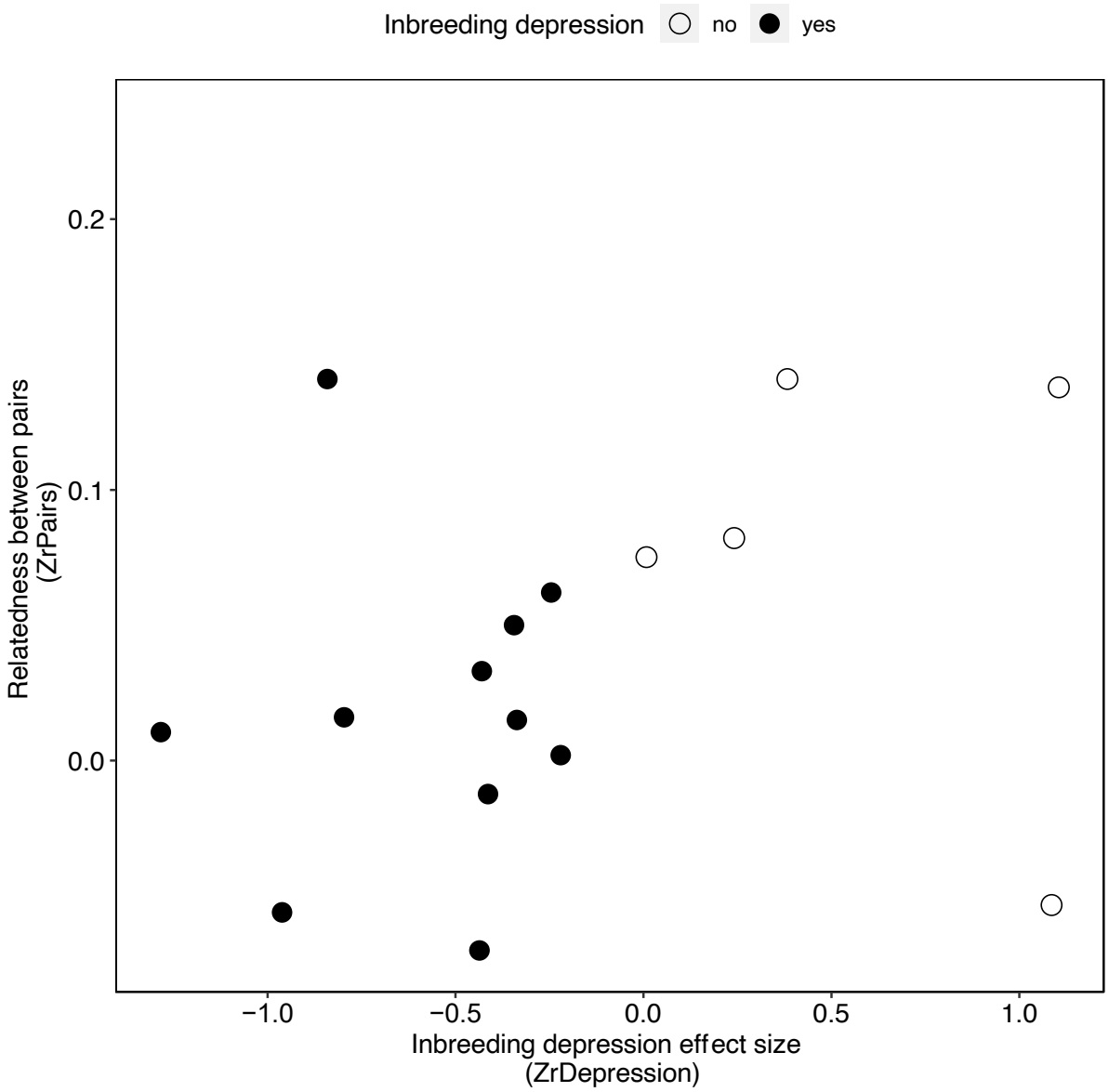


Figure S7: Relationship between average relatedness between males and females in the population (*ZrAverage*) and relatedness between pairs (*ZrPairs*) grouped by the value of inbreeding depression effect size (*ZrDepression*). Where *no* (open circles) represents species with *ZrDepression* >= 0 and *yes* (filled circles) represents species with *ZrDepression*<0. Dashed line represents y=x.

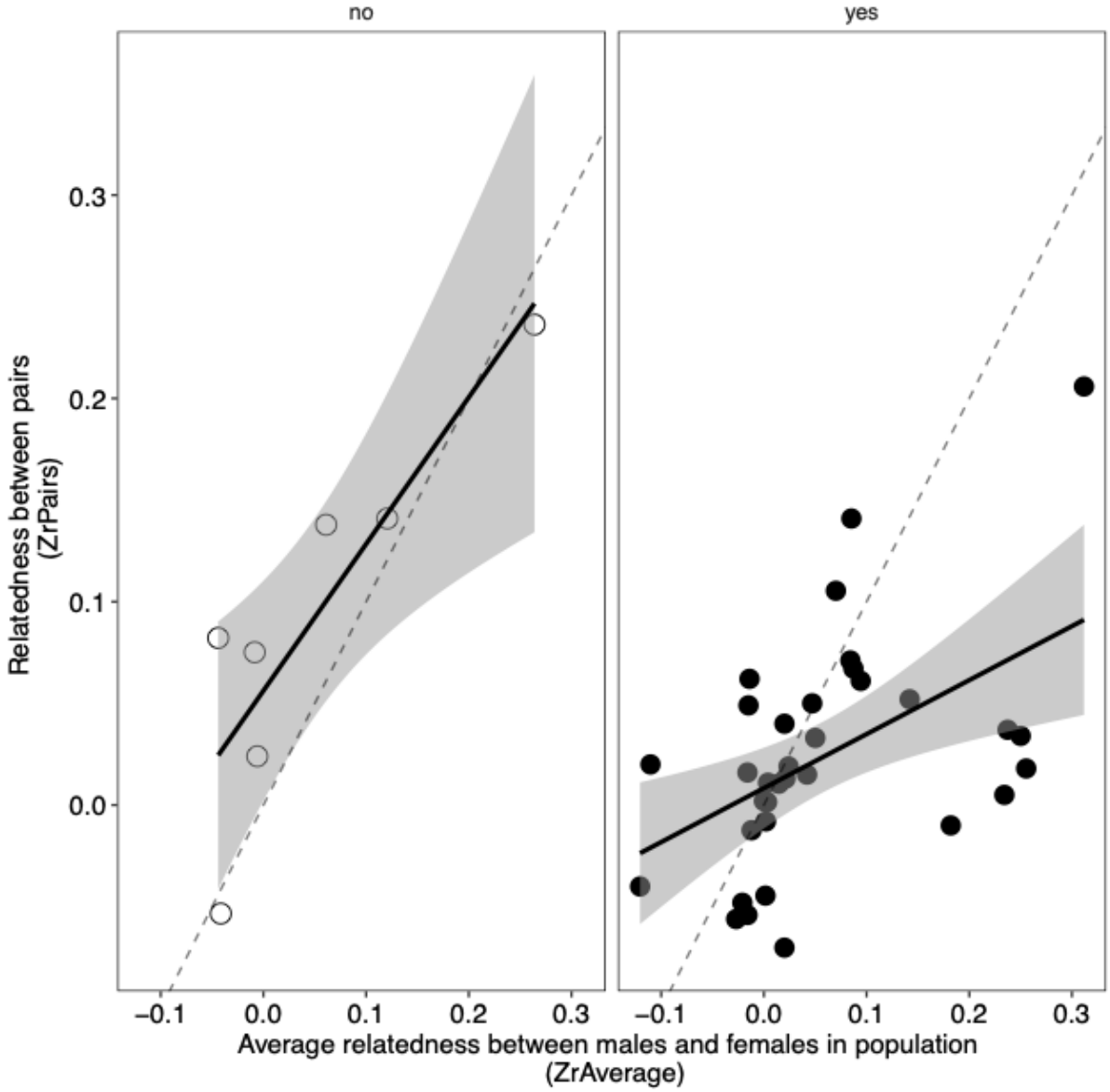


Figure S8: A) Relatedness between pairs (*ZrPairs*) and B) relatedness between males and females in the population (*ZrAverage)* across species with different mechanisms of inbreeding avoidance. Points represent means +/- standard errors.

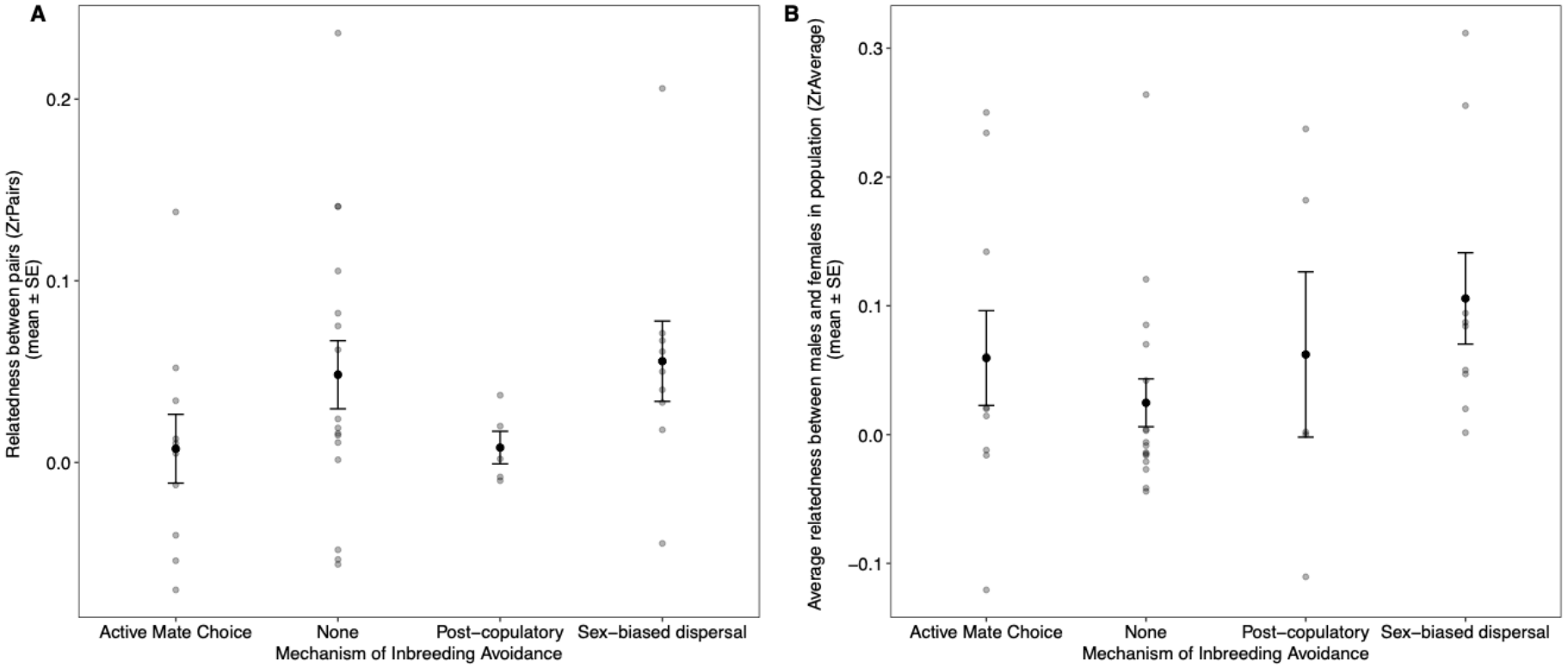
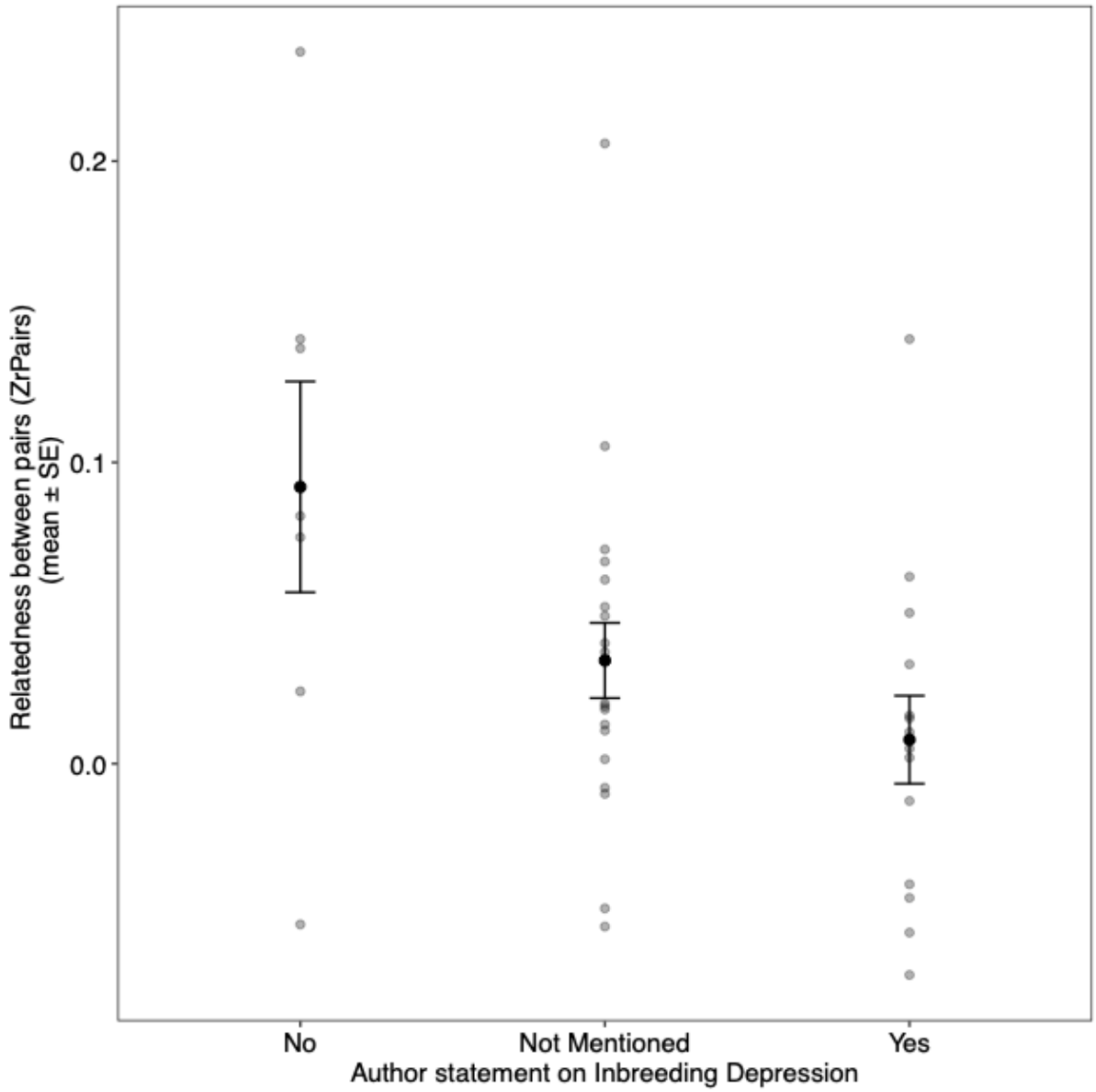


Figure S9: Relatedness between pairs (*ZrPairs*) across species for the different classification of authors statement on inbreeding depression. Points represent means +/- standard errors.



Full references of studies included in the analysis

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Chapter 6
Thesis discussion

Thesis discussion

In this thesis, I investigated the role of nutrition and parasite-parasite interactions on infection outcomes, with a particular focus on virulence and transmission. Below I summarise each of the chapters and highlight some potential avenues for further research.

Chapter summaries

Chapter 2: On the diverse and opposing effects of nutrition on pathogen virulence

In Chapter 2, I broadly investigated whether variation in host nutrition affects pathogen virulence across the tree of life. I carried out two meta-analyses based on different aspects of nutrition, namely: *i)* quantity and *ii*) quality. Overall, there was not a consistent quantitative effect of variable host nutrition on pathogen virulence. I hypothesised that this lack of a general pattern came from a balance between the hosts immune response and resources available for the pathogen to utilise, which I found support for when I qualitatively assessed the studies included in the analysis. In addition, I detected a difference in the response of vertebrate and invertebrate hosts to changes in nutrition. In invertebrate systems, changes to host nutrition tended to affect pathogen growth whereas in vertebrate systems changes to host nutrition tended to affect the host immune response. This study identified that changes to nutrition may have diverse effects across species and highlights the need for further longer-term experiments and consideration of between-host effects.

Chapter 3: A meta-analysis of experimental work on the virulence of coinfections

In Chapter 3, using a similar approach to Chapter 2, I investigated the extent to which coinfections of a divergent range of parasites affected virulence. Using data from experimental studies, I compared virulence in single infections and coinfections to quantify an overall trend in the virulence trajectory associated with multiple parasites within a host.

Across host-parasite systems, I found broad support for the hypothesis that coinfections are more virulent than single infections. The magnitude by which virulence increased in coinfections vs. single infections varied depending on several factors: *i)* the divergence between coinfecting parasites, *ii)* the mechanism of interaction between coinfecting parasites, *iii)* parasite transmission mode, and *iv)* the sites of infection. In this study, I demonstrate the importance of studying hosts infected with multiple parasites for predicting and controlling coinfection outcomes.

Chapter 4: Within- and between-host dynamics of producer and non-producer pathogens

In Chapter 4, I extended the work of Chapter 3 by considering how pathogen traits affect within- and between-host dynamics of coinfecting pathogens. Specifically, I used the nematode host *Caenorhabditis elegans* and pathogenic bacterium *Pseudomonas aeruginosa*; a 'producer' strain and two 'non-producer' strains of quorum-sensing and siderophore traits. I compared the ability of producer and non-producer strains to colonise the nematode host and transmit between new hosts, in single infections and coinfections. Producers were consistently better at colonising and transmitting between hosts, in both single infections and coinfections. Non-producers were unable to successfully cheat (Ghoul et al. 2014), as was also the case in a previous study using this experimental system (Rezzoagli et al. 2019), and were poor at colonising and transmitting between hosts. Overall, I empirically demonstrate, in a field dominated by theory, that within-host pathogen interactions affect between-host infection dynamics. Investigating pathogen dynamics across these multiple levels will help to improve our ability to predict the spread of diseases and thus aid in controlling them especially in response to ecological change.

Chapter 5: Why don't all animals avoid inbreeding?

On a different theme to the preceding chapters, in Chapter 5 I took a comparative approach to investigate inbreeding avoidance across the Animal Kingdom. It is assumed that, due to the deleterious consequences associated with inbreeding (Charlesworth and Charlesworth 1987; Charlesworth and Willis 2009), inbreeding avoidance should be favoured by selection and a lack of inbreeding avoidance is often presented as surprising (Blouin and Blouin 1988; Pusey and Wolf 1996; Keller and Waller 2002). Yet inbreeding avoidance is not ubiquitous in animals (de Boer et al. 2021). In this chapter I demonstrated that inbreeding avoidance only arises when related individuals frequently encounter each other and thus the chance of inbreeding is high. Similarly, I found that inbreeding avoidance mechanisms were only found in species for which inbreeding depression was costly. This helps to explain why some species avoid inbreeding whilst others do not; inbreeding avoidance only evolves when there is a risk of inbreeding depression and when related sexual partners frequently come into contact. In a broader context this study highlights that rather than assuming behaviours are adaptive we should take into account their cost to benefit ratio within specific ecological contexts.

6.1 General themes and future work

6.1.1 Harnessing non-immunological defences

In Chapter 2, I demonstrated that variation in host nutrition can have diverse effects on parasite virulence (Pike et al. 2019). I focussed on the role of host nutrition on parasite virulence, from the perspective that resource provision was outside of the host's control. However, hosts across the Animal Kingdom have been shown to manipulate nutrition to control infections (Adamo et al. 2010; Parker et al. 2011; Hite and Cressler 2019). Somewhat counterintuitively, hosts often decrease their food intake in response to parasitism, known as

illness-induced anorexia (Exton 1997; Parker et al. 2011; Hite and Cressler 2019). This reduction in food intake may directly affect a hosts immune response (Adamo et al. 2010; Cotter et al. 2011; Mason et al. 2014), as in crickets (*Gryllus texensis*) where a trade-off occurs between digestion (specifically lipid transport) and immune function, thus a reduction in food intake can bias the direction of the trade-off towards favouring an immune response. Likewise, the reduction in host resources limits those available for parasite growth, for example this was demonstrated in a mouse model infected with *Listeria monocytogenes* (Murray and Murray 1979; Exton 1997).

Antiparasitic drugs are not always effective at treating parasites in the wild (Pedersen and Fenton 2015). Similarly, with a rise in antibiotic resistance (MacLean and San Millan 2019), there is a timely need to explore a range of alternative treatment strategies. Future work could investigate how manipulating and monitoring host nutrition could combat infection. A potential model system for this work is *Caenorhabditis elegans,* as I used in Chapter 4. The availability of infected *C.elegans* food could be increased or decreased whilst measuring the mortality rate of the infected *C.elegans*. In addition, the exact feeding rate of *C.elegans* could be measured by monitoring pharynx grinder movements [e.g.(Schulte et al. 2012)]. Likewise, other abiotic conditions such as temperature could be explored. Hosts may utilise their external environment to their advantage to eliminate infections. For example, when desert locusts (*Schistocerca gregaria*) are infected with the fungus *Metarhizium anisopliae* they have been shown to seek higher temperatures to reduce fungal growth (Elliot et al. 2002). Experiments could involve manipulating host temperature and tracking any corresponding changes in parasite virulence or host immune function, in a similar approach to research carried out on the fruit fly *Drosophila melanogaster* (Linder et al. 2008).

6.1.2 Investigating the combined role of abiotic and biotic interactions in infection outcomes

In Chapter 3, I emphasised the need for further experimental work to deepen our understanding of the relationship between coinfection and parasite virulence. This statement has been made in other studies too (Pedersen and Fenton 2007; Telfer et al. 2010; Rigaud et al. 2010; Eswarappa et al. 2012). Many of the studies investigating coinfections take place under homogeneous laboratory conditions [e.g.((Mosser et al. 2015; Sheehan et al. 2020))] which may not be representative of natural systems. Understanding how the dynamics of coinfections vary across heterogenous conditions is necessary, as human activities are having a major impact across global ecosystems (Becker et al. 2015), and the primary focus of studies to date has been single infections (Wolinska and King 2009).

We could expand our knowledge of the combined role of abiotic and biotic conditions by investigating how the virulence of coinfections is affected by host nutrition (Stevenson et al. 2015; Civitello et al. 2018) or different temperatures (Fenoy et al. 2009; Milbrath et al. 2015). Experimental work could expand on that of a recent study which investigated both coinfection and nutrient availability using a parasitic plants species (*Prosopsis velutina*) and a mistletoe (*Phoradendron californicum*) host (Nabity et al. 2021). The authors found that host resource availability altered the strength of competition between coinfecting strains of *P.velutina* and the overall virulence of infection (Nabity et al. 2021). When within-host resources were scarce, the coinfecting parasites lowered their virulence by increasing their own autotrophy (production of their own resources) (Nabity et al. 2021). This study introduced an interesting plant host model system that can be easily manipulated and thus well suited for directly testing the combined role of abiotic and biotic factors in infection outcomes.

Experimental studies could also help to uncover whether there are transgenerational effects to variable abiotic and biotic conditions. Such a question could be tackled using a meta-analytic approach. Stressful maternal environments may increase offspring resistance to infection. Experiments with *Daphnia magna* infected with *Pasteuria ramosa* have shown that manipulation of maternal nutrition (Mitchell and Read 2005) and temperature (Garbutt et al. 2014) can affect the susceptibility of their offspring to infection. Mothers in a more stressful environment produced offspring which were more resistant to *Pasteuria ramosa* (Mitchell and Read 2005; Garbutt et al. 2014). Alternatively, in other species, infected mothers that experience a stressful environment may have fewer resources to allocate to their offspring, resulting in poorer offspring quality [e.g. bank voles (*Clethrionomys glareolus*) infected with *Eimeria sp* (Hakkarainen et al. 2007)] that may be more susceptible to parasite infection. Conducting a meta-analysis across a phylogenetically diverse range of host-parasite systems would enable us to detect any broad patterns and gain important insight into the role of transgenerational effects in the spread of disease. It would also be interesting to compare whether maternal host infected with multiple parasites had more extreme transgenerational responses than singly infected mothers. Studies which find transgenerational effects of maternal external environment highlight the importance of controlling for any potential maternal effects when planning experiments investigating disease dynamics (Bernardo 1996).

6.1.3 Between-host dynamics

Empirical work has shown that highly cooperative strains of *P.aeruginosa* often initiate infections (Jiricny et al. 2014) and others suggest that cheating strains could negatively impact between-host transmission and so could be a potential disease intervention strategy (Brown et al. 2009; Jiricny et al. 2014; Rezzoagli et al. 2020). In Chapter 4, I investigated

how parasite interactions (specifically between producer and non-producer strains of *Pseudomonas aeruginosa*) affected between-host dynamics within single infections and coinfections. I found that non-producers were worse at colonising nematode hosts and between-host transmission than producers. However, this may have been influenced by the non-producers inability to benefit from the presence of producers (i.e. cheat (Ghoul et al. 2014)), as in (Rezzoagli et al. 2019). Longer-term evolution experiments [e.g. (Fiegna et al. 2006; Martin et al. 2017)] have demonstrated shifts in the cheating ability of bacteria. Thus, rather than utilising frequently used cheating strains of *P.aeruginosa* as I did in Chapter 4 (quorum sensing and pyoverdine non-producers e.g. (Griffin et al. 2004; Rezzoagli et al. 2019)) a longer-term experimental study could attempt to evolve nematode *de-novo* cheats. Experimental evolution is a widely used technique to study the processes of parasite evolution in real time [e.g. (King et al. 2016)], and thus may be a good approach here. These long-term evolution experiments could start with a single clone of a producer strain of *P.aeruginosa* within the context of the nematode host. If *de-novo* cheats emerged within the nematode host, the experiments carried out in Chapter 4 could be replicated with the *de novo* cheats to explicitly test whether cheating affects between-host dynamics.

Furthermore, in Chapter 4, I maintained standard abiotic conditions for the nematode hosts (Hope 1999). A potential avenue for further research using this system is investigating the role of nutrition on between-host dynamics. Studies across the animal kingdom have shown that natural fluctuations in nutrition [e.g. seasonal changes in resource availability (Leroy et al. 2004) and supplementary feeding (e.g. bird feeders (Robb et al. 2008)] can lead to aggregation and increased contact rate between infected individuals, resulting in high parasite transmission (Oro et al. 2013; Civitello et al. 2018). Similarly some species, such as Eurasian blackbirds (*Turdus merula*), may feed in high densities resulting in consistently high contact

rates (Galbraith et al. 2017). High resource availability can lead to increases in the number of susceptible hosts in the population with increased birth rates or immigration and is likely to influence parasite transmission dynamics (Boutin 1990; Erazo et al. 2021).

The degree to which the quality and distribution of nutrition provisions might influence between-host transmission could be experimentally investigated using *C. elegans.* Specifically, we could ask: how does gathering at sites of high resource density influence parasite transmission? *C.elegans* feed on a variety of bacterial strains (Gravato-Nobre and Hodgkin 2005; Clark and Hodgkin 2016; Schulenburg and Félix 2017) with different nutritional values (Tan et al. 1999), making it possible to manipulate diet quality and quantity. Spatial heterogeneity could be created by having small patches of food available versus a standard evenly distributed lawn of food (Hope 1999). Nematodes could be pre-exposed to parasites, or parasites could be mixed in with standard food. The colonisation ability and transmission between nematode hosts with different resource distributions could then be compared by calculating the number of colony forming units across a sample of nematodes, as in Chapter 4. Furthermore, if the parasites were fluorescently labelled, the proportion of colonised nematodes could be compared for the two types of resource distribution. The extent to which resource distribution affects the transmission of parasites with different transmission modes could also be investigated. Parasites transmitted by direct contact between nematodes, such as bacterium of the genus *Leucobacter* (Hodgkin et al. 2013; Bates et al. 2021), may have a greater increase in transmission around patchy resources compared to those spread by faecal oral transmission, such as *Pseudomonas aeruginosa* (Diaz and Restif 2014).

Alternatively, tackling the question from the perspective of the host, we could ask; how do parasites affect the evolution of host social behaviour? *C. elegans* could be used as a model system, to directly test whether parasite infection can shape the evolution of host social behaviour. Social feeding, or clumping, is found in the majority of natural isolates of the nematode species *Caenorhabditis elegans* (De Bono and Bargmann 1998), often in response to oxygen gradients (Gray et al. 2004). Despite the cost of increased susceptibility, in *C. elegans* social feeding appears to persist in the presence of parasites in short-term studies (Reddy et al. 2009). However, it is unknown whether this social behaviour is influenced by long-term exposure to parasites. Experiments may use strains of *C.elegans* which exhibit the clumping behaviour either a *npr-1* mutant (Reddy et al. 2009) or a range of genetically diverse populations which clump for example the Hawaiian strain CB4856, the Californian strain CB4858 and the Canadian strain KR314. These clumping strains could then be compared to non-clumping strains, such as the standard canonical laboratory strain N2. Competition assays could be carried out on clumping vs non-clumping strains in the presence or absence of parasites.

6.2 Concluding remarks

Ours is a changing world. Human-induced climate change is having a vast effect on ecosystems (Cahill et al. 2013; Stevenson et al. 2015) leading to shifts in abiotic conditions (Becker et al. 2015; Altizer et al. 2018; Gehman et al. 2018) and biotic interactions between species (Parmesan et al. 1999; Hance et al. 2007; Cahill et al. 2013; Longdon et al. 2015). The work in this thesis uses the disease triangle (McNew 1960) as a tool to tackle drivers of host-parasite interaction outcomes and dynamics. Such a conceptual approach is timely. The meta-analyses and experimental work in this thesis highlight the importance of studying host-parasite dynamics within an ecological context. Future work could expand on this thesis work

by investigating the combined effects of biotic and abiotic factors across within- and between-host dynamics.

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