Genetic covariance in immune measures and pathogen resistance in decorated crickets (*Gryllodes sigillatus*) is sex and pathogen specific

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Running Title: Immune measures and disease resistance

Abstract

1. Insects are important models for studying immunity in an ecological and evolutionary context. Yet, most empirical work on the insect immune system has come from phenotypic studies meaning we have a limited understanding of the genetic architecture of immune function in the sexes.

2. We use nine highly inbred lines to thoroughly examine the genetic relationships between a suite of commonly used immune assays (haemocyte count, implant encapsulation, total phenoloxidase activity, antibacterial zone of inhibition, pathogen clearance) and resistance to infection by three generalist insect pathogens (the gram-negative bacterium *Serratia marcescens*, the gram-positive bacterium *Bacillus cereus* and the fungus *Metarhizium robertsii*) in male and female *Gryllodes sigillatus*.

3. There were consistent positive genetic correlations between haemocyte count, antibacterial and phenoloxidase activity and resistance to *S. marcescens* in both sexes, but these relationships were less consistent for resistance to *B. cereus* and *M. robertsii*. In addition, the clearance of *S. marcescens* was genetically correlated with the resistance to all three pathogens in both sexes. Genetic correlations between resistances to the different pathogen species were inconsistent, indicating that resistance to one pathogen does not necessarily mean resistance to another. Finally, while there is ample genetic (co)variance in immune assays and pathogen resistance, these genetic estimates differed across the sexes and many of these measures were not genetically correlated across the sexes suggesting that these measures could evolve independently in the sexes.

4. Our finding that the genetic architecture of immune function is sex and pathogen specific suggests that the evolution of immune function in male and female *G. sigillatus* is likely to be complex. Similar quantitative genetic studies that measure a large number of assays and resistance to multiple pathogens in both sexes are needed to ascertain if this complexity extends to other species.

Key words: ecological immunity, insect, immune assay, disease resistance, pathogen, sexual dimorphism.

Introduction

Animals are under constant attack from a diversity of pathogen species that reduce the fitness of their host in a number of ways, including exploiting their available resources, interfering with their normal behaviour, damaging tissues or organs and/or causing death (Janeway *et al.*, 2001; Moore, 2002). Not surprisingly, hosts have evolved various defences to help minimise these costs, ranging from behavioural adaptations to physical and chemical barriers (Janeway *et al.*, 2001; Moore, 2002). Given the importance of these defences to fitness, immune function is often a central component of the host's life-history, especially where greater fitness is achieved through enhanced longevity (Zuk & Stoehr, 2002) and when the diversity of pathogens is sufficiently low to ensure that defences remain effective (Jokela *et al*., 2000). Consequently, theory predicts that immune function should be subject to strong natural selection (e.g. Jokela *et al.*, 2000; Medley, 2002; Miller *et al.*, 2007), although empirical data suggests that positive directional selection is actually more common in the wild populations (Seppälä, 2015). Both forms of selection are expected to erode the variation in immune function (Endler, 1986), yet hosts continue to remain susceptible to pathogenic infection and immune responses vary widely both within and between individuals (Schmid-Hempel, 2003).

Explaining the persistence of this variation in immune function remains a key focus of ecological immunology, and centres around the notion that mounting an immune response is costly to the host (Sadd & Schmid-Hempel, 2009). These costs prevent the host from maximising all aspects of immune function simultaneously (Reznick *et al.*, 2000) and can manifest at different individual or evolutionary scales (Rolff & Siva-Jothy, 2003). There are direct costs associated with maintaining and using the immune system (termed *usage costs,* Sadd & Schmid-Hempel, 2009), including energetic costs (e.g. Ardia *et al.*, 2012) and damage caused to self through auto-reactivity and auto-immunity (e.g. Sadd & Siva-Jothy, 2006). There may also be fitness costs of evolving an efficient immune system (termed *evolutionary costs*, Sadd & Schmid-Hempel, 2009) mediated by trade-offs between immunity and other important life-history traits (e.g. reproduction, Hosken, 2001), as well as between different immune components (e.g. Cotter *et al.*, 2004) or defence against different pathogens (termed *multiple fronts costs*, McKean & Lazzaro, 2011).

There is considerable empirical evidence suggesting that the costs of mounting an immune response are prevalent (Rolff & Siva-Jothy, 2003) and vary in different ecological and evolutionary contexts to shape the optimal investment in immune function (Schmid-Hempel, 2003; Seppälä, 2015). For example, age (e.g. Leech *et al*., 2019), physiological state (e.g. Adamo, 2009), reproductive status (e.g. Short & Lazzaro, 2013), diet (e.g. Kutzer *et al*., 2018; Cotter *et al*., 2019), social environment (e.g. Leech *et al*., 2019), as well as the interaction between the genotype of the host and the specific pathogen species (e.g. Schmid-Hempel & Ebert, 2003; Hall *et al*., 2017), have all been shown to influence the immune function of the host. One factor that is known to have a particularly large effect on immune function is the sex of the host (Nunn *et al*., 2009; Wilson & Cotter, 2013; Kelly *et al*., 2018). Sexual selection theory predicts sexual dimorphism in immune function due to divergence in the way that immune function is traded against reproduction in the sexes (Zuk & Stoehr, 2002). In most species, male fitness is limited by the number of mates fertilised, whereas female fitness is limited by the number of offspring produced (Trivers, 1972). Males are therefore expected to adopt a “live hard, die young” strategy where reproduction is traded against immune function, whereas females are not expected to make this trade-off as they can maximise their reproductive success by living longer which is, in turn, promoted by stronger immune function (Zuk & Stoehr, 2002).

In agreement with sexual selection theory, sex differences in immune function are taxonomically widespread and in most cases, females have superior immune function (Nunn *et al*., 2009; Wilson & Cotter, 2013; Kelly *et al*., 2018). However, the majority of empirical support for sexual dimorphism in immune function comes from phenotypic rather than genetic studies (Nunn *et al*., 2009; Wilson & Cotter, 2013; Kelly *et al*., 2018). Understanding how immune function evolves in the sexes requires estimation of the genetic variance in and covariance between immune traits within each sex and genetic covariance between these traits across the sexes, which can only come from detailed genetic studies (Lande, 1980). For example, male mealworm beetles (*Tenebrio molitor*) have a different pattern of genetic variance and covariance for three immune traits (cuticular darkness, haemocyte counts and phenoloxidase activity) than females suggesting the potential for different evolutionary trajectories in the sexes (Rolff *et al*., 2005). However, there were also strong positive genetic correlations for these immune traits across the sexes suggesting that there is likely to be a genetic constraint that prevents immune function from evolving independently in the sexes (Rolff *et al*., 2005).

Insects have proven to be valuable models for studying immunity in an ecological and evolutionary context, in part because they possess a relatively simple innate immune system that lacks components of the adaptive immune system of vertebrates (Rolff & Siva-Jothy, 2003; Sadd & Schmid-Hempel, 2009). The immune system of insects consists of both cellular and humoral responses (Siva-Jothy *et al*., 2005). Cellular responses are mediated by circulating haemocytes and include nodulation, encapsulation and phagocytosis, whereas humoral responses include the production of antimicrobial molecules, complement-like proteins, and enzymatic cascades that regulate cytotoxic molecule production, melanin formation and clotting (Siva-Jothy *et al*., 2005). There are a number of assays available to assess both cellular and humoral immunity in insects (reviewed in Moreno-García *et al.,* 2013). Frequently used assays measuring cellular responses include counts of circulating haemocytes and quantification of the encapsulation response to a sterile implant, while phenoloxidase activity (PO), cell-free anti-microbial activity, including lytic activity of the haemolymph, are commonly used to measure humoral responses (Moreno-García *et al*., 2013). However, it is important to note that many of these assays represent a baseline of potential immune function as they are often measured in the absence of any immune system activation by an infective agent or immune elicitor (Moreno-García *et al*., 2013). Well established protocols also exist for a more direct assessment of immune function in insects and have the advantage of assessing the immune system as a whole, incorporating the complex interactions within the immune system (Adamo, 2004a). Frequently used measures include quantifying the ability of the host to clear a pathogen from the haemocoel (referred to as *pathogen clearance*) or to survive pathogen infection (known as *pathogen resistance*) (e.g. Haine *et al*., 2008; Hunt *et al*., 2016). These measures, however, are often highly pathogen specific (e.g. Faria *et al*., 2015; Duneau *et al*., 2017) meaning that it is critical to use an ecologically relevant pathogen species (Adamo*,* 2004a).

The decorated cricket *Gryllodes sigillatus* has proven an excellent model to study the evolution of insect immune function, especially in the context of its interaction with reproduction (Gershman *et al.,* 2010a; Kerr *et al.,* 2010; Galicia *et al*., 2014; Rapkin *et al*., 2018). Previous work on this species has shown that many immune assays are sexually dimorphic, with females having higher PO activity and encapsulation ability than males (Gershman *et al.*, 2010b; Galicia *et al.,* 2014), but the reverse pattern exists for lytic activity (Galicia *et al.,* 2014, but see Gershman *et al.*, 2010b). There is considerable evidence suggesting that immunity is traded against reproduction in *G. sigillatus*, especially in males (e.g. Gershman *et al.*, 2010a; Kerr *et al.*, 2010; Galicia *et al.*, 2014; Rapkin *et al.*, 2018). There is a negative phenotypic correlation between the size of male decorated cricket's endogenously produced nuptial gift and lytic activity (Gershman *et al.*, 2010a). This trade-off is further supported by the fact that males injected with a benign bacterially-based immune elicitor produce smaller nuptial gifts than sham-injected males, and males producing more nuptial gifts have lower lytic activity but not lower encapsulation ability or PO activity (Kerr *et al.*, 2010). There is also evidence for a trade-off between encapsulation ability and reproduction that is larger in males than females and is regulated by sex differences in the nutritional demands for proteins and carbohydrates (Rapkin *et al.*, 2018). However, we currently know little about the genetic architecture of immune function in *G. sigillatus*, especially involving measures of pathogen resistance, and whether this differs across the sexes.

In this study, we use nine highly inbred lines of *G. sigillatus* to provide a comprehensive examination of the genetic relationship between a suite of immune assays and resistance to infection by three pathogens in males and females. We start by quantifying circulating haemocytes, total PO activity, general antibacterial zone of inhibition (ZI) activity against *Micrococcus luteus,* and implant encapsulation responses in male and female crickets from each inbred line. These measures of immunity span cellular and humoral components (Siva-Jothy *et al.,* 2005). Next, we quantify the ability of male and female crickets from each inbred line to clear from their haemolymph the gram-negative bacterium *Serratia marcescens*, the gram-positive bacterium *Bacillus cereus*, and the fungus *Metarhizium robertsii*. These pathogen species were chosen because of their ecological relevance for orthopterans (Gouli *et al.,* 2011). Finally, we examine the resistance of male and female crickets from our inbred lines to each of these pathogen species. This design therefore allows us to quantify the genetic variance in and genetic covariance between immune assays and pathogen resistance in males and females, as well as the genetic covariance for these immune traits across the sexes. These genetic parameters are central to understanding the potential for immune function to evolve in the sexes, as well as the extent to which immune function will evolve independently in male and female *G. sigillatus*.

Materials and Methods

This research did not require ethical approval.

Cricket maintenance and inbreeding protocol

G. sigillatus used in this study were descended from approximately 500 adult crickets collected in Las Cruces, New Mexico, USA in 2001. These founding animals were used to initiate a laboratory culture allowed to breed panmictically (hereafter, the outbred population). Crickets were distributed across twelve, 15L transparent plastic containers and housed in an environmental chamber (Climatron, Thermoline Scientific) at 32 ± 1°C on a 14 h: 10 h light:dark cycle. Crickets were provided *ad libitum* with cat food pellets (Friskies 7, Nestle Purina PetCare, Rhodes, NSW, Australia), rodent chow (Specialty Feeds, Glen Forrest, WA, Australia) and water in 60mL glass tubes plugged with cotton wool, and egg cartons for shelter. When adults were detected, moistened cotton wool was provided in a petri dish as an oviposition substrate. Hatching nymphs were collected *en masse* and approximately 500 nymphs were allocated at random to each container to establish the next generation. This process ensures gene flow each generation to promote the maintenance of genetic variation in this outbred culture.

Nine inbred lines were created from this outbred population. To create these inbred lines (designated A to I), crickets selected at random from the outbred population were subjected to 23 generations of full-sib mating (Ivy *et al.*, 2005), followed by 44 generations of panmixis within each line. Each inbred line was housed in two, 15L containers and maintained following the protocols outlined above, with the obvious exception that individuals were not mixed between different inbred lines each generation.

Two weeks post-hatching, 220 nymphs from each of the nine inbred lines and from the outbred population were isolated and established in individual plastic containers (5 cm^3) containing a piece of cardboard egg box for shelter. Each nymph was provided weekly with fresh cat food pellets and water. Final instar nymphs were checked daily for eclosion to adulthood.

Experimental design and measures of immunity

On day 8 post-eclosion, crickets from each of the nine inbred lines and from the outbred population were randomly allocated to one of eight groups to measure different immune parameters or resistance to pathogenic infection (Fig. 1): (*i*) Haemolymph immune assays (i.e. haemocyte count, zone of inhibition (ZI) and PO activity), (*ii*) Implant encapsulation response, (*iii*) Host clearance of *S. marcescens*, (*iv*) Host clearance of *B. cereus*, (*v*) Host clearance of *M. robertsii*, (*vi*) Resistance to *S. marcescens*, (*vii*) Resistance to *B. cereus* and (*viii*) Resistance to *M. robertsii*. With the exception of group (*i*), where three assays were performed on the haemolymph taken from each cricket, different crickets were used in the remaining seven groups. In total, we sampled 10 males and 10 females from each inbred line and the outbred population for all eight groups (total n = 800 crickets per sex). Immediately prior to assessing immunity, we measured each cricket's body mass using an analytical balance (Mettler Toledo AE260).

We estimated the repeatability of our immune assays (groups *i*-*v*) in 20 crickets of each sex taken at random from our outbred population. Assays were conducted using the same protocols as described below for our experimental crickets. For our three haemolymph assays (haemocyte count, ZI and PO activity) and encapsulation response, two assays were conducted per cricket. For clearance of *S. marcescens*, *B. cereus* and *M. robertsii*, three assays were conducted per cricket. We estimated the repeatability of each assay, as well as the 95% confidence intervals (CIs) for these estimates, using the "ICC" package (Wolak *et al.*, 2012) in R (version 3.6.2). All immune assays were highly repeatable in both sexes, with estimates ranging from 0.78 to 0.98 in males and from 0.82 to 0.99 in females (Table 1).

Haemolymph immune assays (group i)

To collect haemolymph, crickets were cold-anaesthetised for 5 min in a tube on ice. The membrane was pierced under the dorsal pronotum plate with a sterile 25G needle and 4 µl of outflowing haemolymph was collected by positioning a prechilled glass microcapillary tube (Wiretrol® II Micro Dispenser, Drummond Scientific, PA, USA) at the puncture site. Collected haemolymph was then expelled into 11 µl of Grace's insect medium (GM, Sigma-Aldrich, G8142, Australia) to be used in zone of inhibition (ZI) assays; 4 µl of this mixture was then added to 12 µl of GM, which was immediately used for total circulating haemocyte counts (final dilution 1/15); another 4 µl of the mixture was added to 20 µL of GM to be used in PO assays. The samples for ZI and PO activity assays were snap-frozen in liquid nitrogen and stored at -80°C to induce cell lysis and prevent enzymatic reactions from proceeding until later analysis (Gershman *et al.*, 2010b).

Circulating haemocyte counts are routinely used to measure cell-mediated responses (Ryder & Siva-Jothy, 2001). Total haemocytes were counted under an optical microscope (400x) with a haemocytometer (FastRead 102® plastic counting chamber).

The ZI assay evaluates the capacity of cell-free haemolymph samples to inhibit the growth of bacteria seeded in agar petri dishes (Kurtz *et al.*, 2000). The gram-positive *Micrococcus luteus* is routinely used, including in *G. sigillatus* assays (Duffield *et al.*, 2018), due to its sensitivity and resolution in detecting differences in general humoral antibacterial activity, even though it is not typically considered pathogenic to insects (Gouli *et al.*, 2011). *M. luteus* (ATCC® 4698) from a single colony were incubated at 30°C for 48 h with agitation (250 rpm) in 5 mL of nutrient broth (Oxoid, Thermo Fisher Scientific, VIC, Australia). Bacteria from this culture were added to liquid medium containing 1% agar held at 40°C to achieve a final density of 1.5 x 10^5 cells/mL. Six mL of seeded medium was poured into a 10-cm diameter petri dish to solidify. Sample wells were made using a Pasteur pipette (Volac D810) fitted with a ball pump. Samples were thawed on ice and 2.5 µL of sample solution were added to each well. Negative control wells (GM) were included on each plate. Plates were inverted and incubated for 48 h at 30°C. For each inhibition zone, two diameter measurements, perpendicular to one another, were measured using ImageJ (version 1.8.0_112; http://rsbweb.nih.gov/ij) and averaged. Measurements were performed blind to treatment. Each haemolymph sample was tested in duplicate, with the mean of the duplicates being used in subsequent analyses.

The prophenoloxidase (ProPO) cascade is central to the melanisation reaction. ProPO is an inactive, haemolymph-bound molecule. When microbes enter the haemolymph, a series of proteases are activated and transform ProPO into its active form, PO. After activation, PO catalyses the production of melanin, as well as phenols, quinones and other cytotoxins (Nappi & Vass, 1993; Sugumaran *et al.*, 2000) to defend against multicellular pathogens and parasites, bacteria, fungi, and viruses (Sugumaran *et al.*, 2000; González-Santoyo & Córdoba-Aguilar, 2012). PO activity can be measured *in vitro* by incubating haemolymph samples with α-chymotrypsin to activate all of the active zymogen, pro-PO, and allow for total PO capacity to be measured. Samples are then incubated with an excess of an exogenous PO substrate, 3,4-dihydroxy-L-phenylalanine (L-DOPA), with PO activity represented by a rate change in optical density of the sample during the linear phase of the reaction (Reeson *et al.*, 1998; Adamo *et al.*, 2001). Briefly, 10 µl of haemolymph samples were combined with 135 µl of H_2O, 20 µl of phosphate-buffered saline (PBS, Gibco, ThermoFisher Scientific) and 5 µl of bovine pancreas α-chymotrypsin (5 mg/mL, Sigma-Aldrich, CAS: 9004-07-3) in each well of a spectrophotometer microplate. The mixture was incubated for 15 min at room temperature. 20 µl of L-DOPA (4 mg/ml, Sigma-Aldrich, CAS: 59-92-7) were then added and the optical density (OD) was recorded at 490 nm at 30°C (SPECTROstar nano, BMG LabTech, Thermofisher Scientific). The total change in OD over the course of the reaction was determined using MARS data analysis software (version 2.10). This method estimates the total change in OD during the linear phase of the reaction, with OD readings taken every 40 seconds over a 45-min period. Preliminary tests indicated that readings taken between 5 and 40 min best described the fastest rate of change in OD over time. The average slope of the change in OD per min was calculated for the control wells (GM) and subtracted from the slope of a given haemolymph sample to extract the corrected slope, with a larger slope indicating more PO activity. Each haemolymph sample was tested in duplicate and all samples were randomised within and across plates.

Implant encapsulation response (group ii)

The encapsulation response to an artificial implant is commonly used to evaluate the melanisation pathway *in vivo* (Siva–Jothy, 2000; Simmons *et al.*, 2005). Crickets were cold-anaesthetised and implanted with a 3 mm-long segment of 0.25 mm-diameter nylon monofilament abraded with sandpaper and sterilised in 70% ethanol as previously described (König & Schmid-Hempel, 1995; Siva-Jothy *et al.*, 1998). A small hole was made ventrally between the 5th and 6th abdominal segments using a sterile 30G hypodermic needle, and the implant was inserted into the wound with forceps until completely contained within the abdomen. After implantation, crickets were returned to their individual containers with fresh food and water. Exactly 48 h post-implantation, an ideal time-point to detect variation in melanisation in *G. sigillatus* (Gershman *et al.*, 2010b), crickets were frozen at -80°C. Implants were dissected from frozen crickets and clumps of tissue removed. Each implant was photographed three times from different angles next to a clean control implant using a digital camera (KY-F1030, JVC with Image-Pro Plus software version 7.0) mounted on a dissecting microscope (Leica MZ12). Each implant and control was outlined using the ImageJ polygon tool. The darkness of each experimental and control implant (which reveals how well haemocytes encapsulate and melanise a foreign body) was measured as the average grayscale value of all pixels within each image. The darkness score for each individual was calculated as the average grayscale of the three implants' darkness scores subtracted from the average grayscale of the three control implants' darkness scores. Therefore, darker implants yielded higher darkness scores.

Pathogen cultures and injections (groups iii-viii)

The strains *S. marcescens* subsp. *marcescens* BS 303 (ATCC® 13880, Manassa, VA, USA), *B. cereus* Frankland and Frankland 1887 (ATCC® 14579) and *Metarhizium robertsii* (formerly classified as *M. anisopliae* strain ME1) isolate ARSEF 2575 (ATCC® MYA-3093) were used. *S. marcescens* and *B. cereus* were grown on nutrient agar plates (Oxoid) at 30°C for 16 h. Isolated colonies were used to inoculate 7 mL (*S. marcescens*) or 30 mL (*B. cereus*) of nutrient broth incubated for 15 h (*S. marcescens)* or 16 h (*B. cereus*) at 30°C with agitation. Late-logarithmic bacteria were washed in sterile ringer saline (Sigma-Aldrich, Australia) and diluted to the desired concentration. *M. robertsii* was cultured at 28°C in continuous light on one quarter strength Sabouraud dextrose agar with yeast extract (SDAY/4): 10 g/L dextrose, 2.5 g/L neopeptone, 2.5 g/L yeast extract, 15 g/L agar (Oxoid), as previously described (Hunt *et al.*, 2016). Conidia from each plate were harvested at 15-days post-inoculation by gently scrapping the surface of the mycelium with a disposable L-shaped spreader and suspended in sterile saline containing Tween 80 (0.05% v/v, Sigma-Aldrich) as wetting agent. The suspension was vortexed for 30 s and conidia were enumerated using a disposable haemocytometer. The suspension was adjusted to a final concentration of 5×10^7 conidia/mL and stored at 4°C until later use (Goettel & Inglis, 1997; Butt, 2000). Conidial viability was assessed routinely prior to the preparation of each inoculum. Briefly, 100 µl of the conidia suspension was spread onto an SDAY/4 plate. After 20-

22 h incubation at 28°C, a 1 cm^2 piece of inoculated agar was placed on a microscope slide and covered with a drop of methylene blue and a glass coverslip. Germinated and non-germinated conidia were counted over different microscope fields (400x), with more than 200 conidia counted per plate on average. Conidia with germ tubes longer than their diameters were considered germinated (Goettel & Inglis, 1997; Oliveira *et al.*, 2015). Viability was > 95% for all inocula.

Injections were performed as previously described (Duffield *et al.*, 2018) using the Wiretrol® II Micro Dispenser (Drummond Scientific, PA, USA) fitted with hollow-tipped heat-pulled glass capillary tubes. Crickets were injected with 2 µl of the bacterial or conidial suspension corresponding to the optimal dose determined for each pathogen in a preliminary study (Table 2; Fig. S1). Capillaries were cleaned in 70% ethanol and rinsed with nanopure water between injections. Treatments were applied at the same time (0900h ± 1h) throughout the experiment. After the injection, crickets were returned to their individual containers with food and water.

The cuticle is the first line of defence in insects against pathogenic fungi. Experimental fungal infections are therefore usually performed topically (Goettel & Inglis, 1997). However, here, we injected our crickets to evaluate host resistance at the internal level; this allowed for comparisons with our *S. marcescens* and *B. cereus* infections and testing of more relevant associations with haemolymph immune assays.

Host clearance assays (groups iii-v)

Host clearance refers to the removal/neutralisation of pathogens after initial establishment has occurred. Clearance is commonly measured as the number of CFUs recovered from a host at specific time-points post-injection, where low pathogen loads are interpreted as a strong clearance ability (see Haine *et al.*, 2008; Hunt *et al.*, 2016). Here, pathogen load was measured 6 h post-infection (bacteria) or 24 h post-infection (fungus). Briefly, crickets were cold-anaesthetised for 10 min on ice before being transferred to a 7 mL tube containing 2.4 mm-diameter metal beads (Omni International, Kennesaw GA, USA), 4 mL of PBS and N-phenylthiourea (PTU; Sigma-Aldrich, CAS: 103-85-5). PTU inhibits PO activity and melanisation (Ryazanova *et al.*, 2012) and was used here to avoid further bacterial inactivation *in* *vitro*. Crickets were homogenised for 45 s using a Bead Ruptor 12 Homogenizer (Omni International). 100 µl of a 1/10 dilution of the homogenate was then spread onto appropriate selective medium plates. For *S. marcescens,* selective medium plates containing erythritol as sole source of carbon for growth and energy were used (Slotnick & Dougherty, 1972). This basal medium contains 7 g/L K2HPO4, 3 g/L KH2PO4, 0.5 g/L Na3-citrate, 0.1 g/L MgSO47H20, 1 g/L (NH4)2SO4, 5 g/L meso-erythritol (CAS: 149-32-6), 15 g/L agar, pH 7.0 (Sigma-Aldrich). For *B. cereus,* selective medium Brilliance™ *Bacillus cereus* (Oxoid) plates were used. For *M. robertsii,* homogenates were spread onto 'CTC' selective medium plates consisting of potato dextrose agar (PDA; Oxoid) supplemented with 2 g/L yeast extract, 0.5 g/L chloramphenicol, 0.001 g/L thiabendazole and 0.25 g/L cycloheximide (Sigma-Aldrich). CTC medium selectively supports the growth of *M. robertsii* (Fernandes *et al.*, 2010). Homogenates of naive crickets were routinely plated to confirm media selectivity.

After incubation (24 h at 30°C for the bacteria and 4 days at 28˚C for the fungus), CFUs were counted using a colony counter Scan®1200 (Interscience, Woburn, MA, USA). CFU counts were multiplied by the dilution factor to obtain total CFUs recovered per cricket.

Clearance rate was obtained by subtracting CFUs recovered from CFUs injected, divided by CFUs injected. Positive clearance rates represent superior clearance ability of the host compared to the bacterial proliferation rate, whereas negative clearance rates represent faster bacterial proliferation compared to the rate at which the host cleared the bacteria. As higher clearance rates reflect a stronger ability to control bacterial proliferation, they are assumed to represent increased immunity.

Host resistance to infection (groups vi-viii)

Experimental *S. marcescens* and *B. cereus* infections typically cause mortality within 4 days in outbred crickets, and survivorship beyond 4 days post-infection has previously been used to differentiate "disease-susceptible" from "disease-resistant" individuals (Adamo, 2004b; Kerr *et al.*, 2010). In this study, survival was monitored daily for 7 days (bacteria) or 14 days (fungus) to determine the mean time to death (i.e. death directly caused by infection), which provides an estimate of disease resistance. The number of days survived was recorded for each individual and averaged to obtain the mean time to death for each line. Individuals that survived the 7 to 14 day observation period were given an arbitrary 8 day survival time (bacteria) or 15 day survival time (fungus). Our preliminary work showed that the survival of male and female crickets receiving the optimal dosage of each pathogen was much lower than the saline control (Table 2, Fig. S1). This time-frame is relatively short compared to *G. sigillatus* average lifespan (58.45 ± 0.94 days in males and 43.70 ± 0.60 days in females from these inbred lines) (Archer *et al.*, 2012) and the differences detected are therefore unlikely to be confounded by line or sex differences in lifespan. Moreover, to further confirm that death occurred as a direct result of infection, *M. robertsii*-infected cadavers were transferred into tubes containing a damp cotton ball and incubated until sporulation occurred, as previously described (Goettel & Inglis, 1997). Mycosis was confirmed in all cases. Unfortunately, there is no such validated methodology to confirm the cause of death of bacterially-infected crickets, to the best of our knowledge. Therefore, all bacterially-infected crickets that died within 7 days post-infection were considered to have succumbed to the disease.

Statistical analysis

As we conducted our three haemolymph immune assays on the same samples, we used a Multivariate Analysis of Covariance (MANCOVA) to determine how these assays varied across inbred lines and the sexes while controlling for differences in body mass. In this model, inbred line, sex and their interaction were included as fixed effects, body mass was included as a covariate, and our three haemolymph assays were included as response variables. We included body mass as a covariate as it is known to affect immunity and differs across the sexes and inbred lines in *G. sigillatus* (Gershman *et al*., 2010b). Univariate Analysis of Covariance (ANCOVA) using the same model structure was used to determine which haemolymph assays contributed to any overall multivariate effects observed. As our remaining immune measures (encapsulation response, host clearance of, and resistance to, *S. marcescens*, *B. cereus* and *M. robertsii*) were measured on separate male and female crickets, we used the same univariate ANCOVA model to examine how these measures varied across inbred lines and the sexes. We compared our immune measures between outbred and inbred crickets for each sex using a univariate Analysis of Variance (ANOVA). In this analysis, our different inbred lines were all denoted as “inbred” and our outbred population as “outbred” and included as a fixed effect in the model. Although not genetically homogenous, we include the mean immune measures estimated for the outbred population in all of our figures to serve as a baseline.

We estimated the broad-sense heritability (H^2) (the genotypic variance divided by the phenotypic variance) of our immune assays and resistance measures and the genetic correlations (r_G) using standard quantitative genetic analyses. We estimated the H^2 of each immune measure by calculating the coefficient of intraclass correlation (t) (David *et al.*, 2005) as:

$$t = \frac{nV_b - V_w}{nV_b + (n-1)V_w}$$

where n is the number of inbred lines (in our case, 9 lines) and V_b and V_w are the between-line and within-line variance components, respectively, estimated directly from an ANOVA including inbred line as the main effect. The standard error of the intraclass correlation [SE(t)] was calculated according to Becker (1984) as:

$$\mathrm{SE}(t) = \sqrt{\frac{2(1-t)^2[1+(k-1)t]^2}{k(k-1)(n-1)}}$$

where k is the number of individuals sampled within each inbred line (in our case, 10 individuals per sex). The H^2 of each measure was then calculated according to David *et al.* (2005) as:

$$H^2 = \frac{2}{\left(\frac{1}{t} - 0.5\right)}$$

The SE of this estimate [SE(H^2)] was calculated according to David *et al.* (2005) as:

$$\mathrm{SE}(H^2) = \frac{2}{\left(1 - \frac{t}{2}\right)^2}\mathrm{SE}(t)$$

Genetic correlations and their SEs were estimated using the delete-one jackknife method of Roff & Preziosi (1994). In brief, this procedure first estimates the genetic correlation between the two immune measures using the mean estimates for each inbred line. A sequence of N (in our case 9) pseudo-values are then calculated by dropping, in turn, each of the inbred lines and estimating the resulting genetic correlations using the formula:

$$S_{N,\mathrm{i}} = Nr_{\mathrm{N}} - (N-1)r_{\mathrm{N-1,i}}$$

where $S_{\mathrm{N,i}}$ is the ith pseudo-value, r_{N} is the genetic correlation estimated using the means of all N inbred lines and $r_{\mathrm{N-1,i}}$ is the genetic correlation obtained when the ith inbred line is dropped. The jackknife estimate of the genetic correlation (r_{j}) is simply taken as the mean of these pseudo-values. An estimate of the SE for r_{j} is given by:

$$\mathrm{SE} = \frac{\sum_{i=1}^{i=N}\left(S_{N,i} - r_{\mathrm{j}}\right)^2}{N(N-1)}$$

Roff & Preziosi (1994) used simulation models to demonstrate that this jackknife approach provides better genetic estimates than those based purely on inbred line means when the number of inbred lines used is small (< 20 inbred lines). It is important to note that estimates of genetic (co)variance based on inbred lines contain variance due to dominance and/or epistasis and therefore should be considered broad-sense estimates (Falconer & Mackay, 1996). Our estimates of H^2 and r_{G} for immune measures were considered statistically significant if the estimates divided by their SE exceeded 1.96, the critical value for a two-tailed *t*-distribution with infinite degrees of freedom.

Using the above procedures, we estimated the heritability of, and genetic correlations between, immune measures in males (H^2_{M} and r_{M}, respectively) and females (H^2_{F} and r_{F}, respectively), as well as the genetic correlations between these immune measures across the sexes (r_{MF}). We used a randomisation procedure to determine if estimates of r_{M} and r_{F} were correlated across the sexes. In short, we first ran a bivariate Pearson correlation analysis to determine the real correlation coefficient between estimates of r_{M} and r_{F}. Next, we shuffled estimates of r_{F} at random across different combinations of immune measures to create an expected distribution where estimates of r_{M} and r_{F} were not correlated. We then used a Monte Carlo simulation to repeat this process 10,000 times and determine the proportion (p) of times (out of the total number of permutations) that the correlation coefficient obtained from this randomised data exceeded that for the real data. This proportion was used to provide a two-tailed significance test for the correlation coefficient using the protocol outlined in Manly (1997). We examined the normality of our estimates of r_{M} and r_{F} using a Shapiro-Wilk test and compared the average magnitude of differences in these estimates using a paired *t*-test.

It is important to note that, for all of our immune assays (groups *i*-*v*), higher values are assumed to represent increased immunity. If these assays predict disease resistance, we expect a positive genetic correlation between these measures.

Results

The effects of inbred line, sex and their interaction on haemolymph immune assays

There was an overall multivariate effect of inbred line, sex and their interaction on haemolymph immune assays (haemocyte count, ZI and PO activity), but body mass did not influence these assays (Table 3). Univariate ANCOVA showed that the overall multivariate effect of inbred line was driven by all three haemolymph assays, whereas the overall multivariate effect of sex was due to females having higher haemocyte count and PO activity than males, but not for ZI (Table 3, Fig. 2). The overall multivariate effect of the interaction between inbred line and sex was also driven by all three haemolymph assays, as evidenced by the fact that the reaction norms for these assays across the sexes were not parallel (Table 3, Fig. 2). None of the individual haemolymph assays varied with body mass (Table 3). Haemocyte count ($F_{1,98} = 0.49$) and ZI ($F_{1,98} = 2.62$, $P = 0.11$) were similar for inbred and outbred male crickets, although PO activity was slightly higher in inbred than outbred males ($F_{1,98} = 3.06$, $P = 0.08$) (Fig. 2). Haemocyte count ($F_{1,98} = 2.00$, $P = 0.16$) and PO activity (female: $F_{1,98} = 0.04$, $P = 0.84$) were similar for inbred and outbred female crickets but ZI was slightly higher in inbred than outbred females ($F_{1,98} = 3.41$, $P = 0.07$) (Fig. 2).

The effects of inbred line, sex and their interaction on encapsulation response and pathogen clearance ability

There were also significant differences in encapsulation ability and clearance of *S. marcescens*, *B. cereus* and *M. robertsii* across inbred lines (Table 4). Encapsulation ability was higher in females than males (Fig. 3A), but the sexes did not differ in their clearance of all three pathogens (Table 4; Fig. 3B-D). The interaction between inbred line and sex was significant for the clearance of *B. cereus* and *M. robertsii* and approached significance for encapsulation ability and clearance of *S. marcescens* (Table 4). Accordingly, there was considerable intersection of the reaction norms for these immune measures across the sexes (Fig. 3). Encapsulation ability and clearance of all pathogens were not influenced by body mass (Table 4). Male and female inbred and outbred crickets did not differ in encapsulation ability (male: $F_{1,98} = 1.02$, $P = 0.32$; female: $F_{1,98} = 1.15$, $P = 0.29$) or clearance of *B. cereus* (male: $F_{1,98} = 0.33$, $P = 0.57$; female: $F_{1,98} = 2.25$, $P = 0.14$) or *M. robertsii* (male: $F_{1,98} = 0.00$, $P = 0.98$; female: $F_{1,98} = 0.05$, $P = 0.83$) (Fig.

3A,C-D). Inbred female crickets, however, showed higher clearance of *S. marcescens* than outbred female crickets ($F_{1,98} = 8.88$, $P = 0.004$), but this effect was not as great in males ($F_{1,98} = 3.21$, $P = 0.08$) (Fig. 3b).

The effects of inbred line, sex and their interaction on host resistance to infection

There were significant differences in host resistance to *S. marcescens*, *B. cereus* and *M. robertsii* across inbred lines (Table 5). Males showed higher resistance to *S. marcescens* than females (Fig. 4A), but there were no sex differences in resistance to *B. cereus* and *M. robertsii* (Fig. 4B-C). There were significant inbred line by sex interactions for resistance to *S. marcescens* and *B. cereus,* while this interaction approached significance for resistance to *M. robertsii* (Table 5). There was substantial intersection of the reaction norms for resistance to all pathogens across the sexes (Fig. 4). Resistance to *S. marcescens*, *B. cereus* and *M. robertsii* was not influenced by body mass (Table 5). Male and female inbred and outbred crickets showed similar resistance to *S. marcescens* (male: $F_{1,98} = 0.05$, $P = 0.83$; female: $F_{1,98} = 0.56$, $P = 0.46$) and *M. robertsii* (male: $F_{1,98} = 0.01$, $P = 0.93$; female: $F_{1,98} = 0.00$, $P = 0.98$) (Fig. 4). Similarly inbred and outbred males had similar resistance to *B. cereus* ($F_{1,98} = 0.50$, $P = 0.48$) but inbred females had a slightly higher resistance to this pathogen than outbred females ($F_{1,98} = 3.60$, $P = 0.06$) (Fig. 4).

Heritability and genetic covariance of immune measures within the sexes

Consistent with the significant effects of inbred line, we found that all immune assays and our measures of disease resistance were heritable in both sexes (Tables 6, 7). Estimates of H^2, however, varied greatly across the different immune measures, ranging from 0.55 to 0.87 in males and 0.53 to 0.92 in females (Tables 6, 7). There were no obvious patterns in the magnitude of H^2_M and H^2_F estimates, with the possible exception that H^2_F for disease resistance was lower than for immune assays (Table 7). Estimates of H^2_M and H^2_F were of similar magnitude in males and females (H^2_M: 0.71 ± 0.03; H^2_F: 0.74 ± 0.04; $t_9 = 0.70$, $P = 0.50$) and were not correlated across the sexes ($r = 0.16$, $n = 10$, $p = 0.35$, $P = 0.71$).

There was also substantial genetic covariance between our different immune measures in both males and females (Tables 6, 7). In both sexes, estimates of r_M and r_F did not deviate from a mean of zero (r_M: $t_{44} = 0.08$, $P = 0.93$; r_F: $t_{44} = 0.63$, $P = 0.53$) and were normally distributed (r_M:

Shapiro-Wilk = 0.98, $df = 45$, $P = 0.67$; r_F: Shapiro-Wilk = 0.97, $df = 45$, $P = 0.23$) indicating that positive and negative estimates were as common and similar in magnitude. Moreover, the average magnitude of r_M and r_F did not differ (r_M: -0.004 ± 0.05, r_F: -0.04 ± 0.07; $t_{44} = 0.53$, $P = 0.60$) and were not correlated across the sexes ($r = 0.23$, $n = 45$, $p = 0.07$, $P = 0.13$).

Genetic correlations between immune measures in males

There were positive estimates of r_M between haemolymph immune assays, with the notable exception of a negative r_M between haemocyte count and PO activity (Table 6). Likewise, estimates of r_M between encapsulation ability and haemolymph assays were all positive, although only significant between encapsulation ability and ZI. There was a positive r_M between the clearance of *S. marcescens* and haemocyte count and negative estimates between the clearance of this bacterium and PO activity, as well as encapsulation ability. Meanwhile, r_M was positive between the clearance of *B. cereus* and PO activity. Positive r_M estimates were observed between the clearance of *M. robertsii* and ZI, as well as encapsulation ability. There was a positive r_M between the clearance of *S. marcescens* and *B. cereus* and negative r_M between the clearance of *S. marcescens* and *M. robertsii*, as well as between the clearance of *B. cereus* and *M. robertsii*.

There were positive estimates of r_M between all haemolymph assays and resistance to *S. marcescens*, as well as resistance to *B. cereus* (except for haemocyte count, Table 6). In contrast, estimates of r_M between haemolymph assays and resistance to *M. robertsii* were not significant. Moreover, none of the estimates of r_M between encapsulation ability and resistance to *S. marcescens*, *B. cereus* and *M. robertsii* were significant. Estimates of r_M were all positive between the clearance of *S. marcescens* and resistance to *S. marcescens*, *B. cereus* and *M. robertsii*. Similar results were obtained between the clearance of *B. cereus* and resistance to all three pathogens. r_M was positive between the clearance of *M. robertsii* and resistance to *B. cereus*, but negative between the clearance of *M. robertsii* and resistance to the same pathogen. There was also little consistency in the genetic covariance between resistance to both bacteria and the fungus, with only a positive r_M between resistance to *S. marcescens* and resistance to *M. robertsii*.

Genetic correlations between immune measures in females

There were positive estimates of r_F between all haemolymph immune assays, as well as between these assays and encapsulation ability (Table 7). There were no significant r_F between the clearance of *S. marcescens* and haemolymph immune assays, but a negative r_F was observed between clearance of this bacterium and encapsulation ability. Positive estimates of r_F existed between the clearance of *B. cereus* and haemocyte count, ZI and encapsulation activity, as well as between the clearance of *M. robertsii* and haemocyte count, ZI and PO activity. There was also a positive r_F estimate between the clearance of *S. marcescens* and *M. robertsii,* and a negative r_F between the clearance of *S. marcescens* and *B. cereus*. There was no significant r_F between the clearance of *B. cereus* and *M. robertsii.*

There were positive estimates of r_F between all haemolymph assays and resistance to *S. marcescens,* as well as between ZI and resistance to *B. cereus* (Table 7). Meanwhile, estimates of r_F between haemolymph assays and resistance to *M. robertsii* were all negative. Estimates of r_F were similarly all negative between encapsulation ability and resistance to the three pathogens. Positive r_F were detected between the clearance of *S. marcescens* and resistance to the three pathogens. In contrast, negative r_F estimates were observed between the clearance of and resistance to *B. cereus,* as well as between the clearance of and resistance to *M. robertsii.* There was a positive r_F estimate between clearance of *M. robertsii* and resistance to *B. cereus,* however. Finally, there was a positive r_F between resistance to *S. marcescens* and resistance to *B. cereus* and a negative r_F between resistance to *S. marcescens* and *M. robertsii.*

Genetic covariance of immune measures across the sexes

There was also considerable genetic covariance between the same immune measures across the sexes, although this was not as large as expected if immune function was regulated by the same genes in the sexes (Table 8). For example, there was a negative r_{MF} for haemocyte count and positive estimates of r_{MF} for ZI, encapsulation ability, clearance of *S. marcescens* and resistance to *B. cereus*, but all other immune measures were not genetically correlated across the sexes (Table 8). Likewise, while there was ample genetic covariance between different immune measures across the sexes, there was no clear pattern observed in the estimates of r_{MF} (Table 8). That is, estimates of r_{MF} above (female-male) and below (male-female) the diagonal in Table 8 were both normally distributed (female-male: Shapiro-Wilk = 0.97, *df* = 45, *P* = 0.41; male-female: Shapiro-Wilk = 0.99, *df* = 45, $P = 0.84$) and did not deviate significantly from a mean of zero (female-male: $t_{44} = 0.75$, $P = 0.46$; male-female: $t_{44} = 0.50$, $P = 0.62$) indicating that positive and negative estimates were as common and similar in magnitude. Moreover, the average magnitude of these estimates did not differ (female-male: -0.05 ± 0.06, male-female: 0.03 ± 0.06, $t_{44} = 0.89$, $P = 0.38$) and were not correlated ($r = -0.01$, $n = 45$, $p = 0.52$, $P = 0.96$).

Discussion

Here we provide a comprehensive examination of the genetic architecture of seven immune assays and resistance to three pathogens in male and female *G. sigillatus*. Although we show considerable genetic variance in and covariance between immune assays and pathogen resistance in *G. sigillatus*, the nature of these relationships are sex and pathogen specific. While both sexes exhibit substantial genetic (co)variance in immune assays and resistance, these genetic estimates differed across the sexes. In addition, many of these immune assays and measures of disease resistance were not genetically correlated across the sexes suggesting the potential for them to evolve independently in the sexes. The sign and magnitude of genetic correlations between resistances to the different pathogens were also inconsistent in each sex, indicating that resistance to one pathogen does not necessarily confer resistance to another. The nature of the genetic relationships we demonstrate here illustrates that the evolution of immune function in male and female *G. sigillatus* is likely to be complex. Whether or not this degree of complexity exists for other species will require similar quantitative genetic studies that measure a large number of assays and resistance to multiple pathogens in both sexes.

Despite the large number of genetic relationships between immune assays and disease resistance that we quantified in *G. sigillatus*, only two patterns were consistent in both sexes. First, we found that haemocyte count, ZI and PO activity all exhibited positive genetic correlations with resistance to *S. marcescens* in males and females. The relationships between these immune assays and resistance to *B. cereus* and *M. robertsii*, however, were far less consistent. While there were also positive genetic correlations between these immune assays and resistance to *B. cereus*, only half of these estimates were statistically significant (ZI and PO activity in males and ZI in females). There were significant negative genetic correlations between these immune assays and resistance to *M. robertsii* in females but not in males. These patterns suggest that while internal immune defences, such as those captured by our haemolymph assays, may be relatively effective in providing resistance against bacteria, they appear less effective against attack by pathogenic fungi where the main line of defence is likely to occur at the cuticle (Goettel & Inglis, 1997). Likewise, our finding that encapsulation ability exhibited negative genetic correlations with resistance to all three pathogens in females but not in males also suggests that the encapsulation response is unlikely to be effective against bacterial or fungal infection. This is perhaps not unexpected given that the encapsulation response in insects predominately serves to protect against larger attackers, such as macro-parasites and parasitoids. Second, we showed consistent significant positive genetic correlations between the clearance of *S. marcescens* and the resistance to all three pathogens in males and females. While other positive genetic correlations between pathogen clearance and resistance were found, these were far less consistent across the sexes. Exactly why the clearance of *S. marcescens* provides an accurate predictor of resistance to all three pathogens is currently unknown, but the fact that negative clearance rates (i.e. *in vivo* microbial growth) were obtained across most genotypes for *S. marcescens,* but not for *B. cereus* and *M. robertsii* (Fig. 3), suggests that *S. marcescens* proliferates relatively faster in *G. sigillatus.* Therefore, genotypes capable of controlling the fast proliferation of *S. marcescens* might also be more efficient in controlling the proliferation of *B. cereus* and *M. robertsii,* conferring greater resistance to all three pathogens. Clearly more work is needed to understand this relationship, but it does suggest that the clearance of *S. marcescens* may capture an aspect of "general" immune function in *G. sigillatus*.

The general lack of consistency that we show in the relationships between immune assays and pathogen resistance in *G. sigillatus* largely supports the mixed results reported in other insects. In some species, immune assays are positively correlated with resistance to pathogens (Kraaijeveld *et al.,* 2001; Wilson *et al.,* 2001; Tucker & Stevens, 2003), whereas in others, no such relationship exists (Adamo *et al.*, 2001; Adamo, 2004b; Leclerc *et al.*, 2006). The same inconsistency also exists for the relationship between pathogen clearance and disease resistance, where contrasting results have even been documented within the same host species (Corby-Harris *et al.*, 2007; Sleiman *et al.*, 2015). Interestingly, studies on vertebrates have shown more certainty in this relationship, with most confirming a positive relationship between immune assays and disease resistance often with a surprisingly high degree of predictability (e.g. Keil *et* *al.,* 2001; Biard *et al.,* 2015). While this suggests that the relationship between immune assays and disease resistance may be more labile in insects than vertebrates, more thorough testing across a wider range of species is needed to confirm this.

In nature, most organisms are attacked by multiple pathogen species, often coming from very different taxonomic groups. Despite this, only two studies have examined this relationship in insects and have yielded mixed results. In *D. melanogaster*, there is a positive genetic correlation between resistance to the bacterium *P. aeruginosa* and the fungus *M. anisopliae* (Wang *et al.,* 2017). However, 25 generations of artificial selection for resistance to the pathogenic fungus *Beauveria bassiana* in the greater wax moth *Galleria mellonella*, resulted in individuals being more resistant to this fungus but not to the fungus *M. anisopliae* (Dubovskiy *et al*., 2013). In *G. sigillatus*, we found that disease resistance was highly heritable, with H^2 estimates being similar in magnitude for each pathogen examined and for both sexes. The sign and strength of the genetic correlations, however, differed across the sexes. In females, there was a significant positive genetic correlation between resistance to *S. marcescens* and *B. cereus* and a significant negative genetic correlation between resistance to *S. marescens* and *M. robertsii* but the genetic correlation between resistance to *B. cereus* and *M. robertsii* was not significant. In males, there was a significant positive genetic correlation between resistance to *S. marcescens* and *M. robertsii*, but all other genetic correlations were not significant. Collectively, these findings show that resistance to one pathogen does not always mean resistance to another and highlights the need to examine multiple pathogen species in ecological immunity studies.

Our results provide partial support for general prediction of sexual selection theory that females will have a superior immune function to males (Zuk & Stoehr, 2002). We show in *G. sigillatus* that haemocyte count, PO activity and encapsulation response were all higher in females than males across our inbred lines. However, ZI, clearance of all pathogens and resistance to *B. cereus* and *M. robertsii* did not differ across the sexes and resistance to *S. marcescens* was actually higher in males than females. The lack of sexual dimorphism observed for pathogen clearance and disease resistance may reflect the fact that these measures provide a more integrative view of the immune system that involves more immune pathways than the targeted immune assays we examined (Charles & Killian, 2015). As such, it is possible that sex differences may be lost when examining immune measures that encompass a greater number of immune pathways. More importantly, our findings caution against only using single, targeted immune assays to study sexual dimorphism in insect immunity, as sex differences in these assays may not always be accompanied by sex differences in pathogen clearance and/or disease resistance.

Although we observe ample genetic (co)variance in both immune assays and disease resistance in both sexes, males and females were shown to have different genetic architecture for these immune measures. That is, estimates of heritability (H^2_{M} and H^2_{F}) and genetic correlations (r_{M} and r_{F}) for immune assays and disease resistance were not correlated across the sexes. Furthermore, these immune measures were not always significantly genetically correlated across the sexes (r_{MF}), as would be expected if the genes governing them were shared by the sexes. Indeed, only five out of the ten immune measures we examined (haemocyte count, ZI, encapsulation response, clearance of *S. marcescens* and resistance to *B. cereus*) showed significant estimates of r_{MF}, whereas the remaining five measures were not genetically correlated across the sexes. The high estimates of H^2, r_{M} and r_{F} shown for immune measures, as well as the fact that these estimates vary across the sexes, is consistent with previous work on this species, but it should be noted that far fewer immune measures were examined and no formal comparisons between the sexes were conducted (Gershman *et al.,* 2010a, b). Our findings also show striking similarities to work on the mealworm beetle *Tenebrio molitor* that showed high H^2 estimates for PO activity, cuticular darkness and haemocyte density in both sexes and estimates of r_{M} and r_{F} that statistically differed between the sexes (Rolff *et al.*, 2005). This work, however, showed significant positive estimates of r_{MF} for each immune measure suggesting that they are genetically constrained from evolving independently in the sexes (Rolff *et al.,* 2005). Likewise, there are significant negative estimates of r_{MF} in *Drosophila melanogaster* for tolerance and resistance to the bacterial pathogen *Pseudomonas aeruginosa* suggesting that antagonistic pleiotropy is likely to constrain the evolution of sexual dimorphism (Vincent & Sharp, 2014). Contrary to these studies, our estimates of r_{MF} in *G. sigillatus* suggest that while some immune measures are likely to be constrained in this species, others have the potential to evolve independently in the sexes. This is likely to have important implications for the evolution of sexual dimorphism in immune function in *G. sigillatus*, although estimates of selection for these individual immune measures are needed for each sex to better understand this process (Lande, 1980).

It is important to recognize a number of caveats with our current study. First, our genetic estimates for immune assays and pathogen resistance are based on only 9 inbred lines. It is therefore possible that our findings are not generalizable to the population (or species) as a whole. This is a concern for all studies using inbred lines as the inbreeding process can reduce genetic variation as genotypes are selectively lost from the population. While possible, this is unlikely in our study as only one out of ten lines (10%) established at random from the outbred base population went extinct during the inbreeding process and most of our measures of immune function were similar for inbred and outbred crickets. Second, we examined bacterial clearance 6 hours post-infection, so it could be argued that a longer timeframe would have provided a better assessment of immune function. We selected this timeframe as it has been used in other insect studies (Haine *et al.*, 2008; Miller & Cotter, 2018) and the significant estimates of H^2_{M} and H^2_{F} we show for bacterial clearance indicates this timeframe is sufficient to detect differences in immune function between genotypes. However, it is entirely possible that if we measured bacterial clearance over a longer timeframe (as we did for our fungal pathogen), our genetic estimates would be different. Third, while it would have been ideal to have saline control individuals for each sex and genetic line to account for any potential sex and line responses to wounding in our host resistance assay, this would simply not have been logistically possible in our experiment. However, despite not having saline controls for males and females in each inbred line, we did extensively test the optimal dosage of each pathogen in male and female outbred crickets and have found that for the dosages of each pathogen used, it is the pathogen rather than the inoculation procedure that reduces mortality (compare the survival of the saline control to the optimal dosage in Fig. S1). Finally, in our measurements of the clearance and resistance to bacterial and fungal pathogens we injected a known quantity of pathogen into crickets rather than administering this dosage topically. Consequently, this approach bypasses the host's behavioural, physical and local immune defences (Adamo, 2004a), which can often be effective component of immune function in insects (e.g. Hunt *et al.*, 2016; Cini *et al.*, 2020). Our reason for using injections was to ensure that the immune function of crickets from each line and for both sexes was assessed when a standardized quantity of pathogens entered their body, which cannot be guaranteed with topical application. Ideally, future studies would examine both applications simultaneously to better understand the complexity of processes that help prevent pathogens from entering the body of the host in the first place.

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Authors' contributions

CL, KRD, BMS, SKS, CMH and JH conceived the ideas and designed methodology; CL collected the data; CL, BMS, SKS and JH analysed the data; CL and JH led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Data availability statement

Data will be deposited in Dryad on acceptance.

Non-discrimination statement

WSU, UoE, ISU and USDA are equal opportunity providers and employers.

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Tables

Table 1. Estimates of repeatability, measured as the intraclass correlation coefficient (ICC) and 95% confidence intervals (95% CIs) for immune assays in male and female outbred crickets. Repeatability estimates and 95% CIs were estimated following the protocols of Wolak et *al*. 2012.

	Males			**Females**	
Immune assay	ICC	95% CIs		ICC	95% CIs
Haemocyte count	0.85	0.72, 0.97		0.87	0.76, 0.98
Zone of inhibition	0.92	0.86, 0.99		0.98	0.95, 1.00
PO activity	0.78	0.61, 0.95		0.82	0.69, 0.97
Encapsulation response	0.85	0.68, 1.03		0.86	0.69, 1.03
Clearance of *S. marcescens*	0.97	0.94, 0.99		0.99	0.98, 1.00
Clearance of *B. cereus*	0.98	0.96, 0.99		0.98	0.97, 1.00
Clearance of *M. robertsii*	0.92	0.86, 0.98		0.92	0.86, 0.98

Table 2. Dose selection for *Serratia marcescens*, *Bacillus cereus* or *Metarhizium robertsii* in *G. sigillatus*. For each bacterial and fungal species examined, 20 crickets of each sex were injected per dosage (including the saline control). All crickets were injected at 8 days post-eclosion with 2 µl of saline containing various concentrations of live bacteria or fungal spores. In each instance, CFUs refers to colony forming units. Survivorship (measured as a %) of each injected cricket was monitored daily for 7 days in bacteria and 14 days in the fungus. For each pathogen, the dosage highlighted in bold was selected for subsequent experiments.

	% Survival	
Treatment	Females	Males
Serratia marcescens		
Saline	85%	95%
10^2 CFUs (live)	85%	75%
10^3 CFUs (live)	55%	90%
10^4 CFUs (live)	**55%**	**75%**
10^5 CFUs (live)	25%	5%
Bacillus cereus		
Saline	70%	100%
10^2 CFUs (live)	65%	80%
10^3 CFUs (live)	50%	85%
10^4 CFUs (live)	**30%**	**70%**
10^5 CFUs (live)	20%	15%
Metarhizium robertsii		
Saline	65%	80%
10^3 CFUs (live)	75%	75%
10^4 CFUs (live)	50%	80%
10^5 CFUs (live)	**15%**	**5%**
10^6 CFUs (live)	5%	0%

Table 3. MANCOVA examining the effects of inbred line, sex and their interaction on three hemolymph immune assays (haemocyte count, zone of inhibition and PO activity) in crickets. Univariate ANCOVAs are used to determine how each immune assay contributes to the overall multivariate effect.

	MANCOVA			
	Model term	**Pillai's Trace**	***df***	***P***
	Line	0.55	24,483	0.0001
	Sex	0.61	3,159	0.0001
	Line × Sex	0.55	24,483	0.0001
	Body mass	0.03	3,159	0.19
	Univariate ANCOVAs			
Trait	**Model term**	***F***	***df***	***P***
Haemocyte count	Line	3.22	8,161	0.002
	Sex	10.75	1,161	0.001
	Line × Sex	6.14	8,161	0.0001
	Body mass	0.10	1,161	0.75
Zone of inhibition	Line	4.59	8,161	0.0001
	Sex	2.10	1,161	0.15
	Line × Sex	2.74	8,161	0.007
	Body mass	1.07	1,161	0.30
PO activity	Line	7.40	8,161	0.0001
	Sex	244.59	1,161	0.0001
	Line × Sex	7.75	8,161	0.0001
	Body mass	2.98	1,161	0.09

Table 4. Univariate ANCOVAs examining the effects of inbred line, sex and their interaction on the encapsulation response and pathogen clearance of crickets.

	Univariate ANCOVAs			
Trait	**Model term**	***F***	***df***	***P***
Encapsulation response	Line	10.44	8,161	0.0001
	Sex	13.90	1,161	0.0001
	Line × Sex	1.94	8,161	0.06
	Body mass	0.63	1,161	0.43
Clearance of *S. marcescens*	Line	7.40	8,161	0.0001
	Sex	0.29	1,161	0.59
	Line × Sex	1.86	8,161	0.07
	Body mass	0.00	1,161	0.95
Clearance of *B. cereus*	Line	4.33	8,161	0.0001
	Sex	1.13	1,161	0.29
	Line × Sex	2.93	8,161	0.004
	Body mass	1.68	1,161	0.20
Clearance of *M. robertsii*	Line	3.48	8,161	0.001
	Sex	1.08	1,161	0.30
	Line × Sex	5.89	8,161	0.0001
	Body mass	1.56	1,161	0.21

Table 5. Univariate ANCOVAs examining the effects of inbred line, sex and their interaction on resistance of crickets to three pathogens.

	Univariate ANCOVAs			
Trait	**Model term**	***F***	***df***	***P***
Resistance to *S. marcescens*	Line	3.15	8,161	0.002
	Sex	4.26	1,161	0.04
	Line × Sex	2.09	8,161	0.04
	Body mass	0.01	1,161	0.93
Resistance to *B. cereus*	Line	4.52	8,161	0.0001
	Sex	1.78	1,161	0.18
	Line × Sex	1.98	8,161	0.04
	Body mass	1.01	1,161	0.32
Resistance to *M. robertsii*	Line	3.14	8,161	0.002
	Sex	0.32	1,161	0.57
	Line × Sex	1.89	8,161	0.07
	Body mass	0.90	1,161	0.35

Table 6. The heritability (H^2_M) of and genetic correlation (r_M) between different immune measures in male crickets. H^2_M estimates are provided along the diagonal (in italics) and r_M estimates are provided in the off diagonal positions. In each case, estimates provided in bold are statistically significant at $P < 0.05$.

	HC	ZI	PO	ENC	CS	CB	CM	RS	RB	RM
HC	***0.55***									
ZI	**0.29**	***0.77***								
PO	**-0.18**	**0.44**	***0.66***							
ENC	0.11	**0.17**	0.10	***0.87***						
CS	**0.42**	0.01	**-0.35**	**-0.59**	***0.83***					
CB	0.16	0.02	**0.23**	-0.09	**0.59**	***0.70***				
CM	0.29	**0.66**	0.05	**0.34**	**-0.23**	**-0.50**	***0.75***			
RS	**0.35**	**0.45**	**0.29**	0.08	**0.18**	**0.42**	-0.14	***0.66***		
RB	0.20	**0.36**	**0.19**	0.01	**0.18**	**0.20**	**0.73**	0.25	***0.64***	
RM	0.07	-0.15	0.26	0.24	**0.39**	**0.31**	**-0.22**	**0.49**	0.05	***0.62***

Abbreviations: HC = haemocyte count, ZI = zone of inhibition, PO = phenoloxidase activity, ENC = implant encapsulation response, CS = clearance of *S. marcescens*, CB = clearance of *B. cereus*, CM = clearance of *M. robertsii*, RS = resistance to *S. marcescens*, RB = resistance to *B. cereus* and RM = resistance to *M. robertsii*.

Table 7. The heritability (H^2_F) of and genetic correlation (r_F) between different immune measures in female crickets. H^2_F estimates are provided along the diagonal (in italics) and r_F estimates are provided in the off diagonal positions. In each case, estimates provided in bold are statistically significant at $P < 0.05$. Abbreviations as provided in Table 4.

	HC	ZI	PO	ENC	CS	CB	CM	RS	RB	RM
HC	***0.82***									
ZI	**0.78**	***0.70***								
PO	**0.50**	**0.35**	***0.92***							
ENC	**0.65**	**0.33**	**0.26**	***0.81***						
CS	-0.06	-0.16	-0.20	**-0.46**	***0.72***					
CB	**0.34**	**0.47**	-0.04	**0.29**	**-0.66**	***0.76***				
CM	**0.55**	**0.58**	**0.54**	-0.01	**0.52**	-0.14	***0.84***			
RS	**0.51**	**0.71**	**0.50**	**-0.20**	**0.39**	0.00	**0.95**	***0.55***		
RB	0.16	**0.26**	0.06	**-0.47**	**0.50**	**-0.29**	**0.69**	**0.79**	***0.72***	
RM	**-0.77**	**-0.68**	**-0.39**	**-0.63**	**0.29**	-0.05	**-0.23**	**-0.27**	-0.06	***0.53***

Table 8. The across sex genetic correlations (r_{MF}) between immune measures. Estimates of r_{MF} along the diagonal are for the same immune measures in males and females. Estimates of r_{MF} above the diagonal represent the genetic covariance between an immune measure in the female against a different immune measure in the male, whereas estimates below the diagonal represent the genetic covariance between an immune measure in the male against a different immune measure in the female. Estimates of r_{MF} in bold are statistically significant at $P < 0.05$. Abbreviations as provided in Table 4.

		Females									
		HC	ZI	PO	ENC	CS	CB	CM	RS	RB	RM
Males	HC	**-0.49**	**-0.45**	**-0.56**	**-0.44**	**0.63**	**-0.40**	0.14	0.10	**0.50**	**0.67**
	ZI	**0.11**	**0.28**	-0.11	**-0.25**	-0.06	-0.09	**0.24**	**0.50**	**0.83**	-0.33
	PO	0.21	**0.60**	0.15	-0.48	-0.05	**0.35**	**0.17**	**0.47**	**0.40**	-0.09
	ENC	**0.60**	**0.50**	-0.07	**0.69**	-0.23	**0.49**	0.05	0.06	-0.03	**-0.33**
	CS	-0.17	-0.36	0.12	**-0.44**	**0.70**	**-0.55**	**0.62**	**0.45**	**0.54**	**0.32**
	CB	**0.29**	**0.57**	**0.48**	**-0.22**	**0.50**	-0.07	**0.99**	**0.92**	**0.54**	0.01
	CM	0.07	0.10	**-0.77**	0.05	0.06	-0.01	-0.30	-0.12	0.32	**-0.28**
	RS	0.08	**0.11**	-0.19	**-0.33**	**0.75**	**-0.15**	**0.32**	0.23	0.07	**0.35**
	RB	0.12	**0.34**	**-0.69**	**-0.25**	**0.39**	0.05	**0.22**	**0.35**	**0.42**	-0.15
	RM	0.57	0.16	**0.30**	-0.06	**0.34**	**0.36**	**0.55**	**0.44**	0.32	0.16

Figure Legends

Fig. 1. Experimental design showing the immune measures examined, the timeframe over which these immune measurements were taken (indicated by black bars) and the number of male and female crickets sampled from each inbred line for each measurement.

Fig. 2. Reaction norms for mean (A) haemocyte count (cells/mL), (B) zone of inhibition (mm) and (C) PO activity (OD change per min) across the sexes in *G. sigillatus*. Each black line represents a discrete genotype, whereas the grey line represents outbred crickets.

Fig. 3. Reaction norms for mean (A) implant encapsulation response (darkness score) and the clearance rates of (B) *S. marcescens*, (C) *B. cereus* and (D) *M. robertsii* across the sexes in *G. sigillatus*. Each black line represents a discrete genotype (inbred line), whereas the grey line represents outbred crickets.

Fig. 4. Reactions norms for mean resistance (days survived) following inoculation with (A) *S. marcescens*, (B) *B. cereus* or (C) *M. robertsii* across the sexes in *G. sigillatus*. Each black line represents a discrete genotype (inbred line), whereas the grey line represents outbred crickets.

Fig. 1

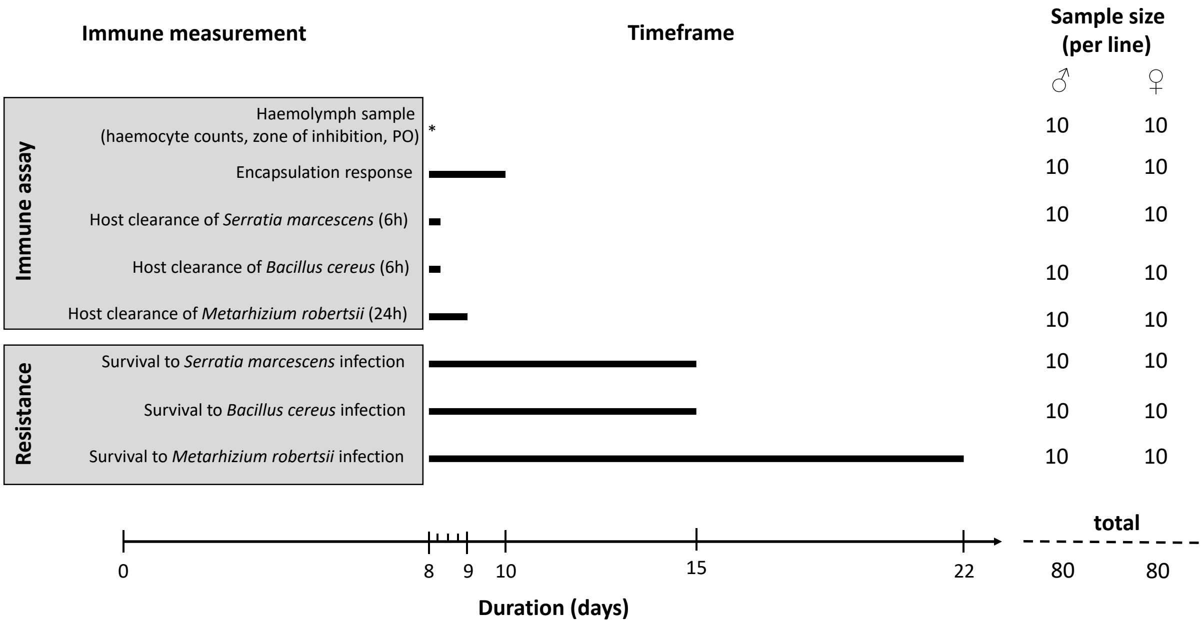
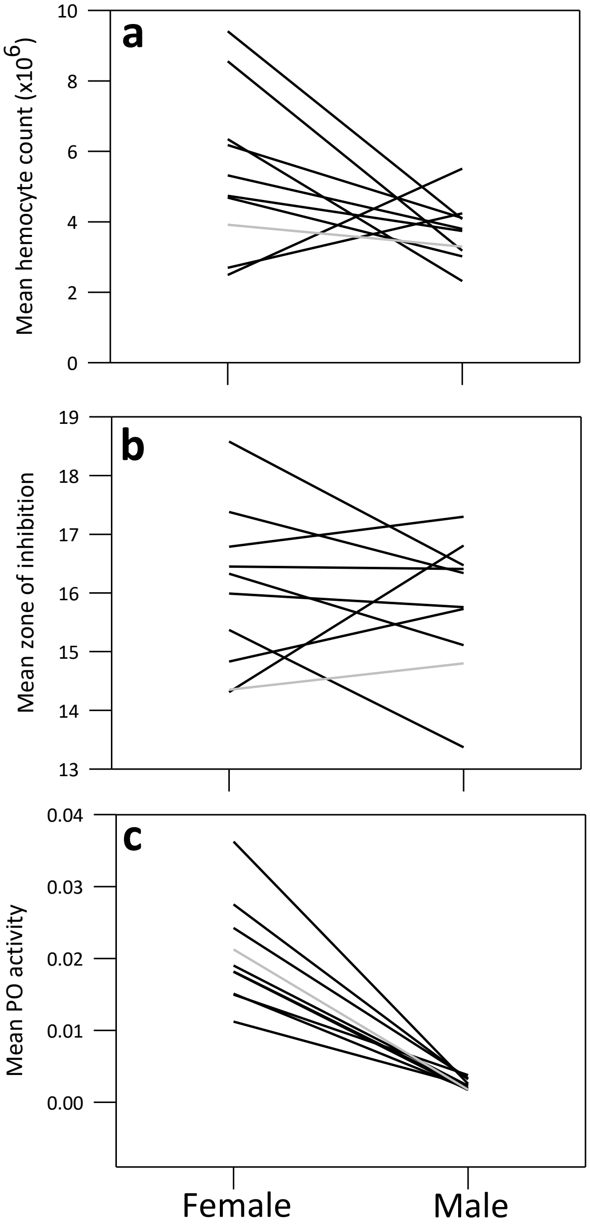

Immune measurement
Timeframe
Sample size (per line)
♂
♀
Immune assay
Haemolymph sample (haemocyte counts, zone of inhibition, PO)
*
Encapsulation response
Host clearance of *Serratia marcescens* (6h)
Host clearance of *Bacillus cereus* (6h)
Host clearance of *Metarhizium robertsii* (24h)
Resistance
Survival to *Serratia marcescens* infection
Survival to *Bacillus cereus* infection
Survival to *Metarhizium robertsii* infection
10 10
10 10
10 10
10 10
10 10
10 10
10 10
10 10
total
80 80
0
8
9
10
15
22
Duration (days)

Fig. 2



a
Mean hemocyte count (x10^6)
0
2
4
6
8
10
b
Mean zone of inhibition
13
14
15
16
17
18
19
c
Mean PO activity
0.00
0.01
0.02
0.03
0.04
Female
Male

Fig. 3

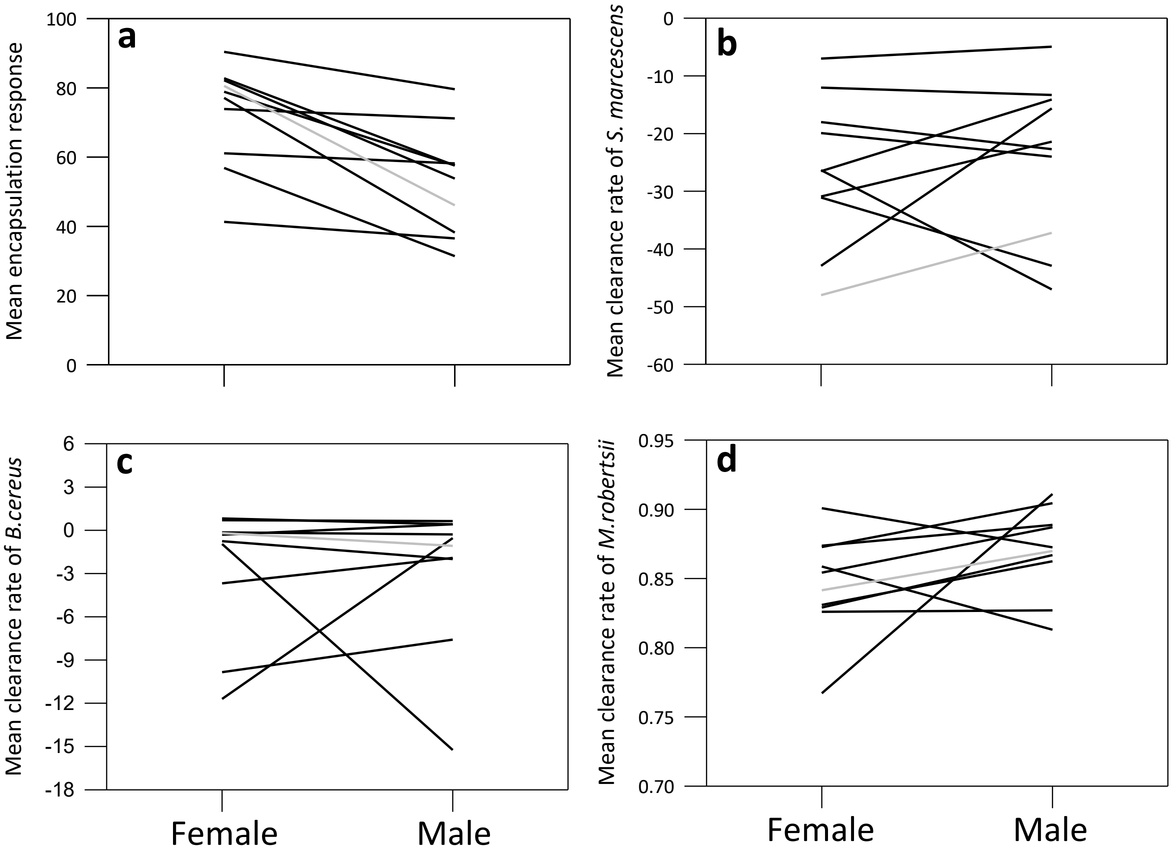
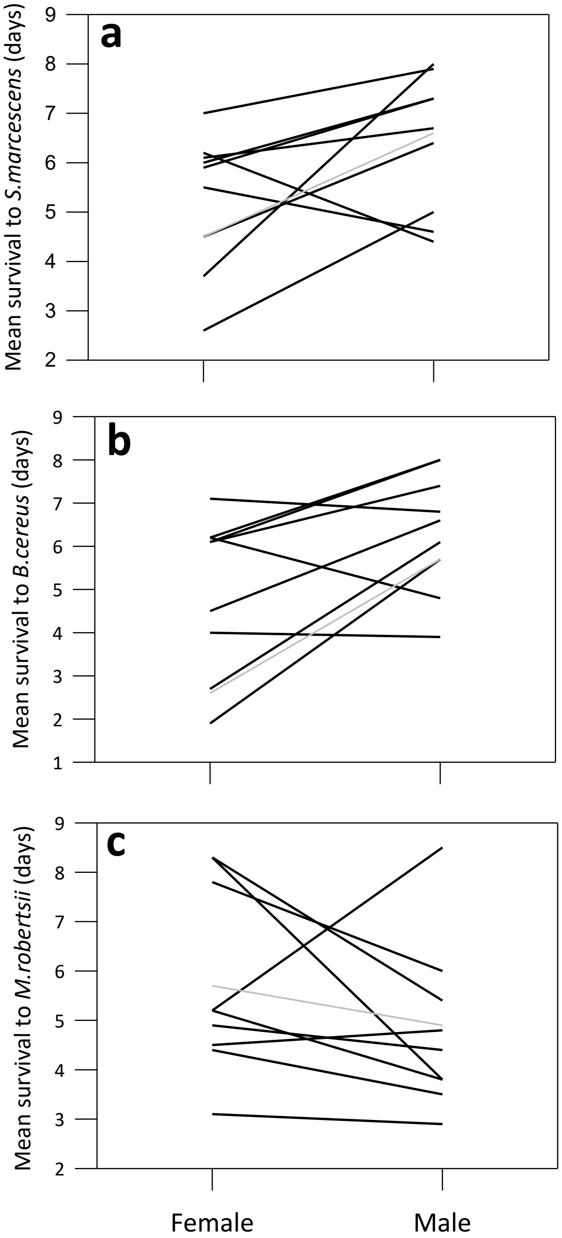

a
Mean encapsulation response
100
80
60
40
20
0
b
Mean clearance rate of *S. marcescens*
0
-10
-20
-30
-40
-50
-60
c
Mean clearance rate of *B.cereus*
6
3
0
-3
-6
-9
-12
-15
-18
d
Mean clearance rate of *M.robertsii*
0.95
0.90
0.85
0.80
0.75
0.70
Female
Male
Female
Male

Fig. 4


a
Mean survival to *S.marcescens* (days)
9
8
7
6
5
4
3
2
b
Mean survival to *B.cereus* (days)
9
8
7
6
5
4
3
2
1
c
Mean survival to *M.robertsii* (days)
9
8
7
6
5
4
3
2
Female
Male