IMMUNE RESPONSES OF WILD BIRDS TO EMERGING INFECTIOUS DISEASES

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SUMMARY

Over the past several decades, outbreaks of emerging infectious diseases (EIDs) in wild birds have attracted worldwide media attention, either because of their extreme virulence or because of alarming spillovers into agricultural animals or humans. The pathogens involved have been found to infect a variety of bird hosts ranging from relatively few species (e.g., *Trichomonas gallinae*) to hundreds of species (e.g., West Nile Virus). Here we review and contrast the immune responses that wild birds are able to mount against these novel pathogens. We discuss the extent to which these responses are associated with reduced clinical symptoms, pathogen load and mortality, or conversely, how they are sometimes linked to worsened pathology and reduced survival. We then investigate how immune responses to EIDs can evolve over time in response to pathogen-driven selection using the illustrative case study of the epizootic outbreak of *Mycoplasma gallisepticum* in wild North American house finches (*Haemorhous mexicanus*). We highlight the need for future work to take advantage of the substantial inter- and intra-specific variation in disease progression and outcome following infections with EID to elucidate the extent to which immune responses confer increased resistance through pathogen clearance or may instead heighten pathogenesis.

INTRODUCTION

The drastic impact that infectious diseases can have on their hosts is illustrated in humans by records of mortality rates resulting from outbreaks like the Spanish flu pandemic of 1918-20 (1), as well as more recently by evidence of the role of pathogens in shaping our genome (2, 3). Emerging and re-emerging infectious diseases (EIDs), which include novel diseases that have spread to a new host species or population and historical diseases which have rapidly increased in incidence (4), are particularly strong selection events (5). They can therefore pose significant threats to wild populations through loss of genetic diversity, population declines, and even localised extinctions of already endangered species (6, 7). Given that risks of disease (re)emergence are thought to be aggravated by anthropogenic factors, ranging from our intensive farming practices to the increased movement of organisms across the globe (8), it is now urgent to improve our understanding of how hosts respond to novel diseases and how immune processes evolve subsequently.

Over the past century, wild birds have been subject to devastating, yet well-documented, wildlife epizootics (Box 1) (9-14), making them valuable models for studying host immune responses to EIDs, as well as how pathogen-driven selection shapes the evolution of host immunity. For example, between December 2002 and January 2003, Hong Kong saw large die-offs of new and old world species of ducks, geese, and swans from Highly Pathogenic Avian Influenza (HPAI) (15), Great Britain lost over half a million greenfinches (*Carduelis chloris*) and chaffinches (*Fringilla coelebs*) within two years of the emergence of *Trichomonas gallinae* (13, 16), and an estimated hundreds of millions of house finches (*Haemorhous mexicanus*) in the eastern United States died following the *Mycoplasma gallisepticum* epizootic that began in 1994 (9, 17, 18).

Similarly, the emergence of West Nile Virus (WNV) in New York (NY) in 1999 was accompanied by more than 17,000 dead bird sightings between May and November of that year, one-third of which were American crows (*Corvus brachyrhynchos*) (19). The causal role of disease in these observed mortality rates was confirmed through testing of carcasses and sick individuals (19, 20) followed by experimental infection studies (21-24). American crows experimentally infected with the NY-1999 WNV strain exhibited 100% mortality with severe clinical symptoms before death, including anorexia, weight loss, encephalitis, and oral and/or cloacal hemorrhaging (21, 22). Likewise, experimental infection with the H5N1 strain of HPAI resulted in 100% mortality of black swans (*Cygnus atratus*), mute swans (*Cygnus olor*), trumpeter swans (*Cygnus buccinator*), whooper swans (*Cygnus cygnus*) (23) and Canada geese (*Branta canadensis*) (24). Of these, some black swans died without ever exhibiting clinical symptoms, while the remaining black swans and mute swans died less than 24 hours after the onset of clinical symptoms that progressively worsened from mild listlessness to severe neurological symptoms including tremors and seizures (23). The severe impact that recent EID outbreaks have had on wild avian hosts therefore raises the question of these hosts' ability to mount immune responses to novel pathogens, as well as the extent to which immune responses may have allowed the host to fight off and/or clear the infection (25).

Despite the high mortality rates observed following these EID outbreaks, there appears to be marked variation in disease development and outcome among and within host species (22, 26). For example, between 2007-2010, wild-caught individuals from 27 of 53 bird species were found to be or to have been infected with *M. gallisepticum* based on PCR and/or testing of serum for antibodies via rapid plate agglutination, but only house finches (*Haemorhous mexicanus*),

American goldfinches (*Spinus tristis*), purple finches (*Haemorhous purpureus*) and black-capped chickadees (*Poecile atricapillus*) exhibited conjunctivitis (26). Experimental infections with NY-1999 WNV of 25 species of birds representing 17 orders also revealed inter-specific differences with mean peak viremias ranging from 10^2.8 to 10^12.1 PFU/ml, as well as highly variable mortality, even amongst species with the greatest viremias (22). In fact, in the same study, pathogen load did not necessarily predict disease outcome: although American crows had 100% mortality with a mean peak viremia of 10^10.2 PFU/ml, three other species, common grackles (*Quiscalus quiscula*), house sparrows (*Passer domesticus*), and blue jays (*Cyanocitta cristata*), reached higher mean viremias yet exhibited mortalities of only 33%, 50%, and 75%, respectively (22). Variation in disease progression and mortality is found not only among species but also within species, even in those species that display noticeably high mortality rates (27-30). For example, the emergence of *Plasmodium relictum* in the Hawaiian islands following the accidental introduction of its mosquito vector (*Culex quinquefasciatus*) in the early 20th century was devastating to populations of some native Hawaiian species, particularly Hawaiian honeycreepers such as the apapane (*Himatione sanguinea*), Hawaii amakihi (*Hemignathus virens*), and iiwi (*Vestiaria coccinea*) (14, 31). Yet experimental exposure of those species to *P. relictum* revealed that some individuals survived and those that did displayed lower levels of infected circulating erythrocytes and lost less mass than birds that died from the infection (28-30). Despite clear evidence of inter- and intra-specific variation in susceptibility to EIDs, our understanding of the precise immune mechanisms by which this variation is achieved remains incomplete.

Here we review the immune responses that are mounted by wild birds to EIDs using data garnered from field studies of live birds and carcasses, as well as laboratory-conducted experimental infections. First, we examine the types of immune responses wild birds are able to mount against novel pathogens at the cellular and molecular level, as well as evaluate how inter- and intra-specific variation in immunity can be linked to variation in disease severity and outcome. Examining such variation is essential for identifying the immune processes associated with differences in disease development and outcome as well as predicting whether and how these immune responses may evolve over time. Finally, we build on the well-documented epizootic outbreak of *Mycoplasma gallisepticum* in North American house finches to illustrate how immune responses can evolve in natural avian populations in response to novel diseases.

IMMUNE RESPONSES OF WILD BIRDS TO EIDS

Evidence from both field and laboratory studies indicate that some individuals are able to mount immune responses against EIDs and that these responses may confer long term protection against secondary exposures. For example, experimental infections of American kestrels (*Falco sparverius*) and dunlin (*Calidris alpina*) with H5N1 HPAI revealed that birds seroconverted and produced detectable levels of specific antibodies by 4 to 5 days post-infection (dpi) (32, 33). Similarly, experimental infection of laughing gulls (*Leucophaeus atricilla*) with H5N1 HPAI revealed that the 2 out of 6 individuals that survived infection produced antibodies against HPAI (27). In addition, these two surviving individuals had no gross lesions at necropsy and only mild encephalitis and pancreatitis due to lymphocytic and heterophilic infiltration, respectively. In contrast, laughing gulls following infection displayed more severe pathology including

widespread petechial hemorrhaging, necrotizing pancreatitis, cerebral neuronal necrosis, and necrotizing adrenalitis (27). This suggests that the humoral response mounted by the two surviving laughing gulls is likely to have enabled them to limit or even clear the infection. Such production of specific antibodies has been found to persist, giving rise to stronger adaptive immune responses upon re-infection. For instance, wild-caught rock pigeons (*Columba livia*) that had been naturally infected with WNV produced antibodies against the virus for at least 15 months after capture (34). Similarly, WNV antibodies have been shown to persist in fish crows (*Corvus ossifragus*) for at least 12 months (35) and in various raptors for at least four years (36), while house sparrows experimentally infected with WNV had detectable antibodies for up to 36 months (37). When re-challenged with WNV at 6, 12, 24, or 36 months post-infection, 52 of 71 house sparrows exhibited ≥ 4-fold increases in antibody titers and only one individual re-challenged at 12 months post-infection became viremic; all individuals given a primary challenge, in contrast, became viremic (37). In the same way, house finches experimentally re-infected with *M. gallisepticum* 219, 314, or 425 days after the primary infection showed reduced conjunctival swelling and duration of clinical symptoms from day 7 dpi onwards relative the response they exhibited upon primary exposure (38).

Differences in the intensity and duration of humoral immune responses to EID may also be associated with variation in disease progression and outcome between avian host species. Experimental infections of American crows and fish crows with WNV revealed that fish crows showed milder and delayed clinical symptoms and lower pathogen loads, and exhibited peak viremias of 10^4.7-6.3 PFU/ml at 3-4 dpi that declined to 10^1.7-2.2 PFU/ml by 6 dpi, whereas American crows had peak viremias of 10^8.22-9.6 at 4-5 dpi that were still high (10^7.3-7.7) at 6

dpi (39). Individuals from both species seroconverted at 5 dpi, but fish crows displayed a greater antibody production that went from 87-90% WNV serum neutralizing activity at 5 dpi to 93-100% at 6 dpi, while antibody production was lower in American crows with only 41-69% WNV neutralizing activity at 5 dpi and 69-79% at 6 dpi (39). Taken together, these results suggest that the stronger antibody response of fish crows to WNV may explain their increased ability to resist WNV infection relative to American crows (Figure 1). Whether this is truly the case is unclear, and explicit links between the intensity of humoral immune responses to EIDs, variation in pathogen load, disease development and outcome remain to be explored further.

The immune responses of wild birds to EIDs do not, however, necessarily give rise to decreased disease severity and a greater ability to clear infection, but may instead be associated with a worsening of clinical symptoms through immunopathology (for example see (40, 41)). This may be particularly true when infections trigger the activation of an inflammatory response, which can damage host tissue and mediate pathogenesis (42, 43). Damage from inflammation was, for e.g., found in HPAI-infected wood ducks (*Aix sponsa*) and laughing gulls that exhibited air sacculitis due to heterophil, lymphocyte, and plasma cell infiltration (27). Geese and swans also displayed mild to moderate heterophilic and lymphoplasmacytic inflammation in locations where HPAI antigen was detected (23). HPAI-infected tufted ducks (*Aythya fuligula*) exhibited encephalitis symptoms that upon necropsy were attributed to gliosis, neuronophagia, and inflammatory lesions associated with macrophage and lymphocyte infiltration (40). Furthermore, heterophilic infiltration was observed throughout the respiratory system of these individuals, yet there was no inflammation associated with the virus in the intestines (40). Patterns of inflammatory responses associated with sites of EID antigen localization have been observed

following both WNV and *T. gallinae* infections (16, 41, 44, 45). For instance, in response to WNV, both blue jays and American crows displayed mixed inflammatory reactions and spleen congestion due to inflammatory cell aggregates and fibrin deposition in areas of inflammation (44). Inflammation in wild finches that succumbed to *T. gallinae* infections in Canada (purple finches) and Great Britain (greenfinches and chaffinches) was found to result from mixed responses of heterophils, macrophages, and lymphocytes (16, 41). Such inflammatory responses were also responsible for the mucosal thickening seen in *T. gallinae*–infected greenfinches and chaffinches in Fennoscandia (45). Finally, post-mortem examination of wild birds naturally infected with H5N1 revealed variation in the distribution and severity of the inflammation of the brain, with species exhibiting some of the highest mortality rates from infection (i.e., swans and geese) also displaying the most severe encephalitis, while other species typically showed only mild to moderate encephalitis (Figure 2) (23, 27, 46-48). All these examples suggest that, in some cases, immune responses (i.e., inflammation) may be detrimental to the host and mediate/accelerate disease progression and outcome. Further support for such a hypothesis comes from the fact that pathogens have been found to benefit from activating inflammatory responses, for e.g., when inflammation disrupts host tissues and facilitates the infiltration and spread of the pathogen (49). Such damages incurred as a result of immune responsiveness are expected to have important consequences for the evolution of immunity to EIDs, with individuals that remain non-responsive or activate other components of the immune system being favoured over time.

While our understanding of the immune responses to EIDs in wild birds mainly consists of measures of antibody production or inflammation, investigations into the transcriptomic changes

following controlled experimental infection reveal a more complex picture. Huang and colleagues recently compared the global gene expression profiles of lungs from mallards (*Anas platyrhynchos*) infected with H5N1 HPAI to control individuals at 1, 2, and 3 dpi (50). The number of differentially expressed genes ranged from 2,257 to 3,066, depending on the day of measurement post-infection and analysis of these genes revealed complex expression patterns of genes known to play roles in immunity. For example, H5N1-infected ducks showed a marked increase (between 2 and 1,414-fold) in the expression of five interferon (IFN), 10 chemokine, and 10 interleukin (IL) or IL-receptor genes. The expression of genes known to be involved in the mammalian response to avian influenza and thought to be involved in the avian response including *DDX58, IFITM3* and *IFIT1–IFIT3,* increased between 6.9 and 440-fold, peaking at 2 dpi (50). Additionally, mallards exhibited increased expression of two RNA helicases, IFN-induced proteins, toll-like receptors (TLRs), and major histocompatibility complex (MHC) genes. In contrast, other genes, including immunoglobulin M (IgM), three T-cell receptor (TCR) genes, and 4 CD molecule-encoding genes were shown to have decreased expression (50). Taken together, these data suggest EIDs elicit altered expression of multiple immune pathways in infected avian hosts.

Such a hypothesis of multiple immune pathways being involved in the responses of wild birds to EIDs is further supported by analyses of H5N1 HPAI-infected jungle crow (*Corvus macrorhynchos*) lung transcriptomes at 6 dpi, which revealed significant differential expression of 2,297 genes between infected and control individuals. Based on gene ontology analysis, the majority of differentially expressed genes were found to have immune-associated functions, with other affected genes being involved in cellular metabolism, transcriptional and translational

regulation, apoptosis, and phagocytosis (51). Vijayakumar and colleagues (51) expanded on these findings by using Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway analysis to refine the specific crow immunological pathways affected by HPAI infection (Figure 3). For instance, crows showed altered expression of multiple innate immune signalling pathways that are involved in viral recognition and influence activation of adaptive responses such as rig-1-like receptor (RLR) and Nod-like receptor (NLR) signalling pathways. Furthermore, infected crows demonstrated altered expression of genes involved in inflammation including cytokines and chemokines as well as adaptive immunity including TCR signalling (51). While gene expression analyses such as those obtained from HPAI-infected mallards and crows represent important advances in our understanding of the immune responses of wild birds to EIDs, they also highlight the complexity of these responses and the gaps in our understanding of the extent to which these responses allow the host to fight and/or clear infection.

EVOLUTION OF AVIAN IMMUNITY TO EIDS: A CASE STUDY OF THE OUTBREAK OF *MYCOPLASMA GALLISEPTICUM* IN HOUSE FINCHES

Evolution of resistance

Few novel EID outbreaks in natural populations are as well documented as the *Mycoplasma gallisepticum* epizootic in North American house finches (52-56). *M. gallisepticum*, an endemic bacterial pathogen of poultry, was first detected in house finches in Maryland in 1994 (17). Although this bacterium readily switches hosts between chickens (*Gallus gallus*) and turkeys (*Meleagridis gallapovo*), a single lineage of poultry origin has since been confirmed to be responsible for the house finch outbreak (57) (58). In house finches, *M. gallisepticum* manifests

as an upper respiratory tract and eye (conjunctivitis) infection (59) that can lead to death, in part, through blindness-induced starvation and predation. Following reports of individuals with swollen eyes at birdfeeders, the Cornell Laboratory of Ornithology set up a Citizen Science program (http://www.birds.cornell.edu/hofi/), through which volunteer birdwatchers could report observations of diseased house finches (60). This allowed for thorough documentation of both temporal and spatial changes in disease prevalence over time (61). Within 4 years, *M. gallisepticum* had spread throughout house finch populations in the eastern United States, killing an estimated tens of millions of house finches (9, 62). Prevalence, however, subsequently declined from epizootic to apparent enzootic levels (54, 61), raising important questions regarding the possible evolution of resistance/tolerance in house finches and underlying changes in host immune processes.

Investigations of host immune responses at epizootic onset, as well as how these responses subsequently evolved, is made possible in this system due to the persistence of unexposed house finch populations with which to compare infected populations (63). In 2007, Bonneaud et al. (63) experimentally infected wild-caught finches from disease-unexposed, western US (Arizona) populations and from disease-exposed, eastern US (Alabama) populations with *M. gallisepticum* to test whether resistance had spread in eastern house finch populations. After verification that the finches had never been naturally infected with *M. gallisepticum*, finches were either inoculated with a contemporary 2007-Alabama strain or sham-inoculated (controls). Two weeks post-infection, finches from disease-unexposed populations harboured nearly 50% greater bacterial loads than finches from exposed populations (Figure 4). Comparison of splenic transcriptional responses to infection of finches from unexposed versus exposed populations

measured before and after the apparent spread of host resistance, confirmed that disease-exposed house finch populations had evolved resistance to *M. gallisepticum* from standing genetic variation in only 12 years of disease exposure (63).

Insights from studies in poultry

M. gallisepticum is an economically-important bacterium known to infect a wide range of hosts of agricultural relevance (64), primarily chickens and turkeys. As a result, studies conducted in poultry have provided important insights into the pathogenesis of *M. gallisepticum*, as well as into the immune processes activated in the poultry host. These, in turn, improve our understanding of the host and pathogen processes taking place in the house finch host. As with other Mycoplasmas (65, 66), *M. gallisepticum* displays the ability to evade and manipulate the immune system of its hosts, with both potentiating and suppressive effects on various components of immunity (67). Teasing apart the immune processes under host and pathogen control and their role in resolving or benefiting infection, is therefore challenging. However, insights into bacterial-driven processes can be obtained, for e.g., by comparing the immune responses elicited in poultry by closely related virulent and attenuated strains of *M. gallisepticum* (68).

The establishment of infection (i.e., colonisation) by *M. gallisepticum* encompasses both adherence to host tissues and initial multiplication and occurs at the mucosal surface of the respiratory epithelium. This is made difficult by the presence of mucus and mucociliary clearance (69). Thus, to facilitate invasion, *M. gallisepticum* can use specific lipoproteins/lipopeptides that bind to host epithelial cells (66) and can induce a misdirected

286 inflammatory response that will disrupt the epithelial membrane (70, 71). Lesions in host tissues
287 have been shown to result from the recruitment, activation and proliferation of heterophils and
288 macrophages initially, and of lymphocytes subsequently, to and at the site of infection (72).
289 Inoculations of chickens with virulent (R_{low}) and attenuated (GT5) strains revealed that this
290 leucocyte chemotaxis is achieved through the release of chemokines by infected tissues (68),
291 including lymphotactin, CXCL13, CXCL14, RANTES, and macrophage inflammatory protein β
292 1(MIP-1β) (68). MIP-1β secretion by chicken monocytes and macrophage-like cells was also
293 confirmed *in vitro* (73) and shown to act as an attractant for many leucocytes, including
294 heterophils, T lymphocytes and NK cells (73, 74). Such findings are consistent with the
295 infiltration of non-specific $CD8^{+}$TCR0 cells (most likely NK cells) in the tracheal mucosa of
296 infected chickens, with infiltration peaking 1 week post-infection and thought to play an
297 important role in disease progression through cytotoxicity (71, 72). Comparison of tracheal
298 expression patterns following infection with R_{low} and GT5 also confirmed that chickens up-
299 regulated pro-inflammatory cytokines (68), such as TNF-α and IL-6, which are responsible for
300 local and systemic inflammation and can also give rise to tissue destruction and local necrosis.
301 While the induction of an inflammatory response may therefore be beneficial to *M. gallisepticum*
302 and facilitate invasion of the host (49, 75), persistence of infection may on the other hand
303 necessitate the suppression of other components of immunity (71). Accordingly, chickens
304 infected with R_{low} down-regulated the tracheal expression of the chemokine CCL20 and
305 cytokines IL-8, IL-1β and IL-12p40 as early as 1 day post-inoculation (68). The fact that these
306 cytokines are also involved in key inflammatory processes (76) highlights the complexity of
307 pathogen-mediated manipulation of the host immune system. Furthermore, chickens infected
308 with *M. gallisepticum* displayed lower T-cell activity 2 weeks post-infection (70, 72) and lower

humoral responses against *Haemophilus gallinarum* (77) or against avian pneumovirus (78) when co-inoculated with *M. gallisepticum*. The ability of *M. gallisepticum* to limit humoral and T-cell responses may be crucial for disease progression, as both local antibody-mediated responses and natural killer and cytotoxic T-cell responses have been suggested to play a role in controlling infection in chickens (71).

Immune processes in the house finch host

Comparison of transcriptional changes in the spleen of infected house finches from disease-unexposed/susceptible and exposed/resistant populations revealed significant differences as early as three days post-infection (79), indicating that the evolution of resistance in exposed populations involved changes in innate immune processes. Two-weeks post-infection, susceptible finches from unexposed populations down-regulated immune-associated genes and, relative to infected finches from exposed populations, exhibited significantly lower levels of transcripts of the following genes (79): T-cell immunoglobulin and mucin domain containing 4 (*tim4*), MHC-class II-associated invariant chain I1 (*cd74*), lectin galactoside-binding soluble-2 (*lgals2*), programmed death ligand 1 (*pd-l1*), TCR beta chain (*tcrβ*), immunoglobulin J (*IgJ*), neutrophil cytosolic factor-4 (*ncf4*), immunoglobulin superfamily member 4A (*Igsf4A*) and parathymosin (*ptms*). The only exception was the complement factor-H (*hCG40889*) gene, whose expression was up-regulated in infected finches from unexposed populations. However, because hCG40889 is known to restrict activation of the complement cascade (80), the overall expression patterns detected suggest that particular components of the immune system were being suppressed in finches from unexposed populations. Infected finches from exposed populations, on the other hand, were able to up-regulate the expression of immune-associated

genes two-weeks post-infection (79). Three of the genes up-regulated were: TIM4, which is involved in the differentiation of naïve CD4+ T cells into Th2 cells and which plays a role in preventing autoimmunity by mediating the clearance of apoptotic (phosphatidylserine-expressing) antigen-specific T cells after infection (81); CD74, which plays a role during the assembly of MHC class II molecules (82); NCF4 which plays a role in phagocytosis-induced oxidant production in heterophils (83). Taken together, these finding suggests that finches from disease-exposed populations have evolved the ability to resist pathogen-induced immuno-suppression and supports a role of both innate (e.g., phagocytosis by heterophils) and acquired (e.g., T-cell activity) immune processes in mediating resistance to pathogen spread (79).

Protective immunity is expected to evolve only when the costs of resisting infection are lower than those incurred by the infection itself (84, 85). Surprisingly, resistance to *M. gallisepticum* was found to have evolved despite the fact that the short-term energetic costs of immunity were greater than those of pathogenesis (86). Disentangling the costs attributable to immune functioning from those incurred from the parasite's presence is challenging in *in vivo* infection studies involving real pathogens (87). As a result, most of our understanding of the costs of immunity stems from studies using inert pathogens (88). However, two unusual features of the *M. gallisepticum*-house finch interaction permitted such a study in this system. First, it is possible to compare the response to infection between finches that are either susceptible or resistant depending on their population of origin (i.e., disease-unexposed or –exposed populations, respectively) (63, 89). Second, only resistant finches from disease-exposed populations are able to mount a protective immune response, as demonstrated both by the greater bacterial load and the overall down-regulation of immune-associated genes at early as 3 days

post-infection in finches from unexposed populations (63, 79). It is important to note, however, that genes associated with innate immunity, and in particular with inflammation, were not specifically examined in this study and hence may have been up-regulated in finches from unexposed populations at the start of the infection. This hypothesis is supported by the findings of Hawley and colleagues (90) showing increased levels of IL-6 in house finches 2 days post-inoculation with *M. gallisepticum*, as well as a 2°C increase in body temperature 1 day post-infection with a ~1°C increase persisting over the entire 2-weeks duration of the experimental infection. Regardless, the greater susceptibility of finches from disease-unexposed population implies that any potential inflammatory response was not protective and therefore likely reflectes pathogenesis.

As expected based on the findings above, infected finches from disease-exposed populations lost 10 times more body mass over the course of two-weeks than uninfected controls from the same populations, revealing a cost of immunity (86) (Figure 5a). Furthermore, infected individuals from the disease-exposed population that lost the most mass and displayed immune-associated gene expression patterns in a direction consistent with greatest protective immunity (i.e., resistance) against *M. gallisepticum*, also harboured the lowest pathogen loads in their conjunctivae (Figure 5b). Conversely, infected finches from disease-unexposed populations lost twice as much body mass as their controls, although this difference was marginal. In addition, in this population, infected individuals that lost the most mass harboured the greatest bacterial load in their conjunctivae, indicating a measurable cost of pathogenesis. Interestingly, the mass lost by infected birds differed significantly between populations, with mass loss being greater in infected finches from exposed populations (Figure 5). This indicates that counter to predictions,

the short-term energetic costs of immunity were greater than those of pathogenesis (86). These results therefore highlight the fact that resistance can evolve despite this, provided the fitness consequences of infection are sufficiently detrimental to the host.

Evolution of tolerance

The consequences of pathogen-driven selection on host evolution in this system are made all the more interesting by the fact that resistance was not the only host trait to evolve following epizootic outbreak. Adelman and colleagues (89) demonstrated that pathogen tolerance, which is the ability to limit the damage incurred from a given pathogen load (91), also spread in eastern house finch populations following disease exposure. To this end, they caught finches from unexposed western US (Arizona) and exposed eastern US (Alabama) populations in 2010 and experimentally infected them with an *M. gallisepticum* isolate collected in Virginia in 1994 (i.e., at epizootic onset). Given that exposed populations were shown to have evolved resistance to *M. gallisepticum* between 2001 and 2007 (63), infection with an isolate sampled 16 years earlier ensured that any immunomodulatory effects of the bacteria would be minimised. Tolerance was then assessed using peak levels of pathology (i.e., eye lesions and mass loss) and bacterial load, as well as measures of pathology and bacterial load that incorporated infection duration and intensity (i.e., by measuring the area under the curves of pathology and pathogen load over time). Results showed that finches from the unexposed population had significantly greater peak eye lesions and mass loss than finches from the exposed population despite similar peak pathogen load. In addition, eye lesions also peaked a week later in finches from the exposed population relative to the unexposed one (89) (e.g., peak eye score; unexposed: 4.13±0.48 on day 7; exposed=5.79±0.14 on day 14) (Figure 6).

The heightened tolerance of finches from the *M. gallisepticum*-exposed population was associated with a lower inflammatory response to infection relative to finches from the unexposed population (89). Specifically, finches from exposed populations displayed significantly lower levels of IL-1β, but marginally higher levels of IL-10, 24-hours post-infection (89) (Figure 7). IL-1β is a pro-inflammatory cytokine secreted by macrophages and that plays a key role in the acute phase response (76). The difference in the expression of IL-1β between finches from exposed and unexposed populations thus is likely to be responsible for the delayed and lowered febrile responses of the former (89) (increase in body temperature on day 1 post-infection; exposed: 0.71°±0.03°C; unexposed:1.44°±0.18°C).

While resistance and tolerance are often thought of as two alternative evolutionary responses to pathogen-driven selection (91, 92), studies on the house finch-*M. gallisepticum* system indicate that this may not necessarily be the case and that both processes can evolve in conjunction to reduce the overall fitness consequences of infection (63, 89). Interestingly, that tolerance reduced both inflammation and the severity of clinical symptoms (i.e., eye lesions and mass loss) without decreasing pathogen load (89) suggests that infection success is not necessarily positively correlated with the level of immunopathology suffered by the host. As a result, the extent to which host lesions can be minimised without impacting pathogen colonisation success or persistence will determine the relative contribution of resistance and tolerance to the evolutionary response of house finches to *M. gallisepticum*, with significant ramifications for the evolution of pathogen virulence (91).

CONCLUSION

Wild birds have been shown to mount immune responses to emerging infectious pathogens, but these responses are not always associated with reduced severity, or even absence, of clinical symptoms, nor do they necessarily allow the host to clear and survive the infection. The extent to which these immune responses help to fight novel pathogens, however, seems dependent on the type of response elicited, with humoral responses conferring some level of protection and inflammatory responses being associated with increased disease severity. The extent to which immune processes allow the host to fight the infection or, on the opposite, facilitate disease progression will have important consequences for the evolution of immune responses over time in response to pathogen-mediated selection. In cases where inflammation underlies disease pathology, a lack of immune responsiveness with or without the involvement of other components of immunity (e.g., humoral immunity) may be favoured by natural selection, thus leading to the evolution of tolerance and/or resistance. The combined spread of tolerance and resistance to EIDs appears to have occurred in house finches following the outbreak of the conjunctivitis-causing *M. gallisepticum*. Whether this evolutionary change was mediated solely by changes in the finches' inflammatory response, with important consequences for more pathogen-specific components of immunity (e.g., T cell and humoral immunity), or whether host evolution has occurred through parallel changes in multiple components of immunity (e.g., inflammation and T cell immunity concurrently) remains to be determined. Finally, further insights into the role of different immune processes can be gained from detailed inter- and intra-specific comparisons linking immune responses to EIDs at a molecular and cellular level with variation in disease progression and outcome. By increasing our understanding of the role of host immune responses in EID outbreaks and persistence, studies conducted on wild bird populations

will have the potential to improve our predictions of species particularly at risk of infection by EIDs.

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BOX 1: The emerging infectious diseases (EIDs) of wild birds discussed in this review (ordered chronologically).

Plasmodium relictum – *P. relictum*, a protist responsible for causing avian malaria, is among the earliest documented EIDs known to significantly affect wild birds. Following the accidental introduction of its mosquito vector, *Culex quinquefasciatus,* to the Hawaiian islands in the early 20th century, this novel disease devastated local populations of honeycreepers including the apapane (*Himatione sanguinea*), Hawaii amakihi (*Hemignathus virens*), and iiwi (*Vestiaria coccinea*) and contributed to the extinction of several others. As a result many native Hawaiian birds could only be found in large numbers in high elevation forests and islands that were free of mosquitos (14, 31). However, based on mist-netting surveys conducted on the island of Hawaii in 2002, Hawaii amakihi have persisted and increased in abundance at low elevations where *P. relictum* is prevalent, such that Hawaii amakihi are more abundant at low elevations than at high elevations (93). Furthermore, these populations have been shown to be genetically isolated from high elevation populations (94), creating a unique system in which the evolution of host immunity to EIDs can be examined (95).

Mycoplasma gallisepticum – In 1994, the poultry pathogen *M. gallisepticum* was found to be the causative agent of a novel conjunctivitis disease observed in house finches (*Haemorhous mexicanus*) in Maryland, United States. Within 3-4 years this bacterial pathogen spread throughout the entire eastern range of the house finch in North America killing an estimated tens of millions of house finches. These deaths resulted in part from the manifestation of *M.*

762 *gallisepticum* as a respiratory disease as well and in part from the conjunctivitis-induced
763 blindness leading to starvation and increased susceptibility to predation (9, 17, 18, 61, 96).
764
765 **West Nile Virus** - In 1999, a novel highly pathogenic strain of West Nile Virus was found to be
766 responsible for the unusually high numbers of bird deaths in New York (NY), United States (19,
767 97-99). While some affected birds displayed no symptoms before death, the most affected
768 species such as American crows (*Corvus brachyrhynchos*) displayed severe symptoms including
769 anorexia, weakness, and mass loss as well as neurological problems such as ataxia, tremors,
770 circling, disorientation, and impaired vision resulting from WNV-induced encephalitis (21).
771 Sequence analysis of WNV isolates from this epidemic found NY-1999 WNV isolates to be most
772 closely related to WNV isolated from a dead goose in Israel in 1998 (100). Combined with a lack
773 of evidence for WNV in the United States before 1999, the epidemic was likely the result of a
774 novel introduction of WNV to the US with a probable Mediterranean origin (99-101).
775
776 **Highly pathogenic avian influenza (HPAI) virus** – Historically waterfowl have been
777 considered asymptomatic carriers of avian influenza viruses. However, H5N1 HPAI was found
778 to be responsible for the deaths of new and old world species of ducks, geese, and swans in two
779 Hong Kong parks between December 2002 and January 2003. Affected birds exhibited
780 symptoms ranging from slight inactivity, inappettence, and ruffled feathers to severe
781 neurological symptoms including paresis, paralysis, tremors, and unusual head tilt, with death
782 often occurring within 24 hours of the onset of symptoms (15). Indeed, H5N1 isolates collected
783 during the outbreak were found to cause systemic disease and similar severe clinical symptoms
784 in mallards (*Anas platyrhychos*) whereas 1997 and 2001 H5N1 isolates from Hong Kong did not

785 (102). Subsequent outbreaks of HPAI affecting waterfowl occurred in China (103, 104), Japan
786 (105) Bangladesh (106) as well as numerous European countries in 2006 (107, 108).
787
788 ***Trichomonas gallinae*** – In 2005, a clonal strain of the protozoan *T. gallinae* spread from wild
789 columbiform birds to chaffinches (*Fringilla coelebs*) and greenfinches (*Carduelis chloris*) in
790 Great Britain, causing the loss of an estimated half a million birds by 2007 (16). While chaffinch
791 populations began to stabilize, greenfinch populations further declined, with an estimated
792 population decrease from 4.3 to 2.8 million, or overall 1.5 million, greenfinches by 2009 (13).
793 Since then, *T. gallinae* has spread to finches in other European countries including Norway,
794 Sweden, and Finland (45) and has been found to cause disease in raptors including
795 sparrowhawks (*Accipiter nusis*) and tawny owls (*Strix aluco*), presumably due to consumption of
796 infected finches (109).
797

FIGURE LEGENDS

Figure 1: Using experimental WNV infections in American crows (*Corvus brachyrnchos*) and fish crows (*Corvus ossifragus*), Nemeth and colleagues (39) show differences in disease progression and outcome between these two species that may be associated with differences in humoral immune responses. (Modified from (39))

Figure 2: Severity of H5N1 HPAI encephalitis in nine naturally infected wild bird species: mute swans (*Cygnus olor*), Canada geese (*Branta Canadensis*), greater scaup (*Aythya marila*), European eagle owls (*Bubo bubo*), tufted duck (*Aythya fuligula*), goosander (common merganser; *Mergus merganser*), common buzzard (*Buteo buteo*), smew (*Mergellus albellus*), and herring gull (*Larus argentatus*). Severity is based on use of immunohistochemistry to assess intensity and area of staining for the following: total area of inflammation, inflammatory components, viral antigen prevalence, neuronal changes, and vascular changes. (From (46))

Figure 3: KEGG pathway analysis of differentially expressed genes in the lungs of non-infected versus HPAI-infected jungle crows (*Corvus macrorhynchos*). (From (51))

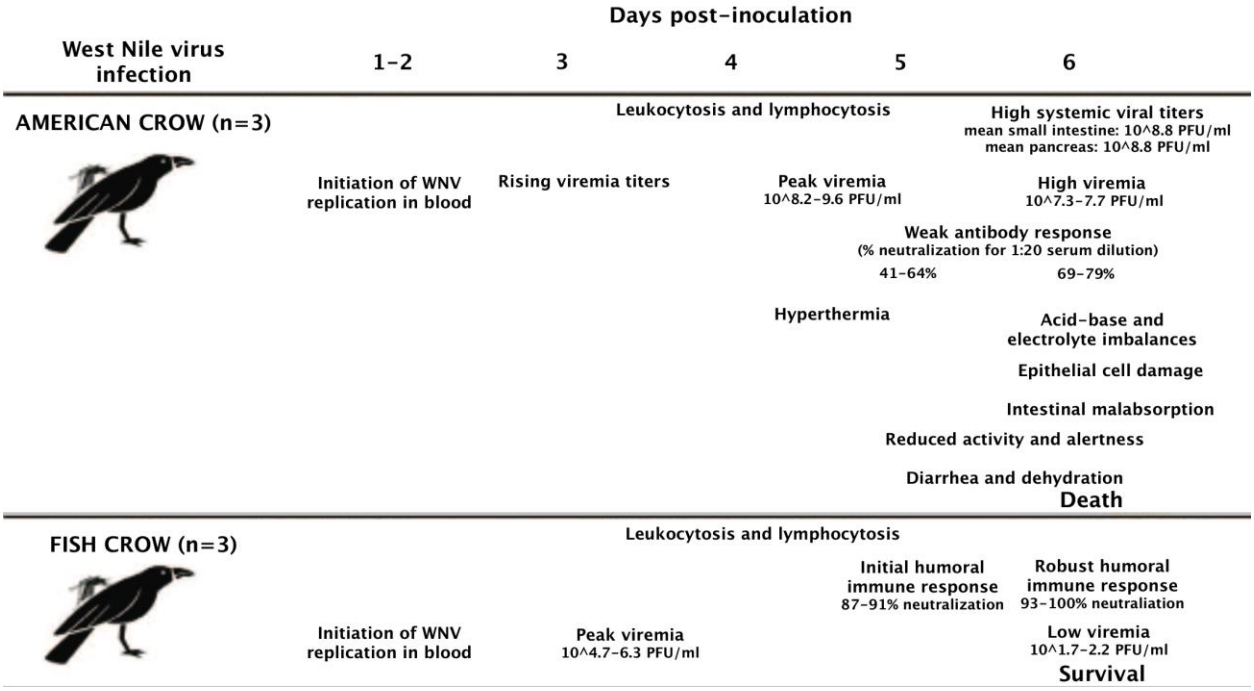
Figure 4: Symptoms of *M. gallisepticum* infection and pathogen load in the conjunctivae of house finches. A) Symptoms of *M, gallisepticum* infection in naturally infected (Left) and healthy (Right) wild house finches. (B) Quantification of bacterial load in the conjunctiva of infected finches from disease-exposed and unexposed populations, 2 weeks post-infection. (From (63)).

Figure 5: Mass loss in *M. gallisepticum*-infected house finches *vs.* sham-inoculated controls, and bacterial load in the conjunctivae of infected finches. (a) Effects of infection with *M. gallisepticum vs.* sham inoculations on mass change (g) between days 0 and 14 post-infection in finches from disease-exposed (Alabama) and disease-unexposed (Arizona) populations. (b) Association between bacterial load 14 days post-infection and mass change (g) between days 0 and 14 post-infection in birds from Alabama (open squares) and Arizona (filled diamonds). (From (86)).

Figure 6: Pathology of house finches infected with *M. gallisepticum* and originating either from disease-exposed (Alabama) or disease-unexposed (Arizona) populations. Finches from the exposed population displayed lower peak eye lesion score (a) and reduced mass loss (b) relative to finches from unexposed populations, despite similar bacterial load (c). (From (89)).

Figure 7: Expression of the inflammatory cytokines in the blood of house finches infected with *M. gallisepticum* and originating either from disease-exposed (Alabama) or disease-unexposed (Arizona) populations. Expression of the pro-inflammatory cytokine IL-1β was significantly lower in finches from the exposed population (a), but expression of the anti-inflammatory cytokine IL-10 was marginally higher in those individuals relative to those from the unexposed population (b). (From (89)).

841 Figure 1



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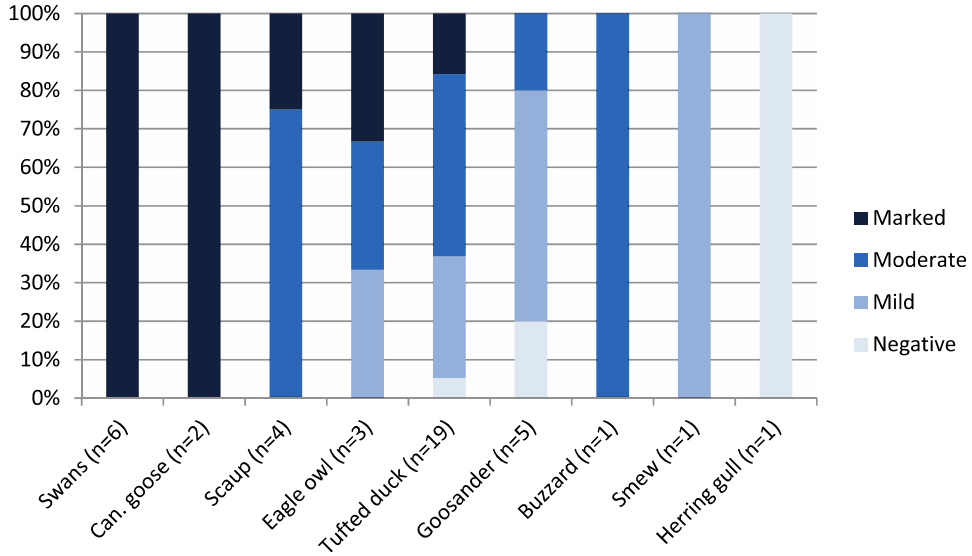
843

844 Figure 2



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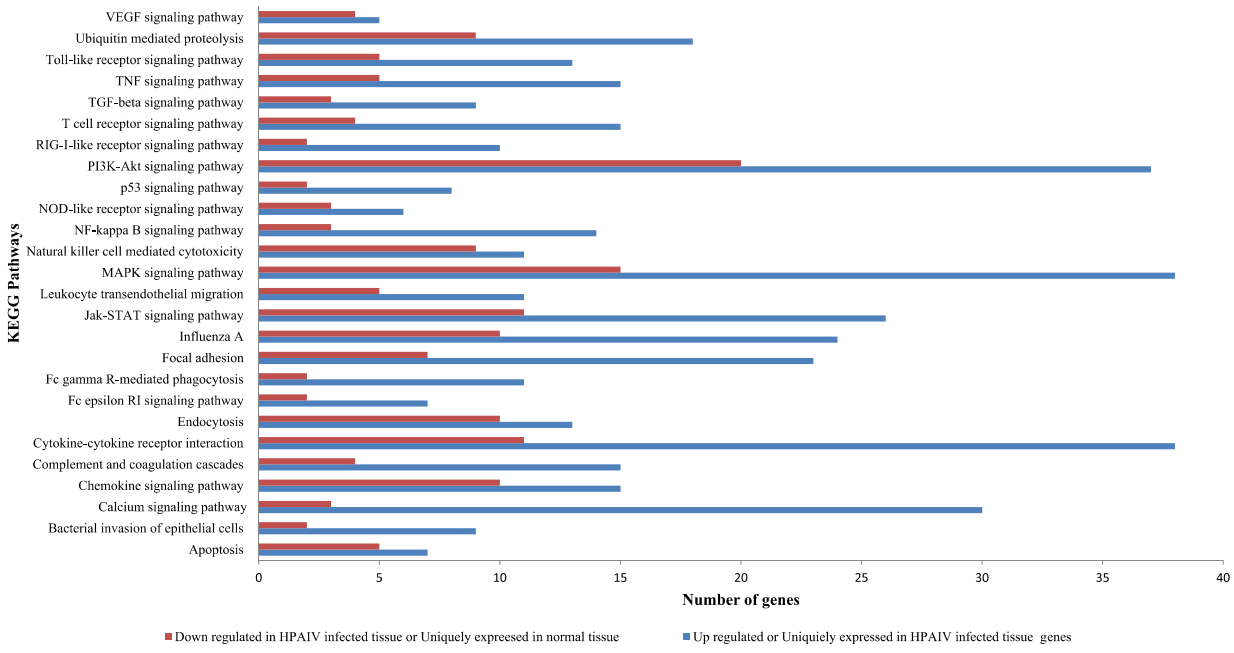
846

847 Figure 3



848

Figure 4

(a)

(b)

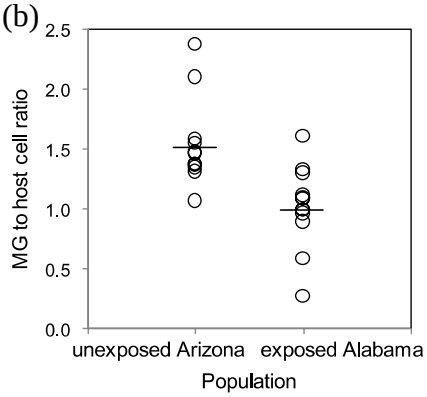

MG to host cell ratio
2.5
2.0
1.5
1.0
0.5
0.0
unexposed Arizona
exposed Alabama
Population

Figure 5

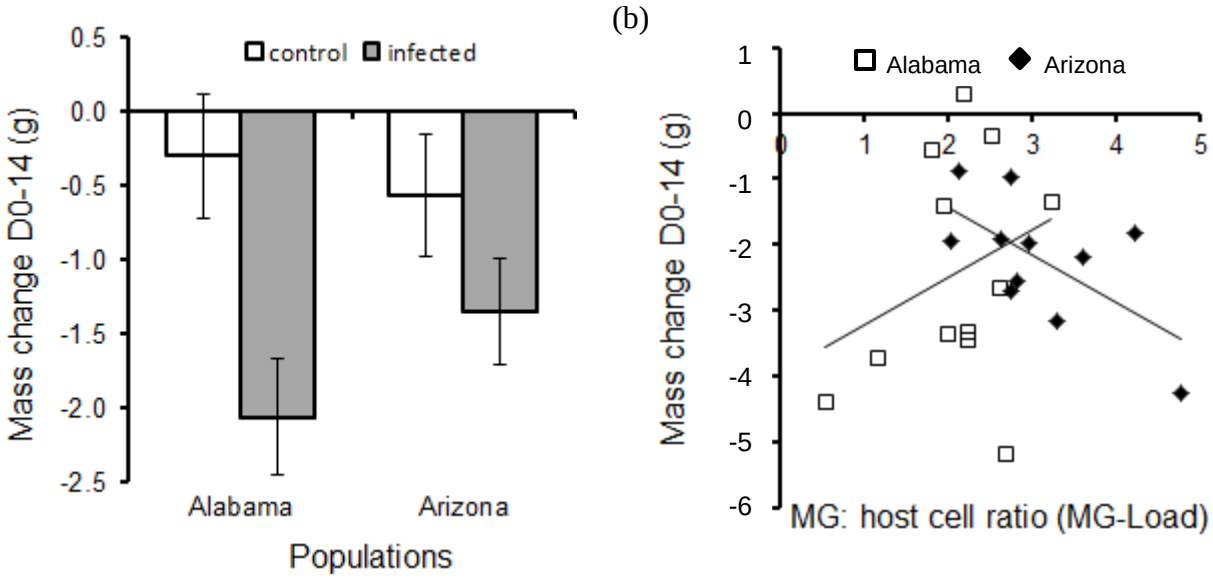
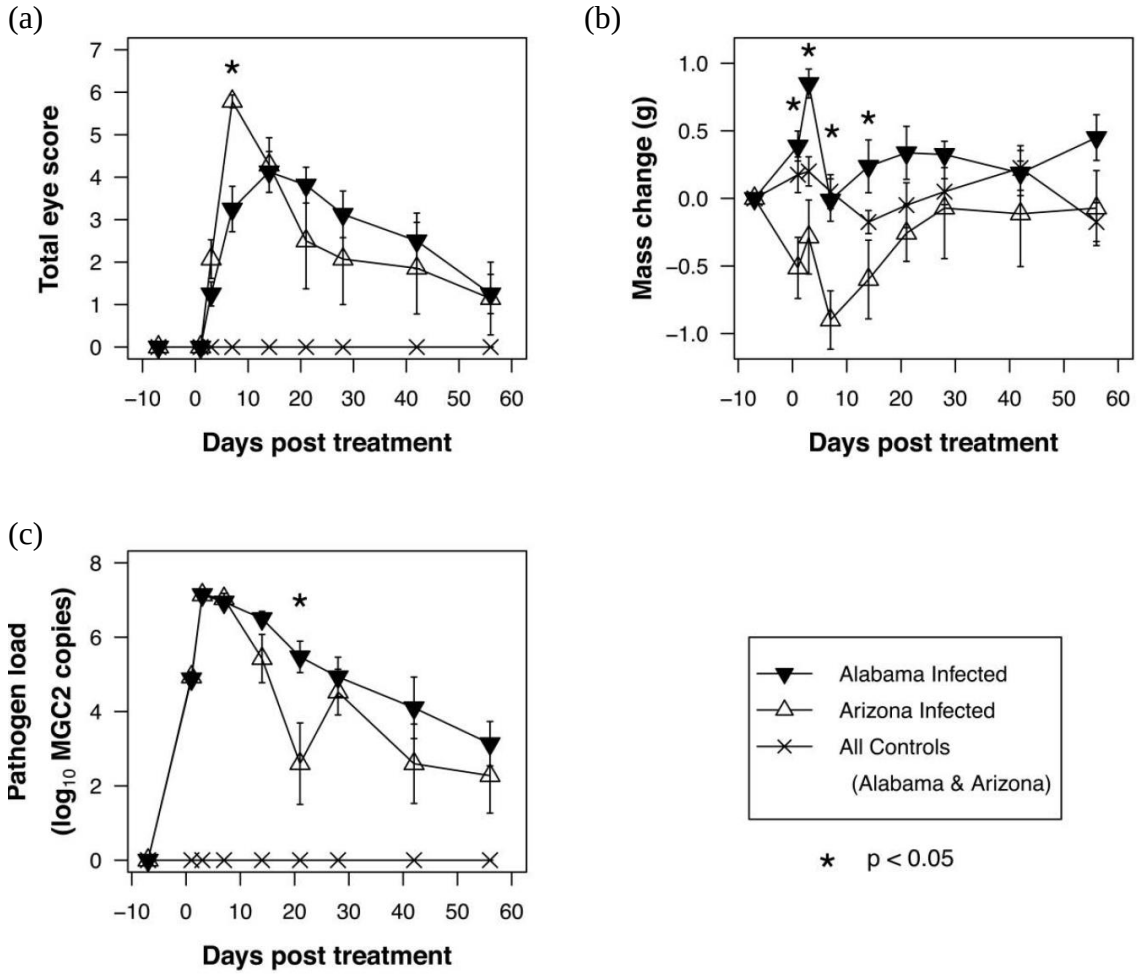

(a)
control
infected
Mass change D0-14 (g)
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
Alabama
Arizona
Populations
(b)
Alabama
Arizona
Mass change D0-14 (g)
1
0
-1
-2
-3
-4
-5
-6
0
1
2
3
4
5
MG: host cell ratio (MG-Load)

Figure 6


(a)
Total eye score
Days post treatment
(b)
Mass change (g)
Days post treatment
(c)
Pathogen load
(log10 MGC2 copies)
Days post treatment
Alabama Infected
Arizona Infected
All Controls
(Alabama & Arizona)
* p < 0.05

874 Figure 7

875 (a) (b)

876