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Pathogen within-host dynamics and disease outcome: what can we learn from insect studies?

David Duneau[1,2] and Jean-Baptiste Ferdy[1]

Parasite proliferations within/on the host form the basis of the outcome of all infectious diseases. However, within-host dynamics are difficult to study in vertebrates, as it requires regularly following pathogen proliferation from the start of the infection and at the organismal level. Invertebrate models allow for this monitoring under controlled conditions using population approaches. These approaches offer the possibility to describe many parameters of the within-host dynamics, such as rate of proliferation, probability to control the infection, and average time at which the pathogen is controlled. New parameters such as the Pathogen Load Upon Death and the Set-Point Pathogen Load have emerged to characterize within-host dynamics and better understand disease outcome. While contextualizing the potential of studying within-host dynamics in insects to build fundamental knowledge, we review what we know about within-host dynamics using insect models, and what it can offer to our knowledge of infectious diseases.

Addresses
[1] Université Toulouse 3 Paul Sabatier, CNRS, IRD, UMR5174 EDB (Laboratoire Évolution & Diversité Biologique), Toulouse, France
[2] Instituto Gulbenkian de Ciência, Rua da Quinta Grande 6, P-2780 Oeiras, Portugal

Corresponding authors: David Duneau (david.duneau@gmail.com), Jean-Baptiste Ferdy (jean.baptiste.ferdy@univ.tlse3.fr)

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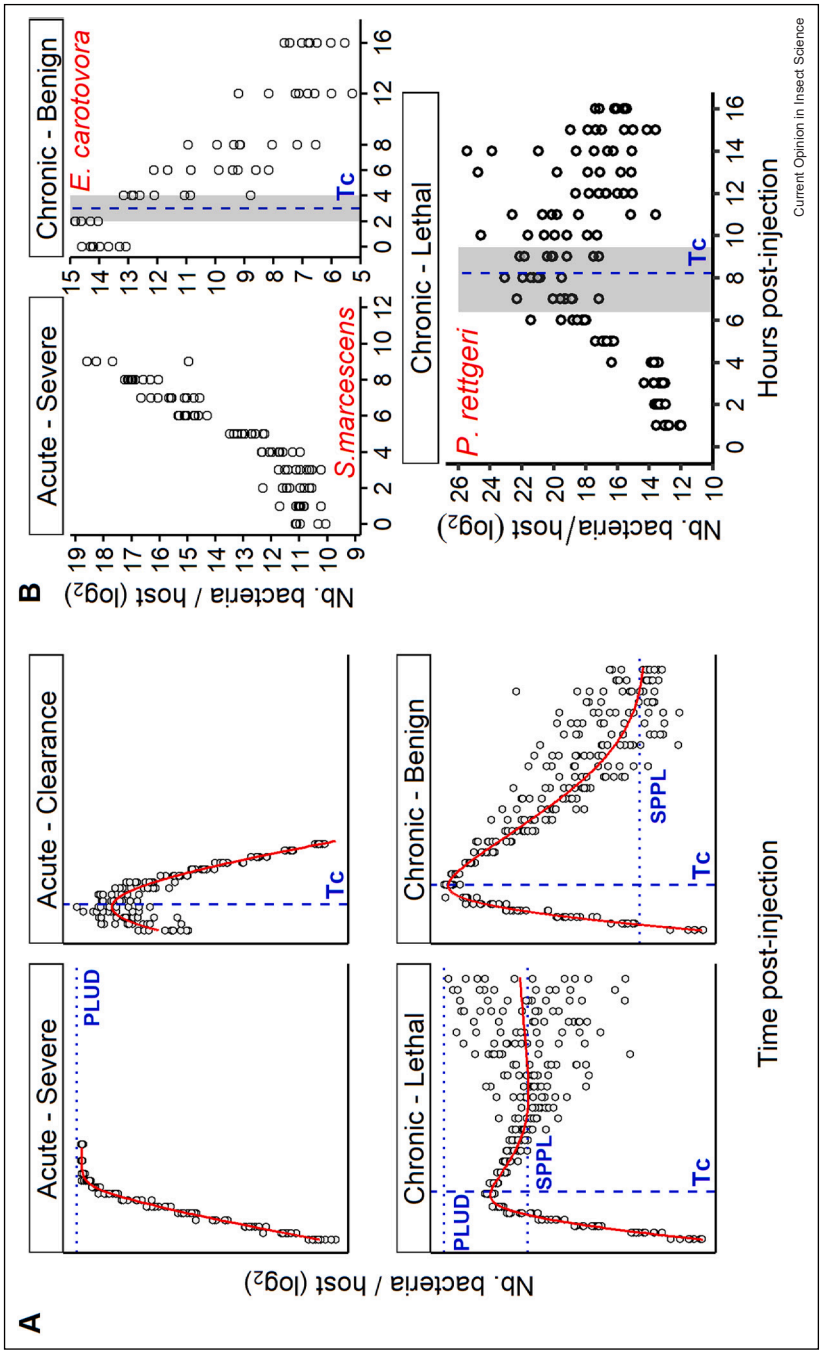
Introduction

The outcome of all infectious diseases is underpinned by interactions between host and parasite. These interactions occur along a succession of steps, which starts with their encounter and contact, and can continue with the proliferation within/on the host, and transmission to a new host [1–3]. The infection itself starts when the parasite begins to use host resources and, depending on both the host and the parasite, the disease progression will end with a rapid/acute clearance (i.e. most benign infections), stabilize with its control at a given load (i.e. chronic infections either benign or eventually lethal), or cease with the rapid/acute death of the host (i.e. severe infections). It is evident that disease outcome is tied to the step where the parasite proliferates, yet this dynamic has often been accepted as a 'black box', especially in evolutionary biology [4], or investigated theoretically (see [5,6]).

Studies of within-host dynamics have proven crucial to our understanding of some major human infectious diseases. More than twenty years ago, Robin Weiss stated that "*the key to understanding [HIV] pathogenesis lies in elucidating the course of infection and the virus-host relation in the years preceding terminal illness*" [7]. Indeed, the characterization of pathogen-load kinetics played an important role in understanding HIV pathogenicity, in identifying patients most at risk, in designing drugs, and preventing the evolution of drug resistance [8], as is the case in several other diseases (e.g. Ebola [9], chicken influenza [10], tuberculosis [11], Salmonella [12], Streptococcus [13], and malaria [14]). Ideally, the number of pathogenic cells/particles should be followed from the beginning of the infection until its end, whatever the outcome. This requires repeatedly sampling individuals at multiple timepoints and, depending on the tissue tropism, can necessitate sacrificing the host. These requirements pose serious challenges with vertebrates where experimental infection is expensive and/or unethical. But as Jacques Monod once stated "*What is true for* E. coli *is true for the elephant*", and insects have for long been used as surrogates that reduce the ethical cost of fundamental research.

Since Elie Metchnikoff discovered phagocytes in starfish in the 19th century [15], invertebrates have been used to study immunity and infectious diseases, achieving Nobel prize recognition for discoveries made on innate immune-system activation in *Drosophila*. Early studies were generally not investigating infection dynamics per se, but rather how loss-of-function mutations in various genes impacted disease outcome. More recently, within-host dynamics have been studied in invertebrates. These encompass a range of pathogens, including intra- and extracellular infections with *Staphylococcus aureus* [16], the intracellular bacteria *Listeria monocytogenes* [17,18], extracellular bacteria of different pathogenicity [19], the microsporidium *Tubulinosema ratisbonensis* [20], the fungus *Candida albicans* [21], the bumble bee

Figure 1



Within-host dynamics and infection outcome. **(a)** Simulated data (using our model in Lafont et al. *BioRxiv* doi:10.1101/2021.10.19.464998) and which can be reproduced at https://plafont.shinyapps.io/WHD_app/ allow for the representation of the within-host dynamics (red curve) for each disease outcome, and the retrieval of parameters that characterize the infection dynamics (e.g. PLUD, SPPL, and time to control (Tc)). In addition, the maximum proliferation rate can be estimated from the steeper positive slope. **(b)** Empirical data to estimate bacterial within-host dynamics for three types of disease outcome. For each case, a population of *Drosophila* is infected with a precise inoculum, and individuals are sampled periodically to determine how the number of pathogens in whole individuals changes over time (data from [19]). *Serratia marcescens* induces acute and severe disease, where no host can control the proliferation. *Erwinia carotovora* does not kill its host, where the average time to control the proliferation (Tc) is 2 h. It will become chronic, but the host will not die from the infection. *Providencia rettgeri* is chronic but eventually lethal for the host. Most hosts will control the proliferation (Tc) at around 8 h after injection (as shown in B), but the host will eventually lose control and die from the infection, several days post injection (beyond the timescale graphed here). The gray area represented the 95% confidence interval of Tc calculated with a bootstrap method.

trypanosome [22], and the Dengue virus [23]. The use of bioluminescent pathogens to follow the proliferation in vivo within an individual host is one of the first emergent approaches to characterize within-host dynamics. Under development in insect models (Ramirez-Corona et al. *bioRxiv* doi:10.1101/2021.08.17.456698), it will ideally allow the tracing of 'personalized health curves' whereby the condition of an individual host can be related to the dynamics of pathogen during the infection [24], as is successfully done in mammals [25,26].

Populational methods to follow within-host dynamics in insect models

The best-defined method for within-host dynamics quantification so far uses a populational approach. A population of hosts, generally from the same genotype, is infected with a precise inoculum, and batches of ~10 individuals are sampled periodically, so that the number of pathogens in whole individuals is followed over time (see Figure 1). This method has the advantage to avoid data autocorrelation tied to repeated measurements on the same individual, as each data point *is* a distinct individual. The technique gives access to the whole pathogen population, independent of tissue tropism, as the whole host body is homogenized before pathogen quantification. This prevents bias in pathogen-load estimation, but also precludes detailed study of differences between tissues (as in the case of Dengue virus infecting mosquito, e.g. [23,27]).

Many of the studies we cite in this review follow pathogen dynamics on a daily basis, which may be suitable for slowly progressing infections. However, sampling hourly bacterial infections in a *Drosophila* model revealed that the first few hours of the infection may be the major determinant of whether a host is going to survive or not [19]. Indeed, the difference in susceptibility between male and female *Drosophila* to *Providencia rettgeri* correlates with a difference in control of the proliferation of only four hours [28]. Therefore, scattered sampling is likely to miss crucial parts of the dynamics, especially in fast-proliferating pathogens, and the regularity of sampling is a key point to consider when designing such experiments.

Statistical analysis of populational studies

Comparing dynamics among host conditions or types can be reduced to a simple comparison of loads at each timepoint. This method has been used successfully in *Drosophila* to show the role of nephrocytes in affecting bacterial proliferation [29] and, that supplementing host diet can favor microsporidium proliferation [20]. Another approach is to use statistical modeling to describe how load varies with time. A linear regression analysis has been used to compare competition of Dengue virus strains within mosquito hosts [27], and to show that transgenerational priming influences bacterial dynamics in flour beetles [30]. A more powerful yet simple approach used a logistic growth model to estimate three parameters (age of the infection, pathogen growth rate, and plateau of pathogen load) and to compare dynamics of pathogen strains in different host genotypes and with different inocula [17,18,23]. One limit of these approaches is that they consider that all individuals follow the same infection dynamics. But some may die over the course of the experiment, while others control the infection; the first group will then experience ever-increasing pathogen loads but not the second, even though the initial conditions of infection seemed to be similar [19,31]. To investigate this type of situation, we have used a mixture-model approach that distinguishes these two groups and estimates for each individual the probability that it belongs to one or the other [19]. With this statistical method, we can determine additional infection parameters that predict different infection outcomes, notably, the maximum proliferation rate, the chance to control the infection, the average time it takes for the host to control, and the load at which it controls (see illustration in Figure 1b).

Modeling dynamics may therefore be better than comparing loads at fixed timepoints (e.g. 24 h post injection), which might correspond to different stages in individual infections that progress at different speeds. A third option is to measure loads at well-defined, key steps of the infection. This is possible either when pathogen load stabilizes (as when chronic infections reach the Set-Point Pathogen Load, SPPL) or when hosts display a conspicuous and characteristic symptom (as they typically do when they die, when infections reach then the Pathogen Load Upon Death, PLUD). We describe these situations and others below, and discuss what we think can be learned from studying within-host dynamics.

The early phase of the infection

Mobilization of hemocytes, the insect analog of vertebrate macrophages, occurs within seconds after infection start in many insect species [32]. Similarly, melanization (which is part of the humoral response) is considered to be an equally rapid mechanism deployed to control infections [33,34]. However, the role of both melanization and hemocytes in controlling early proliferation remains unclear, in part because experimental results are conflicting. In *Tenebrio* beetles, it seems that *S. aureus* is almost cleared before antimicrobial peptides (AMP) expression even begins, which suggests that clearance is permitted by melanization and/or the action of hemocytes during the first stages of infections [35]. In *Drosophila*, the presence of hemocytes in adult flies does not affect the within-host dynamic of the Gram-negative *Providencia rettgeri* [19], but reduces the load of *S. aureus* and *Enterococcus faecalis* 20 h after the start of the infection [36]. These discrepancies not only reflect the diversity of the host species used in the experiments, but

also of the pathogens, as some can suppress cellular immunity (e.g. the bacterium *Xenorhabdus nematophila* infecting the moth *Spodoptera exigua* [37]).

AMPs have long been thought to be active late in the infection [38]. This seems to be supported by some bacterial infections of *D. melanogaster*, such as *P. rettgeri*, where proliferation over 6 h is similar in wild-type and AMP-deficient flies [39], and is in fact comparable to in vitro proliferation rates [19]. This was also observed over the first 8 h of infections with the bacteria *Xenorhabdus nematophila* [40], although this pathogen can manipulate the immune system by inhibiting expression of AMPs [41]. Conversely, *Escherichia coli* and *Erwinia carotovora* (which both cause benign infections in *D. melanogaster*) are controlled within 2 h in wild-type hosts, unlike in AMP-deficient hosts [19]. Similar dynamics have been reported over the first 6–8 h of infections with *Enterobacter cloacae* and *Providencia burhodogranariea* [39,42]. In fact, immune genes encoding for AMPs can be strongly expressed almost immediately after the infection starts (even after peptidoglycan injection) [43] and last for many days, as shown in beetles and bumble bees [44,45]. AMPs are therefore likely to have a key role within the first few hours on within-host dynamics and impose selection on pathogens to adapt to resist to them, potentially at the cost of proliferation in their absence [40,46].

It is becoming evident that despite being triggered by canonical pathways (e.g. IMD and Toll), different AMPs have different dynamics of expression (see [47] for response to several bacterial infections in *Drosophila*). Some AMP genes activate rapidly upon infection, while others respond slowly, some increase gradually, while others turn on in a switch-like manner [17], and some turn off rapidly after activation, while others remain on longer, each probably responding to different cues [48]. The different immune gene expression patterns are even more interesting when considering that each AMP has specific efficacies. For example, Cecropins have an important role in controlling *Providencia heimbachae*, while Drosocin is more important in the control of *E. cloacae* infections [39,42]. In fact, even small amino acid variations in AMPs may change specificity, as demonstrated in the case of Diptericin A in *P. rettgeri* infections [49]. It is becoming clear that looking at the expression of one, or a small number of AMPs, especially at a single timepoint, is insufficient to understand the response to an infection, and that some AMPs have little influence on pathogen proliferation, despite being expressed in response to infection (e.g. Defensin against *E. cloacae* [39]). Although expression data do not always correlate with protein concentrations and the dynamics of AMP released in the hemolymph may be delayed, those results raise many questions [50]. What are the distinct dynamics of individual AMPs within the course of an infection? How does this impact pathogen proliferation? How does this compare between infections with different pathogens? These questions, at the heart of understanding how the innate immune system evolves, remain an uncharted field of research, which studies of early within-host dynamics could shed light on.

Pathogen load during chronicity may not reflect the impact of the immune response on within-host dynamics

While clearance seems to be a common disease outcome in general, it appears to be rare in *Drosophila*. Even avirulent bacteria are generally not cleared and their population is controlled at a low load ([19,51], Acuña Hidalgo et al. *bioRxiv* doi:10.1101/2021.03.29.437521v4). This load at chronicity is the SPPL [19]. Studies with *Drosophila* suggest that pathogen inoculum positively correlates with the SPPL, before the pathogen reasserts itself as an acute, lethal infection ([19,51], Acuña Hidalgo et al. *bioRxiv* doi:10.1101/2021.03.29.437521v4), unless the infection is kept under constant control and is harmless for the whole host life (benign) (Ramirez-Corona et al. *BioRxiv* doi:10.1101/2021.08.17.456698). This load has been used to compare hosts and identify whether differences in susceptibility are due to differences in the ability to control proliferation (i.e. difference in SPPL) or due to differences to tolerate damage (i.e. no difference in SPPL). However, theory suggests that the mechanistic interpretation of the SPPL can be difficult, as high SPPL can also stem from lower damage mitigation, a disease-tolerance mechanism (Lafont et al. *BioRxiv* doi:10.1101/2021.10.19.464998). Mechanisms to repair/sustain damage interact strongly with the ability to mount an effective immune response [52], which consequently means that bacterial load during chronicity depends on both. Another difficulty in the interpretation of SPPL is that it may be stable because pathogens are constantly under control or because they are quiescent [31]. Both are likely to occur, and it will be important to distinguish them. Determining which infections in insects are quiescent, such as tuberculosis in humans, will allow us to study, for example, which conditions trigger pathogens to restart proliferation and kill their host. On the other hand, in infections under constant host control, chronic infections are ideal conditions for strong within-host evolution to occur, and insect models could offer a tractable system to study the evolution of pathogen escape from the innate immune system during chronic infection.

Set-Point Pathogen Load, a measure for transmission potential

From the parasite point of view, within-host dynamics describes how population size increases over time. It consequently relates to the severity of the disease (i.e. the virulence of the pathogen) but also connects to its transmission: large population sizes in chronically

infected hosts increase transmission. Surprisingly, the connection between pathogen load and transmission is rarely well-demonstrated, due to technical limitations. Transmission of *E. coli* in cattle [53] or of SARS-CoV2 [54] in humans appears to be correlated with the pathogen burden carried by the host. One study [55] measured SPPL of C virus in *Drosophila* every day over 3 days. They showed that SPPL is highly variable (with 20% of variation explained by genetic background and sex), but observed no correlation with shedding via fecal excretion. However, pathogen shedding should be distinguished from transmission potential because the detected pathogen particles are not necessarily infective. This is highlighted by data from the current/recent pandemic [56], where despite shedding of SARS-CoV2 particles in feces for up to 126 days, no viable virus has been reported beyond 9 days [57]. In summary, the field of infectious diseases in invertebrate hosts still lacks a system where horizontal transmission between living individuals can be reliably controlled — such a model would be invaluable to understand the parameters that affect the link between within-host dynamics and disease transmission, especially in tractable model systems.

The Pathogen Load Upon Death reveals pathogen pathogenicity and host characteristics

In lethal infections, where death is certain, infections can be quantitatively distinguished either by the time the pathogen takes to kill the host, or by the load it has reached upon host death, PLUD. PLUD is the critical threshold, independent of the time to death, at which individuals die [19,58,59]. Contrary to the time to death, PLUD has been shown experimentally [19,60] and theoretically (Lafont et al. *BioRxiv* doi:10.1101/2021.10.19.464998) to be almost insensitive to inoculum size. It is therefore a good candidate measurement to assess the host capacity to handle infections. Thus far, PLUD has been used as a way to quantify the host's capacity to both sustain and repair the damage caused by the infection ([19,47,58,59], Huang et al. *bioRxiv* doi:10.1101/2020.11.23.394809).

PLUD can also be used to compare pathogens in experimental infections that use the same genotype of insect hosts. In this case, PLUD should vary with pathogen pathogenicity, that is, its capacity to cause damage during the within-host dynamics [19]. For example, wild-type genotypes of the bacteria *X. nematophila* kill *D. melanogaster* faster than mutants but at a similar PLUD, suggesting that wild-type genotypes are more virulent but have similar pathogenicity [40].

A recent study [59] questioned the use of PLUD due to small measured differences between beetle host populations, especially when compared with within-population variation. Similarly, small PLUD differences were found even when different species of *Drosophila* were compared [19]. This raises the general question of what magnitude of PLUD difference should be considered as *biologically*, rather than statistically, significant. PLUD is usually measured on log scales; it may therefore be the case that a small difference on such a scale, which corresponds to large numbers of pathogens, has great biological significance. For example, a 0.3 difference on a decimal log scale is a twofold difference; from a mechanistic point of view, this difference may be negligible when pathogen load is 10^3 but of major importance when it is 10^8. The question of what difference in PLUD reflects significant biological differences in response to infections is still open.

Another concern with PLUD is that, as is the case for the time to death, and to a greater extent the SPPL, it can vary as a consequence of the influence of both defense and damage- repair mechanisms. As for the SPPL, this is because defense mechanisms interact with damage-mitigation mechanisms (e.g. Diptericin can protect from damage generated by reactive oxygen species production [61], and impaired negative regulation of AMPs reduces lifespan, suggesting that AMPs induce damage [62,63]). However, simulations supported by experiments showed that a susceptibility underlined by a higher PLUD will most likely result from an impeded immune response (Lafont et al. *BioRxiv* doi:10.1101/2021.10.19.464998). Therefore, when aiming to understand why a given host is more susceptible to infection than others, we can predict that greater susceptibility comes from impaired damage mitigation, if the most susceptible host has the lowest PLUD, but from defective or insufficient immune response if it has the highest. It is important to note that one cause does not exclude the other (e.g. higher PLUDs indicate a deficiency in immune response, but do not rule out impaired damage mitigation).

Conclusion

Here, we largely compare lethal diseases with chronic ones, as if we are able to neatly classify pathogens into one of these two categories. But experimental infections have revealed that some pathogens can, depending on small differences in how the infection has been initiated, be either lethal or chronic. These initial dynamics, sometimes appearing stochastic, translate into differences in early within-host dynamics, which eventually determine the outcome of the disease [19,31]. For example, just a few hours difference in controlling early bacterial proliferation between males and females can lead to strong sexual dimorphism in survival to infection in flies [28]. The evolution of sentinel immune cells, such as macrophages, and of immune memory and priming, in the animal kingdom, is suggestive of strong selection pressures on organisms to control the within-host dynamics as soon as possible. Yet, it is difficult to

study this crucial, early infection phase in vertebrates. The recent scientific enthusiasm for studying within-host dynamics in insects, using measures we have described, will help us to understand how disease outcome is determined by host and pathogen genotypes, inoculum size, infection route, and varied environmental factors, which we believe has the potential to offer enormous benefits to our understanding of disease progression, their management, and their evolution.

Conflict of interest statement

The authors declare having no conflict of interest.

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- • of special interest
- •• of outstanding interest

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