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Effect of different immune priming doses on survival
after secondary challenge with live bacteria in
Drosophila melanogaster

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Abstract

Invertebrates rely solely in innate immunity, which is believed to always respond to an infection the same way. However, in recent work, phenomenological evidence has been found in arthropods, suggesting that the innate immune system might also provide immune memory under some circumstances, the so-called ‘immune priming’. However, immune priming in arthropods is not fully understood and evidence to support such a phenomenon is not always found. Here I argue that we do not have a very good understanding of some of the basic aspects of these experiments and that many experimental parameters can be adjusted when testing for such a phenomenon, for example, the first dose of parasite that an individual receives (priming dose) before the secondary dose (challenge), to test for a priming effect. In this thesis I used *Drosophila melanogaster* as a host and the bacteria *Lactococcus lactis* as pathogens to try to (i) estimate the 50% lethal dose after 7 days, and to answer whether (ii) we find evidence of immune priming in terms of increased survival after secondary challenge?, (iii) are responses affected by priming dose of heat-killed bacteria? and (iv) if there is priming, is it affected by whether the priming dose is of live or dead bacteria? We have demonstrated that flies can present a significant higher survival after a secondary challenge of live bacteria when previously primed with a different doses of heat-killed and live bacteria, but in an overall view, it is still early to affirm that this response could be due to a priming effect, since the increase in the survival rate was small. This experiment highlights the necessity of more investigation in this field, exploring other parameters as time between priming and challenge, different priming doses and interaction of *Drosophila* with other bacteria.

1. Introduction

Our understanding of the immune system in animals has dramatically changed in the past decades, when the number of publications in this field increased and brought new light to this topic (Kurtz 2004). It's easy to understand why adaptive immunity typically received more attention from researchers. This branch of the immune system possesses as hallmarks immune specificity and memory (Janeway *et al.* 2001), which allowed, for example, the development of vaccinations in the 18th century, even before the mechanisms behind such phenomena were known.

In general lines, we can say that the immune system is typically divided in two categories: (i) innate immunity and (ii) adaptive or acquired immunity. The latter is found exclusively in vertebrates, and is based on somatically rearranging genes encoding B-cell receptors (BCRs), T-cell receptors (TCRs) and antibodies (Janeway *et al.* 2001), which allows the capacity to create billions of clones with distinct antigen receptors (Govind *et al.* 2004). Such factors confer specificity to the adaptive immune response, which means the degree to which immune reactions differentiate between antigens (Kurtz 2005).

As mentioned above, another hallmark of adaptive immunity is immune memory, which is the capacity of the body to “remember” a specific adaptive response in after exposure to specific antigens (Govind *et al.* 2004). This means that the body is able to make a greater and more rapid second response when reinfected with the same pathogen (Govind *et al.* 2004; Janeway *et al.* 2001).

Innate immunity, on the other hand, is believed to act naïvely to each encounter with a pathogen and to always respond to an infection in the same way (Brennan & Anderson 2004; Hoffmann 2003). It depends on the recognition of broadly conserved molecular moieties and exhibits only weak specificity, such as the ability to distinguish between different structural classes of peptidoglycan, the so called pathogen-associated molecular patterns (PAMPS), which are detected by germline-encoded, non rearranging pattern-recognition receptors (PRR) (Kurtz 2005). The innate system is found in both vertebrates and in invertebrates (Janeway *et al.* 2001).

Nevertheless, recent data (Lemaitre *et al.* 1997; Schmid-Hempel *et al.* 1999; Carius *et al.* 2001; Little *et al.* 2003; Kurt & Franz 2003; Witteveldt *et al.* 2004; Vierstraete *et al.* 2004; Zhang *et al.* 2004) suggests that the innate immune system might be more complex than we

previously believed. Phenomenological evidence has been found in arthropods suggesting that the innate immune system might present some characteristics, such as immune memory and specificity, previously assigned solely to the vertebrate adaptive immune system (Lemaitre *et al.* 1997; Schmid-Hempel *et al.* 1999; Carius *et al.* 2001; Little *et al.* 2003; Kurtz & Franz 2003; Witteveldt *et al.* 2004; Vierstraete *et al.* 2004; Zhang *et al.* 2004). It is also suggested that the adaptive arm of the immune system, can additionally depend on innate components of the vertebrate immune system (Paust & von Andrian 2011).

As a general statement we can say that functional immune adaptation can be defined as any case where an immune response differs between a first and a second challenge (Pham *et al.* 2007). In 1972, Boman *et al.* showed that antibacterial activity in *D. melanogaster* males hemolymph persists after challenge with viable *Aerobacter cloacal* and can provide higher survival rates to this flies when they are faced with subsequent challenges with *A. cloacal* as well as with *Pseudomonas aeruginosa* and *Escherichia coli* K12, hence showing a simple form of adaptation, in which the immune system remain activated after an initial challenge.

But beyond these findings, more complex phenomena have been shown in arthropods, such as strain-specific-immunity that is passed from a mother to her offspring, as shown by Little *et al.* 2003 and specific memory, as shown by Kurtz & Franz 2003. In invertebrates we see a initial immune activation that is nonspecific and confers protections against many types of challenges (Pham *et al.* 2007). However, they are also able to mount long-term specific protection where a priming dose - i.e. a first encounter of the host with a pathogen, which can be of low live doses or doses of dead pathogen - of a particular species of bacteria only protects against that species (or class of species), as shown by Sadd & Schmid-Hempel 2006 in bumblebees and Roth *et al.* in the red flour beetle.

Klein, 1989 said that due to the lack of potential molecular mechanisms, invertebrates were considered to lack both specificity and memory. Nevertheless, it is important to consider that the vertebrate immune system has at least 3 different functional aspects: (i) self-non-self recognition, (ii) innate pathogen recognition and (iii) adaptive immune recognition with memory (Janeway *et al.* 2014; Kurtz 2004). These functions did not necessarily evolve from the same origin and under the evolutionary pressures (Kurtz 2005). Kurtz (2005) suggested that this means that evolutionary and mechanistic causes are not always distinguished strictly enough, what leads to the notion that innate defense must be

devoid of specific memory just because the mechanisms establishing such memory within the vertebrate acquired immune system are absent.

Some approaches have helped to understand some of the underlying mechanisms for such phenomena, like Pham *et al* (2007) and Kurtz & Roth (2009), who have shown the role of phagocytosis to mediate specificity in the immune defence of *Drosophila* and *Porcellio scaber* (Crustacea: Isopoda), respectively. Even though there had been many advances in this field and in our understanding of the abilities of the invertebrate immune system, specially for *Drosophila melanogaster* (Lemaitre & Hoffmann 2007), the mechanisms underlying such capacity that functionally matches the abilities of the vertebrate immune system is not fully understood yet.

Here I argue that we do not have a very good understanding of some of the basic aspects of these experiments. Since immune priming is a complex phenomenon, many experimental parameters can be adjusted, for example, the time between priming and challenge, the priming doses and whether the priming dose is of live, as in a variolation, where there is a transfer of low exposure doses of infectious pathogen, or dead bacteria, in a process like a vaccination with an injection of dead or attenuated pathogens, which trigger a specific immune response (Masri & Cremer 2014).

In their study, Pham *et al.* showed evidence that *Drosophila* can modulate its immune response as a result of multiple challenges, and that a priming dose of *Streptococcus pneumoniae* is sufficient to protect the flies from a second challenge of the same bacteria in a lethal dose. They also showed that the fly immune system exhibits the adaptive characteristics of specificity and persistence, and that is really likely that phagocytes are the critical effectors of the primed response (Pham *et al.* 2007).

In their studies, Wu *et al.* 2014 also demonstrated, as Pham *et al.* 2007 that *Galleria mellonella* (Lepidoptera) larvae primed with an appropriate dose of heat-killed bacteria *Photobacterium luminescens* (TT01) induced an increased protection against subsequent infection of a lethal dose of viable TT01 for a period of time after priming. Moreover, the authors showed that the extent and the length of time that protective effect persisted correlated positively to the priming dose (Wu *et al.* 2014).

Both works from Pham *et al.* 2007 and Wu *et al.* 2014 tested for different parameters while investigating priming Immunity, as different priming doses within and different time intervals between priming and challenge. Pham *et al.* 2007 also tested a live bacterial priming

dose. However, the different priming doses are not the main focus in these described experiments.

Bearing in mind that dose-response curve is a key element of understanding host-parasite interactions (Schmid-Hempel 2011) but still we face such problematic aspects, as well as that a systematic assessment of the immune priming may provide information about important facets of this phenomenon, I use the *D. melanogaster* model as a host and the bacteria *Lactococcus lactis* as pathogens to achieve the main goals of my bachelor thesis, which are to (i) Estimate the 50% lethal dose after 7 days (LD50/7), which means, the dose needed to kill 50% of the tested host during a period of 7 days after infection (Schmid-Hempel 2011), and to answer whether (ii) we find evidence of immune priming in terms of increased survival after secondary challenge?, (iii) are responses affected by priming dose of heat-killed bacteria? and (iv) if there is priming, is it affected by whether the priming dose is of live or dead bacteria?

2. Materials and Methods

2.1 Experimental organisms

2.1.1 *Drosophila melanogaster*

All flies used in the experiment were wild type descended from inseminated females wild-collected in Münster in 2007 (stock name: 1-4 WS). The flies were kept in a population cage in an incubator at 25°C and on a 12:12 light dark cycle at 70% relative humidity and feed with standard sugar-yeast-agar medium (Water 970 mL, Agar 15 g, Sugar 50 g, Yeast 100 g, Nipagin 30 mL, Propionic acid 3 mL - recipe for 1 L of fly food). The food was kept in glass bottles inside the population cage and were changed every week.

2.1.2 *Lactococcus lactis*

Lactococcus lactis is a Gram-positive bacterium, used worldwide in the industrial manufacture of fermented milk products (Neves *et al.* 2006). This bacterium is characterized by grouping in pairs, forming short chains, not producing spores and being non-motile. In

nature, *L. lactis* occupies a niche related to plant or animal surfaces and the animal gastrointestinal tract (Bolotin *et al.* 2001).

L. lactis was first isolated from the hemolymph and thoracic muscle of wild fruit flies females in State College, Pennsylvania in 1998 (Lazzaro *et al.* 2002; Lazzaro *et al.* 2006). There are also reports of this bacterium being previously isolated from termite and lepidopteran gut tissues (Bauer *et al.* 2000; Shannon *et al.* 2001). According to Lazzaro 2002, *D. melanogaster* shows a moderate and sustained increase in mortality when infected with *L. lactis*.

2.2 Establishing the concentration of bacteria for a 50 % lethal dose 7 days after infection (LD50/7)

2. 2.1 Obtainment and maintenance of the flies

All the flies used in this experiment were the F₁ generation produced from adults in the population cage. To produce the F₁ generation we put plates with purple grape juice medium (distilled water 27.5 ml, agar 12.5 g, red grape juice 150 ml, nipagin 10.5 ml) with a small amount of fresh yeast paste into the population cage. Flies were allowed to lay eggs on the plates for 12h -14h. After this period the plates were removed from the population cages and left in the incubator for 18 hours, until the eggs eclosed. A total of 1,000 first instar larvae were picked with an entomological needle and distributed in groups of 100 larvae per small glass vials (28.5×95mm), containing standard sugar-yeast-agar medium, until the adults eclosed.

Virgin females and males were separated from each other within a maximal 8-hour-interval after they had eclosed and kept in vials containing standard sugar-yeast-agar medium in groups of 10 males or 10 females. All flies were flipped to vials containing fresh food 5 days after they eclosed, to avoid them to stick in the old food.

When the adults were 9-10-day-old, i.e. one day before the injections, I set a group mating assay with the flies, where 10 males were flipped to a vial containing 10 females. I allowed the flies to mate during a 1.5h interval. After the mating, females were kept in groups of 10 flies per vial and males were discarded.

2.2.2 Injections

D. melanogaster adult females, 10-11-day-old, were infected with 18.4 nl with different doses of live *L. lactis* (2,000, 20,000, 200,000 and 500,000 CFUs). For controls I injected flies with Ringer's solution pH 7.2 (control for wounding) and did not inject anything in the naïve treatment. Females were anaesthetized on CO₂ and pierced in the second thoracal segment in the left ventrodorsal side (except for the naïve treatment).

The injections were performed with a Nanoject (Drummond Scientific Company), and with one capillaries for each group of 10 flies. The pulled glass capillaries (Bo-glass capillaries, Hilgenberg, Art.-No. 1406119, length = 100 mm, outside diameter = 1 mm, wall thickness = 0.21 mm) were prepared in two heating steps (68° C and 100° C) with a pulling machine (Narishige) and the tips of the capillaries were ground to approximately 45° with a capillary grinder (H. Saur), then they were backfilled with mineral oil and afterward load onto the Nanoject. Mortality was recorded every day for the following seven days to estimate the 50% lethal dose after 7 days (LD_{50/7}). 168h after infections, information about whether the flies had offspring or not was recorded to find out if flies were virgins or not. These experiment was conducted in four independent blocks with a total sample size of 240 flies, 60 flies per block.

2.2.3 Bacteria culturing

Bacteria culturing was performed according to the protocol by Sophie Armitage and Barbara Hasert (last update: 19th August 2014). To cultivate *L. lactis* and *P. entomophila*, samples were taken from frozen glycerol stocks (-80 °C). The preparations were done during three days, with the assistance of Dafne Strozake Maximo. In the first day we streaked four LB-agar plates (Luria/Miller, Carl Roth GmbH + Co. KG) with *L. lactis*. We also included an LB-agar plate streaked with *Drosophila* Ringer's solution (KCl 13.57g, NaCl 2.69g, CaC₂H₂O 0.33, Tris-Hcl 1.21g, 7.2 pH, recipe for 1 Liter solution) as a negative control. On the following day we selected four single colony forming units (CFUs) from each of the four plates and grew overnight 4 CFUs per Erlenmeyer flasks filled with 100 mL of LB-medium each, in a 30°C incubation shaker at 200 rpm.

On the third day we checked for possible contamination of the bacteria by putting a 1 μ l droplet on a slide and examining it under the microscope. This would allow us to identify any significant levels of contamination with bacteria of a different morphology to our desired bacteria, but not low levels of contamination or bacteria with similar morphology. The bacteria from the liquid culture were split in 50 ml Falcon tubes and centrifuged (10 min, 2057 RCF, 4 °C), afterwards, the supernatant was discarded and the bacteria pellet was resuspended in Ringer's solution and centrifuged (10 min, 2057 RCF, 4 °C) two times to wash the bacteria.

After the final wash bacteria was resuspended in a small amount of Ringer's solution. A 1:100 and a 1:1000 solution of each bacteria were generated to count the number of cells of bacteria in a volume of 0.008 mm³ under the microscope using a Thoma haemocytometer (0.2 mm deep, 0.0025 mm²). The final volume of bacteria to be added to Ringer's solution to obtain the final concentrations of 1 x 10⁸, 1 x 10⁹, 1 x 10¹⁰ and 2.5 x 10¹⁰ cells/mL of live bacteria. The correspondence of final concentrations prepared and the number of cells to be injected in the flies in a injection of 18.4 nl is shown in Table 2.1.

TABLE 2.1: Concentration of Bacteria cells/mL of solution and corresponding dose of Bacteria cells that were injected in the flies with an injection of 18,4nl.

Bacteria cells/mL	Bacteria cells/18,4nl
1 x 10 ⁷	200
1 x 10 ⁸	2,000
1 x 10 ⁹	20,000
1 x 10 ¹⁰	200,000
5 x 10 ⁹	100,000
2.5 x 10 ¹⁰	500,000

After the injections, dilutions from 1x10⁹ to 1x10⁴ cells mL were prepared and 50 μ l of the lower concentration were plated out on LB agar plates and left to grow overnight in the incubator at 30 °C. The following day the CFU's were counted to make an estimate of the number of bacteria injected into the flies. To correspond to the number of Bacteria that we

injected in the flies, we expected to count ~500 CFUs in each the plate. Plates with Ringer solution were also plated to control for contamination.

2.2.4 Data analysis

The survival rates were plotted in a Survival Curve in RStudio (2012) using the *survival* package (Therneau & Grambsch 2000; Therneau 2015), *coxme* package (Therneau 2015) and *bdsmatrix* package (Therneau 2014).

2.3 Priming-Challenge experiment

2. 3.1 Obtainment and maintenance of the flies

For the Priming-Challenge experiment all flies used were the F₂ generation from the population cage, to control parental age and rearing density. The same procedure as described above in Section 2.2.1 was conducted to obtain an F₁ generation. Three days after the F₁ adults eclosed they were placed in embryo cages (5.6 x 7.6 cm) in groups of 30 females and 30 males with purple grape juice plates and a small amount of fresh yeast.

Over the following day the plates were removed from the embryo cage and left in the incubator for 18 hours, until the eggs eclosed. 1,000 F₂ generation larvae were collected, as described for the LD50/7 experiment, and kept in the same conditions on standard sugar-yeast-agar medium until the adults eclosed, in groups of 100 larvae per vial. Virgin females and males were separated from each other within a maximal 8-hour-interval after they had eclosed and kept in vials containing standard sugar-yeast-agar medium in groups of 10 males or 10 females.

Three days after the adults eclosed, virgin females were primed. When the adults were 9-10-day-old, i.e. one day before the injections, I set a group mating assay with the flies, were 10 males were flipped to a vial containing 10 females. I allowed the flies to mate during a 1,5h interval. After the mating, females were kept in groups of 10 flies per vial and males were discarded. In the following day females were challenged and then allocated in individual vials. Mortality was recorded every day for the following seven days. 168h after infections, information about whether the flies had offspring or not was recorded to find out if

flies were virgins or not. More details about the Priming-Challenge experiment are given in the following sections.

2.3.2 Experimental design

The experimental design of the Priming-Challenge experiment is shown in Figure 1. This experiment was performed in two blocks, the first with an initial sample size of 260 females and 6 sub-blocks, and the second block with an initial sample size of 490 females and 7 sub-blocks. A Priming dose of 200,000 CFU heat-killed *L. lactis* was included in the second block.

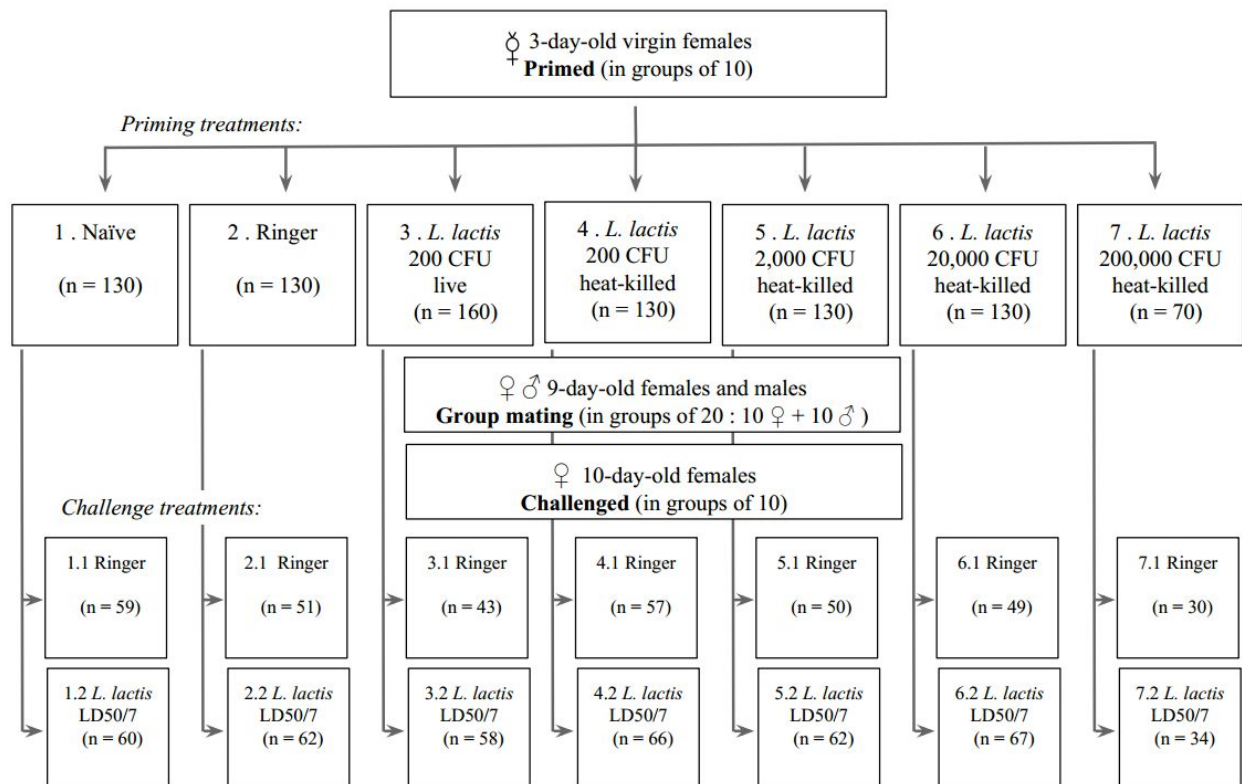


FIGURE 2.1: The design of the Priming-Challenge experiment. 3-day-old virgin females were primed in groups of 10 with one of the seven treatments: (i) naïve, which went through the all the procedure of anaesthetization and handling, but without an injection, (ii) *Drosophila's* Ringer Solution, (iii) dose of 200 CFU live of *L. lactis*, (iv) dose of 200 CFU heat-killed of *L. lactis*, (v) dose of 2,000 CFU heat-killed of *L. lactis*, (vi) dose of 20,000 CFU heat-killed of *L. lactis* or (vii) dose of 200,000 CFU heat-killed of *L. lactis*. Three days after the Priming 10 males were flipped to the vials with the females, and were allowed to mate during 1.5 hour interval. Afterward males were discarded. On the following day, females received a second injection, also in groups of 10. Approximately half of the flies from each Priming treatment received an injection of (i) *Drosophila's* Ringer Solution, as a control for the wounding and handling and the other half received a (ii) Challenge with an LD50/7 dose of live *L. lactis* (100,000 CFU). After the Challenge flies were individualized and kept in a climate chamber for 7 days and survival rates were recorded.

2.3.3 Bacterial infections - Priming

For the priming, *L. lactis* was heat-killed in a heat-block, at 90°C for 30 min. Afterwards, heat-killed bacteria and live bacteria were washed twice with *Drosophila* Ringer's solution at 4 °C and 2057 RCF for 10 min. The pellet was resuspended in a small amount of *Drosophila* Ringer and a 1:100 and a 1:1000 solution were prepared. The bacteria were counted under the microscope using a Thoma haemocytometer and concentrations of 1×10^7 of live bacteria cells/mL, and 1×10^7 , 1×10^8 , 1×10^9 , 1×10^{10} of heat-killed bacteria cells/mL were prepared (Table 2.1). To check for successful heat-killing, 50 µl of the bacteria solution were plated out on LB agar and put in the incubator at 30° C overnight and checked the next day that no bacteria had grown.

Virgin females were anaesthetized on CO₂ and pierced in the second thoracal segment in the left ventrodorsal side. For controls I injected flies with Ringer's solution pH 7.2 to control for wounding (total of 130 flies) and did not inject anything in the naïve treatment (total of 130 flies). For the Priming treatments flies were injected with 18,4nl of bacterial solution in the concentrations of 1×10^7 cells/mL of live bacteria (total of 158 flies) and 1×10^7 (total of 130 flies), 1×10^8 (total of 130 flies), 1×10^9 (total of 130 flies), 1×10^{10} (total of 70 flies) cells/mL of heat-killed bacteria. After Priming the flies were kept in groups of 10 flies per vial. All flies were flipped to vials with fresh food 4 days after primed.

2.3.4 Bacterial infections - Challenge

For the Challenge, bacteria were washed three times with *Drosophila* Ringer's solution at 4 °C and 2057 RCF for 10 min. The pellet was resuspended in a small amount of *Drosophila* Ringer and a 1:100 and a 1:1000 solution were prepared. The bacteria were counted under the microscope using a Thoma haemocytometer and a 5×10^9 cells/mL of live bacteria concentration was prepared. To check the concentrations, dilutions from 1×10^9 to 1×10^4 cells/mL were prepared and 50 µl of the lower concentration were plated out on LB agar, put in the incubator at 30° C overnight and counted in the following day.

Females were anaesthetized on CO₂ and pierced in the second thoracal segment in the left ventrodorsal side. For controls I injected flies of all Priming treatments with Ringer's solution pH 7.2 to control for wounding as follows: flies naïve at priming and challenged

with Ringer solution (total of 59 flies), flies injected with Ringer solution and challenged with Ringer solution (total of 51 flies), flies primed with 1×10^7 cells/mL of live bacteria and challenged with Ringer solution (total of 43 flies), flies primed with 1×10^7 cells/mL (total of 57 flies), 1×10^8 (total of 50 flies), 1×10^9 (total of 49 flies), 1×10^{10} (total of 30 flies) of heat-killed bacteria and challenged with Ringer solution.

For the Challenge, all Priming treatments were injected with 18,4nl of bacterial solution in the concentration of 5×10^9 of live bacteria cells/mL as follows: flies naïve at priming and challenged with live bacteria (total of 60 flies), flies injected with Ringer solution and challenged with live bacteria (total of 62 flies), flies primed with 1×10^7 cells/mL of live bacteria and challenged with live bacteria (total of 58 flies), flies primed with 1×10^7 cells/mL (total of 66 flies), 1×10^8 (total of 62 flies), 1×10^9 (total of 67 flies), 1×10^{10} (total of 34 flies) of heat-killed bacteria and challenged with live bacteria. After Challenge all flies were kept individually in the vials.

2.3.5 Data analysis

To test whether there was (i) an effect of challenge on survival, (ii) an effect of priming on the survival of flies that only had ringer at challenge and (iii) an effect of priming on the survival of flies that had *L. lactis* at challenge, analyses were done in RStudio using Cox proportional hazards models for survival data and Survival Regression with Weibull errors.

Data from both block were analyzed together, excluding the flies primed with a 1×10^9 cells/mL concentration, since this treatment group was present only in the second block. The effect of priming on the survival of flies that had *L. lactis* at challenge was also checked for the second block individually and 1×10^9 cells/mL concentration was included. A Cox mixed-effects model fit by maximum likelihood also was performed where Block was included a random effect.

3. Results

3.1 Establishing the concentration of bacteria for a 50 % lethal dose 7 days after infection (LD50/7)

This experiment was performed in order to know in a first place how *Drosophila* respond to different infection doses with *L. lactis*, since little is known from the literature. Nevertheless, similar experiments using different infections doses with this bacterium in *Drosophila* were previously performed in our Lab by M. Kutzer (unpublished data) and D. S. Maximo (unpublished data), which allows us to make some comparisons between these results. Also, I performed the same experiment in my Project-Module (unpublished data), but as the mortality rates were quite higher than we expected I repeated the experiment.

This experiment also had the goal of defining the 50%-lethal dose (LD50/7). The LD50 was set as the dose of live bacteria to be used as Challenge, since such scenario would provide a dose high enough to kill the flies, but not all of them, so we could see if there was a reduction in the mortality of the flies primed, which could be an indication of a Priming Immunity.

The survival curve for this experiment is shown in Figure 3.1. The mortality rates were lower in general than my previous results (Figure 3.2) and more proximate to what Kutzer had found before. A 2,000 CFU of *L. lactis* dose showed a survival rate of $\approx 59\%$ 168h after infection, much closer to Kutzer's results than in the first replicate.

Doses containing 20,000 CFU, 200,00 CFU and 500,000 CFU of *L. lactis* showed, 168h after injections, survival rates of $\approx 50\%$, $\approx 20\%$ and $\approx 10\%$ respectively. Even though the 20,000 CFU dose had a survival rate close to the LD50/7, I decided to go for a higher dose of 100,00 CFU, taking into account the fact that Kutzer and Maximo's results showed lower mortality rates than mine when infected with *L. lactis*.

86.25 % of the flies used in the experiment produced adult offspring (Figure 3.3). We assumed that flies without offspring did not mate, even though we know that this might not be always the case.

3.2 Priming-Challenge experiment

Estimates of the number of live bacteria cells injected in the Challenge differ between the first and second block. In the plates were I plated the dilutions of 1×10^4 I expected to count 500 CFUs, what would therefore correspond to a injection with 100,000 cells of live bacteria. In the second first block I counted 11 CFUs per plate, which means that I injected,

actually, 2.2×10^2 cells. For the second block, I counted 51 CFUs per plate, which correspond to injections with 1.02×10^3 cells. Therefore, block 1 and 2 were analysed separately.

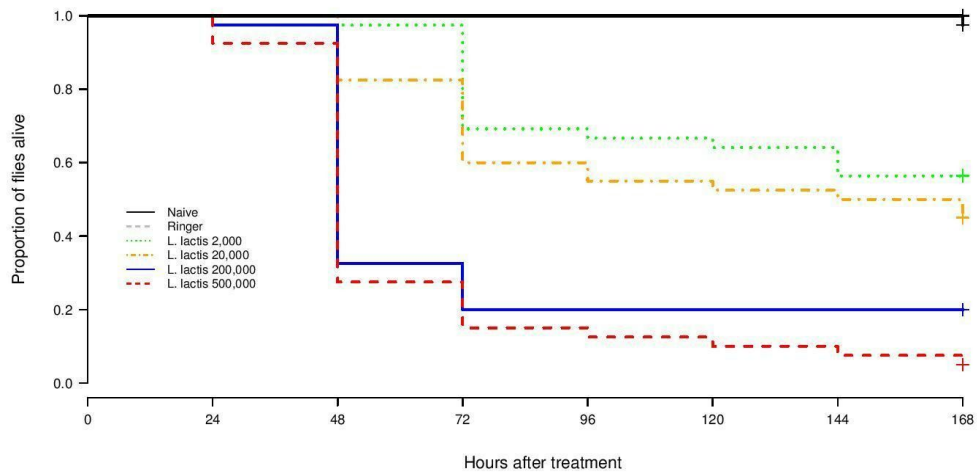


FIGURE 3.1: Survival curve for *D. melanogaster* injected with live *L. lactis* (replicate 2). Flies were injected with 18,4 nl of Ringer solution, 2,000, 20,000, 200,000 and 500,000 cells of *L. lactis*. The survival rate was recorded for a total of 168 hours after treatment, with intervals of 24 hours.

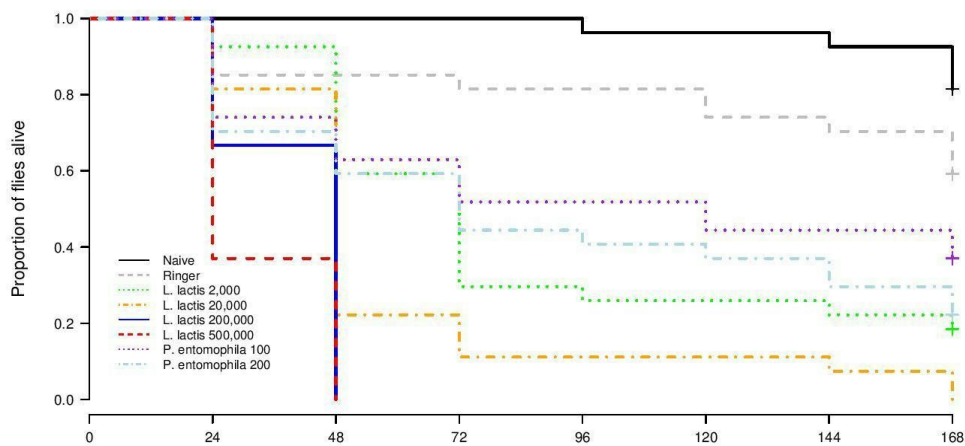


FIGURE 3.2: Survival curve for *Drosophila melanogaster* injected with live *L. lactis* (replicate 1). Flies were injected at time 0 with 18.4 nl of Ringer solution, ~2,000, ~20,000, ~200,000 and ~500,000 cells of *L. lactis* and with ~100 and ~200 cells of *P. entomophila*. The survival rate was checked for a total of 168 hours after treatment, at intervals of 24 hours.

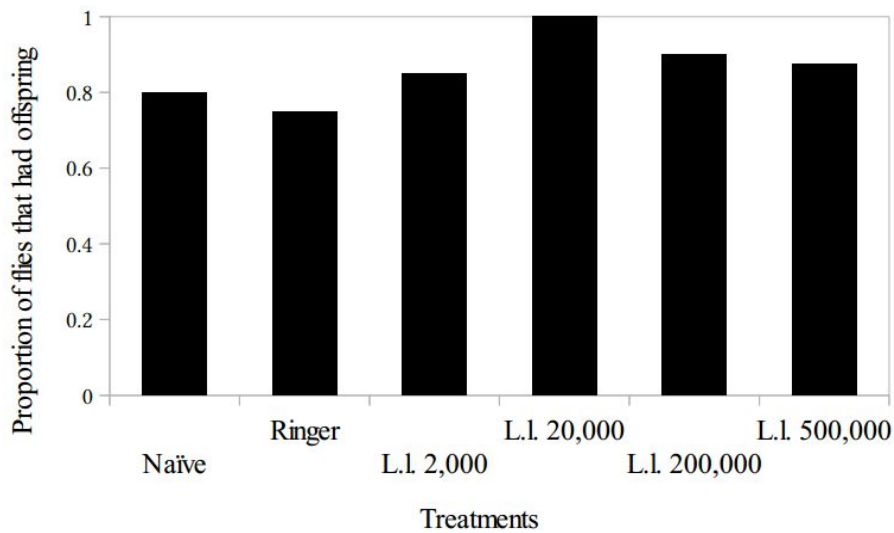


FIGURE 3.3: Proportion of flies that produced adult offspring in replicate 2. Each group (Naïve, Ringer, *L.l.* 2,000, *L.l.* 20,000, *L.l.* 200,000 and *L.l.* 500,00) had a total n flies = 40.

3.2.1 Is there an effect of Challenge on survival?

As expected, there was a significant effect of Challenge (Ringer's vs *L. lactis*) on fly survival, flies injected with *L. lactis* had a statistically significantly higher mortality when both blocks were analyzed together ($z = -14.78$, $df = 1$, $p < 0.0001$).

3.2.2 Is there an effect of Priming on the survival of flies that only received Ringer at Challenge?

There was no significant effect of Priming on survival of flies that only had Ringer Solution at Challenge when both blocks were analyzed together ($\text{Chisq} = 9.43$, $df = 6$, $p = 0.150670$) (Figure 3.4).

3.2.3 Is there an effect of Priming on the survival of flies that had *L. lactis* at Challenge in the first and second Block?

A significant overall effect of priming was found when flies that were challenged with live bacteria from both blocks were analyzed together ($\text{Chisq} = 18.86$, $df = 6$, $p = 0.00441410$) (Figure 3.5).

Flies primed with live *L. lactis* (dose = 200 CFU) had a statistically significant higher survival when challenged with live *L. lactis* ($z = -2.43$, $p = 0.015$) than flies that were naïve at priming. All other priming treatments did not show a statistically significant higher survival than flies that were naïve at priming: 200 cells of heat-killed *L. lactis* ($z = -1.46$, $p = 0.140$); 2,000 cells of heat-killed *L. lactis* ($z = -1.08$, $p = 0.280$); 20,000 cells of heat-killed *L. lactis* ($z = -1.27$, $p = 0.200$); Ringer solution ($z = -0.63$, $p = 0.530$).

3.2.4 Is there an effect of Priming on the survival of flies that had *L. lactis* at Challenge in the second Block?

For the second block no significant overall Priming effect was found (Chi square = 10.2, $df = 6$, $p = 0.116$) when we performed a Cox proportional hazards models, despite the quite highly significant differences between naïve and the priming groups. Nevertheless, when we performed a survival regression with Weibull errors we found a significant overall effect of priming (Chi square = 17.83, $df = 6$, $p = 0.0067$).

Flies primed with 200 cells of live *L. lactis* ($z = 3.65$, $p = 0.00026$), 200 cells of heat-killed *L. lactis* ($z = 2.76$, $p = 0.0058$) and 200,000 cells of heat-killed *L. lactis* ($z = 3.34$, $p = 0.00083$) had a statistically significant higher survival when challenged with live *L. lactis* than flies that were naïve at priming (Figure 3.6).

All other priming treatments did not show a statistically significant higher survival than flies that were naïve at priming: 2,000 cells of heat-killed *L. lactis* ($z = 1.91$, $p = 0.06$); 20,000 cells of heat-killed *L. lactis* ($z = 1.54$, $p = 0.12$); Ringer solution ($z = 1.63$, $p = 0.1$).

82.19 % of the flies of the first block produced adult offspring (Figure 3.7 A), and for the second block this percentage was of 93.46 % (Figure 3.7 B). We assumed that flies without offspring did not mate, even though we know that this might not be always the case. Figure 3.8 brings the proportion of flies that died between the priming treatment and the challenge, in an interval of 168 hours. When primed with live bacteria, flies showed a mortality rate of 38%.

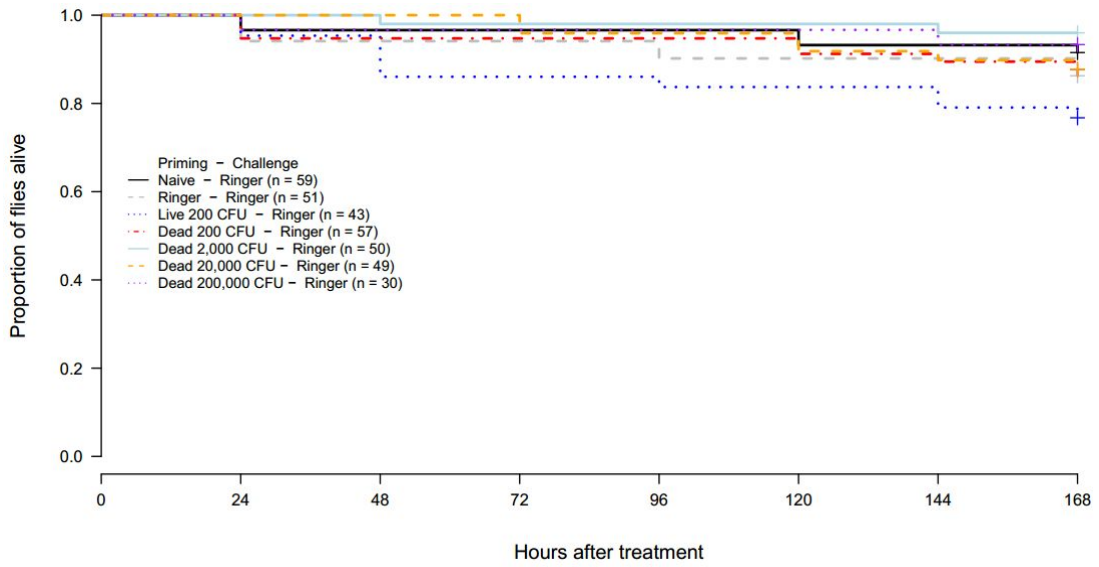


FIGURE 3.4: Block 1 and 2. Survival curve for *D. melanogaster* challenged with Ringer solution. The survival rate was recorded for a total of 168 hours after treatment, with intervals of 24 hours. Values weren't significantly different from the naïve group (df = 6, p = 0.150670) according to Cox proportional hazards.

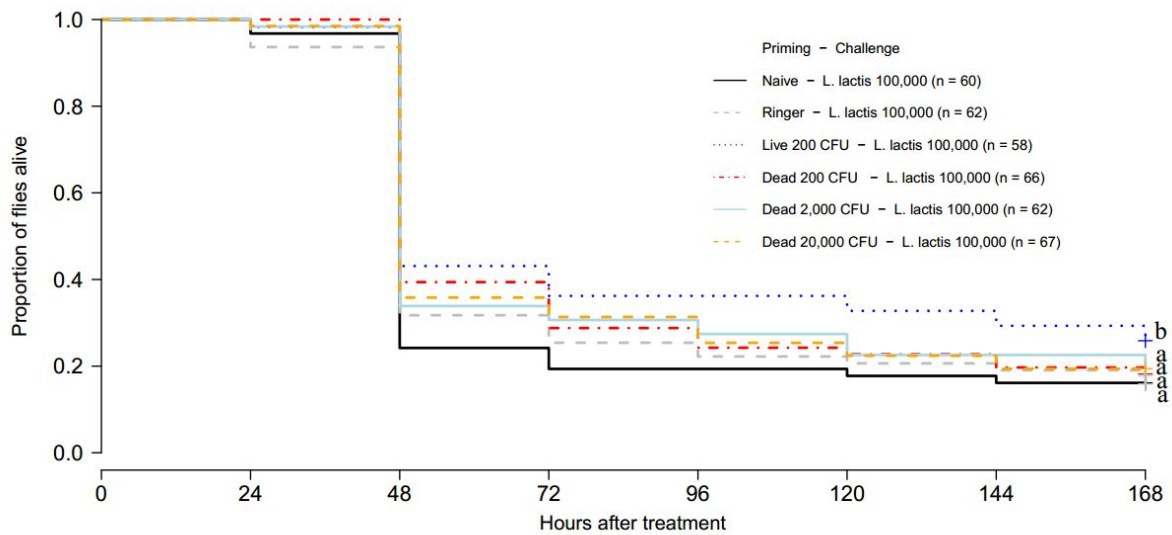


FIGURE 3.5: Block 1 and 2. Survival curve for *D. melanogaster* challenged with 100,000 cells of *L. lactis*. The survival rate was recorded for a total of 168 hours after treatment, with intervals of 24 hours. Values for different groups followed by different letters are significantly different from the naïve group (p = 0.015) according to Cox proportional hazards.

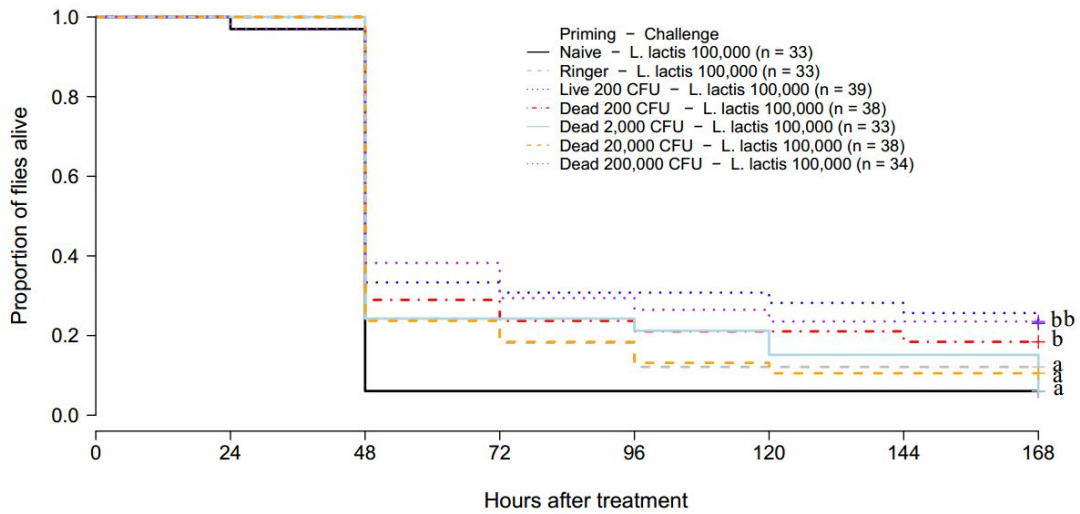


FIGURE 3.6: Block 2. Survival curve for *D. melanogaster* challenged with 100,000 cells of *L. lactis*. Values for different groups followed by different letters are significantly different from the naïve group ($p = 0.00026$, $p = 0.0058$, $p = 0.00083$ respectively) according to survival regression with Weibull errors. Values weren't significantly different from naïve group ($p = p = 0.116$) according to Cox proportional hazards.

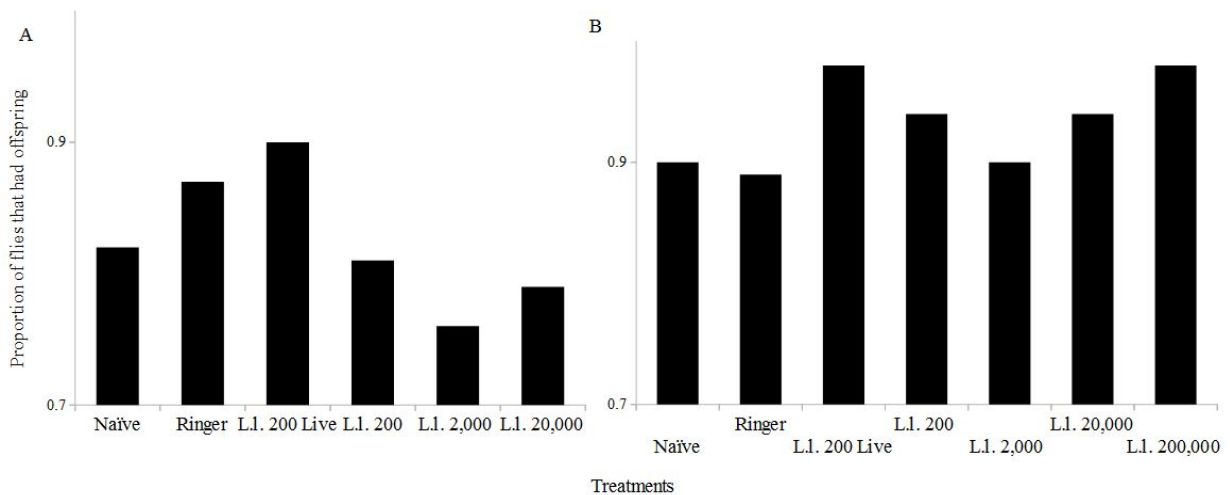


FIGURE 3.7: Proportion of flies that produced adult offspring in the Priming-Challenge experiment. A. Data for flies in the first block. Each treatment group had a total 60 flies. B. Data for flies in the second block. Each treatment group had a total 70 flies, except for the *L.l.* 200 live that had 98 flies.

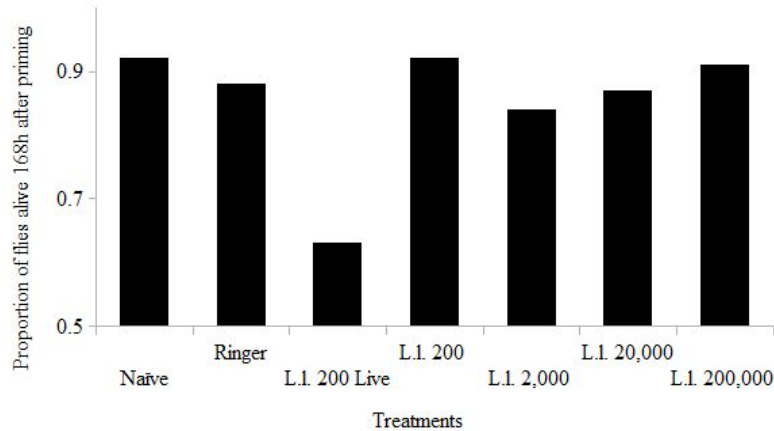


FIGURE 3.8: Proportion of flies alive 168h after the priming treatment.

4. Discussion

As far as I am concerned, this is the first study where such priming effect was tested in this host-pathogen combination. The current study demonstrated that no strong priming effect is seen in flies infected with *L. lactis* when primed with this specific doses, in this specific interval of time.

Such findings contrast with previous works as the one from Sadd & Schmid-Hempel 2006, where they demonstrated, that a priming dose with live bacteria in *Bombus terrestris* can show, even several weeks after the clearance of a first exposure, increased protection and narrow specificity upon secondary exposure. Same for Pham *et al.* 2007, where they showed that priming *Drosophila* with a sublethal dose of *S. pneumoniae* protects against an otherwise-lethal second challenge of the same pathogen, hence demonstrating coarse specificity for this pathogen that could persists for the life of the fly.

Flies primed with 200 cells of live *L. lactis* had a statistically significantly higher survival when challenged with 100,000 cells of live *L. lactis* than flies that were naïve at priming, which is suggestive of a priming effect. However, this increase in survival could also be due to a selection effect, where only more resistant flies survived after priming. As shown in Figure 3.8, the mortality of flies primed with 200 cells of live *L. lactis* were higher than all the other treatment groups 168h after priming.

In addition to that, *L. lactis* can persist in the hemolymph of the flies for some

days after infection (Kutzer, unpublished data). This scenario would probably lead to an upregulation of components of the Toll pathway, which is activated by fungi and Gram-positive bacteria (Rutschmann *et al* 2002). This upregulation of the immune system could confer a small advantage to flies that were primed with live bacteria and afterwards faced with a secondary encounter with the pathogen in comparison with flies that had no previous contact with the bacteria (naïve and Ringer treatments) or with flies that were injected with heat-killed bacteria.

When the second block was analysed separately, flies primed with doses of 200 and 200,000 cells of heat-killed bacteria and challenged with live bacteria also showed an increase of 15-20% in the survival rate when compared with flies that were naïve at priming and challenged with bacteria.

Since the survival of flies primed with 200 and 200,000 cells of heat-killed bacteria (94.3% and 91.4% respectively) 168h after priming were higher than the survival of the flies primed with live bacteria (62,61%), this increase in survival might not be explained by a selection of more resistant flies.

According to Pham & Schneider 2008, *Drosophila* can show a specific primed response that is dependent on phagocytes, where an increase in survival rates is correlated with reduced bacterial load, demonstrating that the immune response is activated to kill bacteria more quickly. Even though this might be the case occurring in interactions between *D. melanogaster* and *L. lactis* at this priming doses and challenge conditions, still, it's early to affirm that this could be a priming effect.

The fact that only the lowest and the highest priming doses of heat-killed showed increase in survival request more careful analysis and investigation and raises questions, as, for example, why higher doses than 200 cells of bacteria, as 2,000 and 20,000 cells, wouldn't show an increase in survival as well,

In summary, taking into account this study and the findings that we achieved so far, we can say that (ii) we find a weak evidence of immune priming in terms of increased survival after secondary challenge for flies primed with heat-killed *L. lactis*, (iii) responses are affected by priming dose of heat-killed bacteria, since just some doses showed enhanced survival, even though this increase was low and (iv) from our observations we can't assume that a priming dose with live *L. lactis* conferred a priming Immunity to flies under the conditions of this experiment.

5. Conclusion

We have demonstrated that flies can present a significant higher survival after a secondary challenge of live bacteria when previously primed with different doses of heat-killed bacteria, but in an overall view, it is still early to affirm that this response could be due to a priming effect, since the increase in the survival rate was small.

We also can observe an increase in the survival rate of flies that were primed with live bacteria and later challenged with an LD50/7 of live bacteria, but again, the causes for such phenomena are more likely to be due a selection of more resistant flies after priming.

In this study no strong evidence of priming or effect of different Immune priming doses was found, still, this experiment highlights the necessity of more investigation in this field, exploring other parameters as time between priming and challenge, different priming doses and interaction of *Drosophila* with different bacteria. Furthermore, other topics of interest are to test whether phagocytosis or Dscam are responsible for immune priming by using mutant flies (previously used in our Lab), test for a lower dose for challenge and examine resistance and tolerance in the flies, hence include a fitness measure and test for transgenerational immune priming in *Drosophila melanogaster*.

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8. Plagiatserklärung des Studierenden

Hiermit versichere ich, dass die vorliegende Arbeit „Phagocytosis after Immune Priming in *Drosophila melanogaster* - An In Vivo and Ex Vivo Approach“ selbstständig verfasst worden ist, dass keine anderen Quellen und Hilfsmittel als die angegebenen benutzt worden sind und dass die Stellen der Arbeit, die anderen Werken – auch elektronischen Medien – dem Wortlaut oder Sinn nach entnommenen wurden, auf jeden Fall unter Angabe der Quelle als Entlehnung kenntlich gemacht worden sind. Des weiteren erkläre ich hiermit, dass ein Teil der praktischen Arbeiten mit Hilfe von Mitstudenten und meiner Betreuerin verrichtet wurde.

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