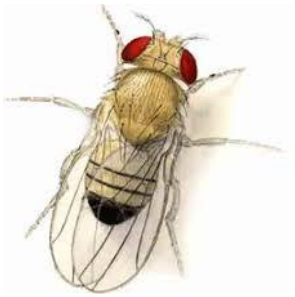
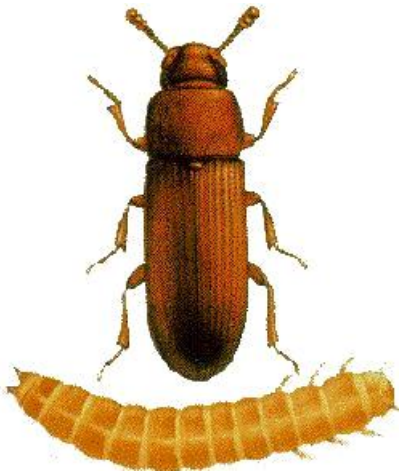
***MSc Advanced –Module***
***Host-Parasite Coevolution***
***November 17. – December 12. 2014***



*Drosophila melanogaster*



*Tribolium castaneum*

Gasterosteus aculeatus

Institute for Evolution and Biodiversity,
Hüfferstr. 1,
D-48149 Münster,
(http://ieb.uni-muenster.de/teaching/hostpara)

**Content**

# 1 Concept of module

## 1.1 Motivation

Parasites are just everywhere! - and they are among the strongest evolutionary forces. Host-parasite interactions are thus central to understand how organisms evolve. Parasites here include all organisms living in or on another organism, the host, and causing a fitness reduction of the host. This course will teach the basic background and current research progress in the fields of host-parasite coevolution, the evolution of immunity and virulence. It comprises a lecture series, a practical methods part, an introduction to experimental design and statistics, the discussion of scientific literature and the design and carrying out of your own research project. Frequent oral presentations about your projects or literature work will give ample opportunity to train analytical and presentation skills.

## 1.2 Communication and Language

Science is a lot about communication - knowledge only exists when it is communicated. Therefore, it is essential that students learn and practise continuously how to present scientific findings. English is the universal language of science. Therefore, and to allow participation of non-German speaking students, the default language of our courses at the Master level is English. Nevertheless, if subjects are really difficult and the language hinders understanding, your questions in German are always welcome.

## 1.3 Integration with Bachelor 'Vertiefungsmodul'

In this year some (8) students from the BSc 'Vertiefungsmodul' Evolutionary Ecology, will partially integrated in the MSc Module 'Host-Parasite-Coevolution'. This mainly refers to the lectures during the module, the IEB seminar and the 'Evolutionary thoughts' lecture, but also to the final presentations of your (and the Bachelor students) the projects results (see schedule 3.2.).

# 2 Overview of the three main parts of the module

## 2.1 Lectures

### 2.1.1 The plant defence system and response to selection (Juliette De Meaux)

The lecture will provide an introduction to plant defence systems and will give some examples of plant immunity. The second part will introduce general aspects of responses to selection, including responses of hosts to their parasites and of parasites to host defence systems.

### 2.1.2 Host-parasite coevolution lecture (Joachim Kurtz)

The lecture will introduce basic concepts of host-parasite coevolution, spanning the field from the evolution of immunity as the major host defence against parasites to

medically relevant aspects, such as the evolution of virulence and resistance. Moreover, the lectures will also confer relevant basic knowledge of parasitology, immunology and evolution.

### 2.1.3 IEB Seminar

This is the seminar series of the Institute for Evolution and Biodiversity which always takes place on Tuesday 16:00 (st). Generally speakers are invited from different fields of evolutionary biology research, but starting in the second week of the Host Parasite module; speakers have been invited who work on topics of host parasite interactions and coevolution. So your attendance of the IEB Seminar is obligatory during the module.
In addition to the lecture you will have the possibility for closer interaction with the invited speakers in a special seminar. In the literature seminar (2.2.3) you will be asked to discuss one paper chosen by each of the seminar speakers before the IEB seminar. On the day of the seminar or the Wednesday there after (see time plan), you will spend about 1h with the speaker to discuss his / her work and to ask questions.

### 2.1.4 The Growth of Evolutionary Thought

This lecture series delivers insight into current ideas and progress of evolutionary research from the perspective of diverse disciplines (geosciences, philosophy, biology, medicine). The lecture is not mandatory for the module, but we strongly encourage you to participate.

## 2.2 Special Seminars

### 2.2.1 Plant versus animal immunity?

This seminar will follow the lectures of J. Kurtz and J. De Meaux and in there you will have the chance to discuss plant and animal immunity with the experts. To motivate the discussion you will be asked already before the lectures to form two parties. The one party should defend the idea, that plant and animal immunity is very similar, while the other party should defend the idea that the plant and animal immune system is very different.

### 2.2.2 Basics and problems of experimental design (Joachim Kurtz)

This seminar will use the article “How To Avoid Seven Deadly Sins in the Study of Behavior” from Manfred Milinski to discuss issues of experimental design. The article is attached to the script. Please make sure you have read the article by 11:00 Wednesday November 19$^{th}$.

### 2.2.3 Introduction to experimental design and statistics (Sophie Armitage)

This seminar will follow on from the basics and problems of experimental design (2.2.2). It will cover an introduction to hypothesis testing, a couple of aspects of experimental design, and an introduction to some statistical tests. The seminar starts at 11:00 on Thursday Nov 20$^{th}$.

### 2.2.4 Paper seminar (Sophie Armitage)

You will first be given a short introduction into how to evaluate papers and into how the paper seminar will be run. Then, you will divide yourselves into three groups - each

group will present and discuss the paper of one of the invited speakers. You will read it thoroughly and discuss it with your group, then present it to the other participants and lead a seminar discussion. The seminar will help you to critically evaluate research papers and to train your presentation and oral communication skills. Based on the discussion of the speaker's paper, you will later have a chance to ask him/her personally about his/her work.

#### 2.2.5 Seminars with invited speakers

In these seminars you will have opportunity to ask questions to and discuss with experienced and established scientists. You will at first discuss one of their papers in the literature seminar. In the seminar with the invited speaker, please feel free to ask any question that interests you and in the discussion, please be critical, no scientist will be offended by a well placed, thoughtful critical question. By contrast, this often helps to develop new ideas and concepts.

### 2.3 Do your own research project

With this part we want you to collect experience how a research project is normally developing. We have only limited time, but we want to address questions with you that are in fact new and relevant for our on going research and we will take you through all the necessary steps:

- Develop, write and defend a research proposal
- Do the experiments
- Analyse the data
- Write a report in paper form
- Present the results

In this part of the course you can plan and carry out your own research project. In a small group of students, you have the choice between three proposed topics. Alternatively, you have also the possibility to propose your own topic in the field of host-parasite interactions, but it has to be feasible with respect to time and available resources. Experiments will be carried out with one of the model systems fruit flies, flour beetles or sticklebacks. From a textbook and key research papers you will learn the basic predictions in this field. You will be able to discuss specific aspects of you project with the projects supervisors and develop you own hypothesis. You will write a proposal and design an experiment to test your hypothesis. You will defend and discuss your project. You will do the experiment. You will analyse your data and sum up the story in a report in paper form. Finally, with a power point presentation you have the opportunity to persuade your colleagues that your experiment is great and that it is worth the money.
Please note that the projects results elaborated by the parallel Bachelor module will be presented and discussed together with the projects from the master module.

## 2.4 Online material:

Please go to the Host-Parasite Coevolution webpage (http://www.uni-muenster.de/Evolution/teaching/hostpara.shtml) for online material, including scripts and lecture slides (password: diplo).

# 3 Time schedule

| | | | | | |
|---|---|---|---|---|---|
| | Week 1 | | | | |
| | MO - 17 November | TU - 18 November | WE - 19 November | TH - 20 November | FR - 21 November |
| 8:45-10:00 | | | Evolutionary thoughts | | 10:00 Tierschutz! |
| 10:00-11:00 | Short Intro (JS) | Animal defense systems (JK) | Read article ...7 sins : | Intro paper seminar (SA) | Write proposal/prepare paper |
| 11:00-12:00 | 10:30 Projects intro (P) | | 7 sins paper (JK) 14:45 | Intro exp design stats (SA) | |
| Lunch | | | | | |
| 13:30-14:00 | S1 intro (GS) | Host Parasite Coevolution (JK) | Proposal intro (JS) | Intro exp design stats (SA) | Write proposal/prepare paper |
| 14:00 | Plant defense systems I (JM) | 15:00 Debate plant versus | Decide for own project (P) | | |
| | | animal immunology (JM, JK) | Write proposal | Write proposal/prepare paper | |
| 17:00 | Plant defense systems II (JM) | IEB seminar (16-17:00) | | | |
| | | Nicolas Gompel | | | |
| | Week 2 | | | | |
| | MO - 24 November | TU - 25 November | WE - 26 November | TH -27 November | FR - 28 November |
| 8:45-10:00 | | | Evolutionary thoughts | | |
| 10:00-11:00 | Write proposal/prepare paper | Write proposal/prepare paper | Discussion Mathias Wegner | Own projects experiments (P) | Own projects experiments (P) |
| 11:00-12:00 | | | Present/discuss proposal (P) | | |
| Lunch | | | | | |
| 13:30-15:00 | Write proposal/prepare paper | Write proposal/prepare paper | Present/discuss proposal (P) | Own projects experiments (P) | Own projects experiments (P) |
| | | | Correct proposal | | |
| 15:00-17:00 | | Paper seminar Wegner (SA) | | | |
| | | IEB seminar (16-17:00) | | | |
| | | Mathias Wegner | Hand in proposal | | |
| | Week 3 | | | | |
| | MO - 1 December | TU - 2 December | WE - 3 December | TH - 4 December | FR - 5 December |
| 8:45-10:00 | | | Evolutionary thoughts | | |
| 10:00-11:00 | Own projects experiments (P) | Own projects experiments (P) | Discussion Eva Stukenbrock | Own projects experiments (P) | Own projects experiments (P) |
| 11:00-12:00 | | | | | |
| Lunch | | | | | |
| 13:30-15:00 | Own projects experiments (P) | Own projects experiments (P) | Own projects experiments (P) | Own projects experiments (P) | Own projects analysis (P) |
| 15:00-17:00 | | Paper seminar Stukenbrock (SA) | | | |
| | | IEB seminar (16-17:00) | | | |
| | | Eva Stukenbrock | | | |
| | Week 4 | | | | |
| | MO - 8 December | TU - 9 December | WE - 10 December | TH - 11 December | FR - 12 December |
| 8:45-10:00 | | | Evolutionary thoughts | | |
| 10:00-11:00 | Intro report poster (JS) | Write report | Write report | Prepare poster | Project Presentation BScs and |
| 11:00-12:00 | Own projects analysis (P) | | | Write report | MSc |
| Lunch | | | | | |
| 13:30-15:00 | Own projects analysis (P) | Paper seminar Kaltenpoth (SA) | Write report | Prepare poster | |
| | | 14:30 Discussion Kaltenpoth | | Write report | |
| 15:00-17:00 | | | Prepare poster | | |
| | Write report | IEB seminar (16-17:00) | | Hand in report | |
| | | Martin Kaltenpoth | | | |
| | | | | | |
| | Lectures obligatory for BSc and | Practical work | Reading/writing | Presentation/discussion | Deadlines |
| | MSc students | MSc only | MSc only | | MSc only |
| | JK = Joachim Kurtz | SA = Sophie Armitage | MF = Momir Futo | | |
| | BH = Barbara Hasert | JM = Juliette De Meaux | FF = Frederik Franke | | |
| | JS = Jörn Scharsack | MK = Megane Kutzer | GS = Gregor Schmitz | | |
| | P = project supervisors = JS, MF, FF, MK, BH | | | | |

## 4 Grade points

| Task | | | Credits |
|---|---|---|---|
| Seminar | | | |
| | Paper presentation | 10 | |
| | Guidance of discussion | 10 | |
| | Participation in discussion | 10 | 30 |
| Own project | | | |
| Proposal | | | |
| | Clarity | 10 | |
| | Content | 15 | |
| | Originality | 10 | 35 |
| Proposal defense | | | |
| | Presentation | 10 | |
| | Participation in discussion | 15 | 25 |
| Practical work | | | |
| | Skills and accuracy | 25 | 25 |
| Report | | | |
| | Improvement to proposal | 15 | |
| | Results and discussion | 20 | 35 |
| Presentation | | | |
| | Clarity | 5 | |
| | Content | 10 | |
| | Originality | 10 | 25 |
| Poster discussion | | | |
| | Participation in discussion | 25 | 25 |
| Total grade points | | | 200 |

## 5 Details of the three main parts of the module

### 5.1 Lectures

#### 5.1.1 The plant defence system and response to selection (Juliette De Meaux)

| Date | Title |
|---|---|
| 17.11.2014 | *The plant defence system I* |
| 14:00 | |
| 15:30 | *The plant defence system II* |

5.1.2 Host-parasite coevolution lecture (Joachim Kurtz)

| Date | Title | Topics |
|---|---|---|
| 18.11.2014 13:30-15:00 | Animal defence systems | Models and examples for h.-p. coevolution; evolution of virulence and resistance |
| 18.11.2014 10:00 -12:00 | Host-parasite coevolution | Evolutionary ecology of immune defences; introduction to parasites |

5.1.3 IEB Seminar

The IEB seminar takes place on Tuesday at 16:00 in the lecture hall, Hüfferstr. 1, IEB.
(For abstract information, please visit the IEB seminar webpage:
http://ieb.uni-muenster.de/seminar)

189.11.2014, Tue 16:00, Nicolas Gompel
25.11.2014, Tue 16:00, Matthias Wegner
02.12.2014, Tue 16:00, Eva Stukenbrock
09.12.2014, Tue 16:00, Martin Kaltenpoth

5.1.4 The Growth of Evolutionary Thought

Wednesday 8:45-10:00, Kavaliershäuschen, Schlossplatz 6.
http://ieb.uni-muenster.de/mgsei/teaching

5.2 Paper seminar (Sophie Armitage)

The introduction to the paper seminar will be held on Wednesday, 20.11. at 11:00 in the course room. The seminars will take place in the seminar room on the attic floor, on the Tuesday before the invited speaker will give his presentation in the IEB seminar. Please see the schedule for the exact timing of the paper seminars and discussions with the invited speakers.

5.3 Do your own research project

5.3.1 Tribolium project: Dose assessment for oral infection of the red flour beetle with different *Bacillus thuringiensis* strains

Momir Futo, Joachim Kurtz
**Background:** During transmission of infectious diseases, the dose of the pathogen which is transmitted from the infection source to the next host is an important determinant whether the disease can spread in the population, i.e. among different hosts, or not. In cases when the dose is too low, the immune defense mechanisms might be strong enough to stop and clear the infection and thus prevent the host from

getting ill but also the spread of the pathogen in the population. Furthermore, there is also the possibility that the initial dose of the pathogen is too low to be detected by the immune system of the host, in which case the immune system might detect the spreading infection too late. On the other hand, too high doses may kill the host as the immune system is swamped with the infection agent. Additionally, different abiotic (*e.g.* temperature) and biotic factors (*e.g*. co-infecting bacteria, different toxins and/or virulence factors) may contribute to the success or failure of the infection.

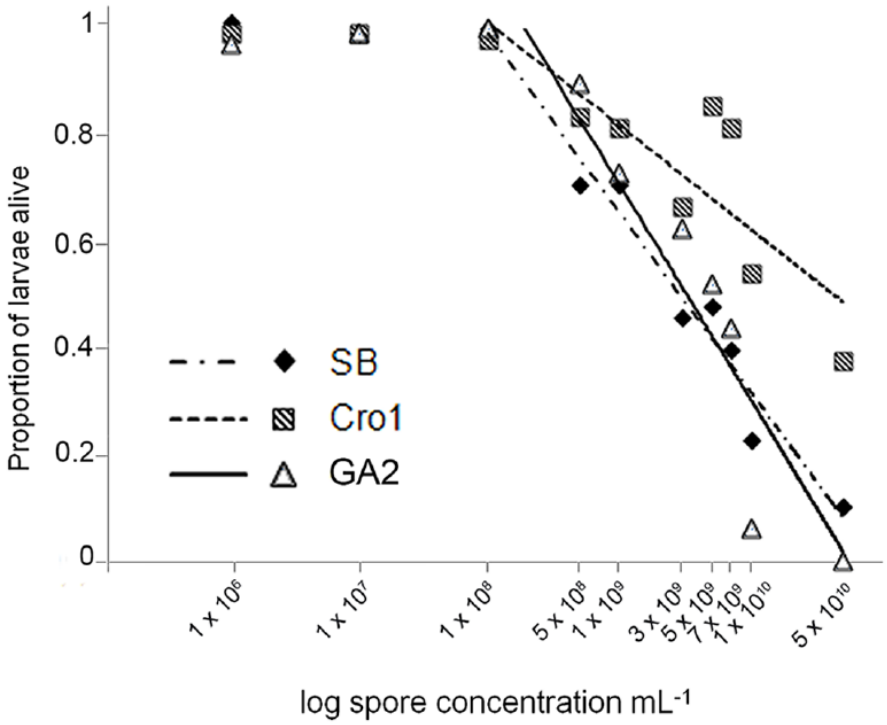



**Figure 1 Dose response curves for *Btt* oral infection.** Survival of three populations of *T. castaneum* larvae at day seven after constant exposure to different concentrations of *Btt* spores in flour (Milutinović *et al.* 2013)

*Bacillus thuringiensis* (*Bt)* is a spore forming, Gram-positive, entomopathogenic bacterium. During the process of sporulation, crystalline proteins (a.k.a. Cry toxins) are being produced. The Cry toxins can be highly strain specific (different *Bt* strains are producing different Cry toxins) (Schnepf *et al.,* 1998*)* but also infectious to different insect species or higher taxonomical levels (for review see Zeigler 1999). As the Cry proteins are plasmid-encoded, pathogenic and non-pathogenic *B. thuringiensis* strains are available. Furthermore, it has been suggested that *B. thuringiensis* is a naturally occurring parasite of the red flour beetle, *Tribolium castaneum,* and their host-parasite interactions have been studied in our lab during the last years.

Recently, an oral way of priming and subsequent infection was proposed for studying immune priming effects in *T. castaneum* by (Milutinović *et al.*, 2013a). Oral priming (a form of vaccination) with filter-sterilized spore-suspension of *Bacillus thuringiensis* and oral infection with the same pathogen lead to increased survival in *T. castaneum*. For this experiment a subspecies *Bacillus thuringiensis* bv. *tenebrionis* (*Btt*) producing a Cry3Aa form of toxin which is especially pathogenic to coleopteran species (Milutinović *et al.*, 2013b) was used. Meanwhile, Contreras *et al.* showed in 2013 that using a

slightly different approach, two other *Bt* strains producing Cry3Ba and Cry23Aa/Cry37Aa have a lethal effect on *T. castaneum.*

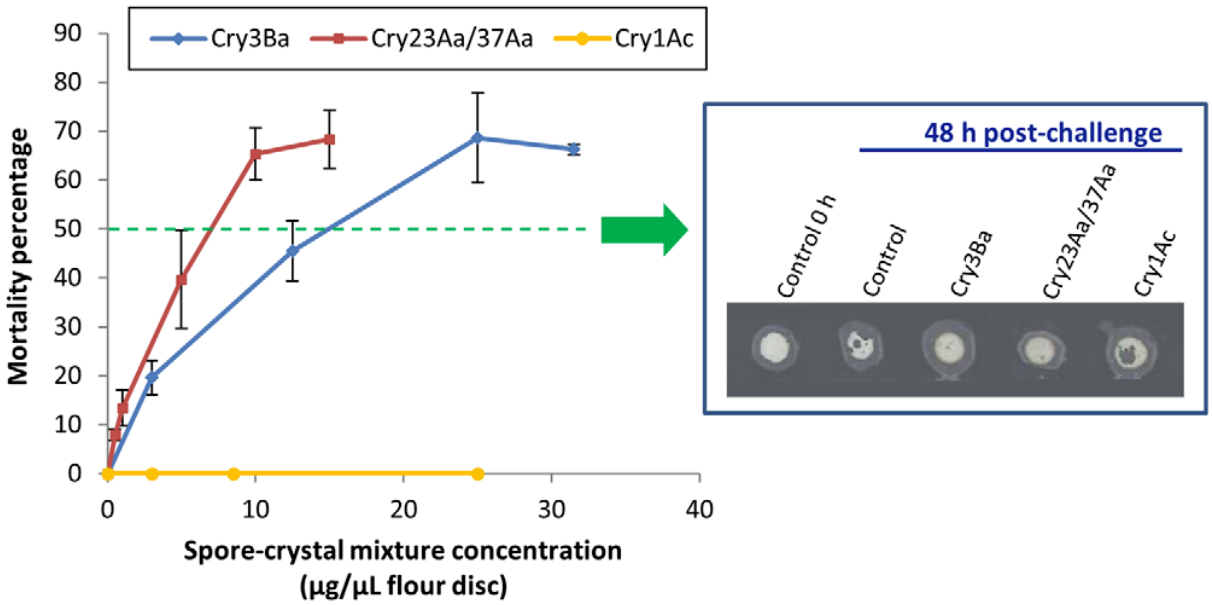



**Figure 2**. Dose-mortality assays with Cry3Ba, Cry23Aa/Cry37Aa and Cry1Ac spore-crystal mixtures in Tc larvae. Bioassays were performed on eight to ten day-old larvae fed on flour discs containing spore-crystal mixtures of Cry3Ba, Cry23Aa/Cry37Aa and Cry1Ac toxin producing Bt strains. Arrow points to images of 10 mL flour discs on which Tc larvae were fed for two days containing water (control), an approximately LC50 of Cry3Ba and Cry23Aa/37Aa spore-crystal preparations (12.5 mg/mL flour disc and 5.0 mg/mL flour disc, respectively), and 25 mg Cry1Ac spore-crystal mixture/mL flour disc (negative control). doi:10.1371/journal.pone.0055330.g001 (Contreras *et al.*, 2013)

**The aims of the project**

The aim of your project would be to design and carry out an experiment which will answer the following question:

1. Is the survival rate of *T. castaneum* larvae as a measure of the infection success of *B. thuringiensis* dependent on the dose applied with an oral uptake?
2. How much do different *B. thuringiensis* strains vary in their virulence and is this reflected in the dose of bacteria?

Literature

Ben-Ami F, Regoes RR, Ebert D (2008) A quantitative test of the relationship between parasite dose and infection probability across different host-parasite combinations. *Proc R Soc* B 275: 853–859

Contreras E, Rausell C and Real MD (2013) Proteome Response of *Tribolium castaneum* Larvae to *Bacillus thuringiensis* Toxin Producing Strains. *PLoS ONE* 8(1): e55330.

Milutinović B, Fritzlar S, Kurtz J (2013a) Increased Survival in the Red Flour Beetle after Oral Priming with Bacteria-Conditioned Media. *J. Innate Immun.* doi:10.1159/000355211

Milutinović B, Stolpe C, Peuβ R, Armitage SAO, Kurtz J (2013b) The red flour beetle as amodel for bacterial oral infections. *PLoS One* 8(5) e64638. doi:10.1371/journal.pone.0064638

Roth O, Sadd BM, Schmid-Hempel P, Kurtz J (2009) Strain-specific Priming of Resistance in the Red Flour Beetle, *Tribolium castaneum*. *Proc. R. Soc. B* 276, 145–151.

Schnepf E, Crickmore N, Rie Van J, Lereclus D, Baum J, Feitelson J, Zeigler DR, and Dean DH (1998)

*Bacillus thuringiensis* and Its Pesticidial Crystal Proteins. *Microbiology and Molecular Biology Reviews* 62(3), p.775-806.

### 5.3.2 Drosophila project: Does being sick affect fly mating behavior and fecundity?

Megan Kutzer, Sophie Armitage

**Background**: There is increasing evidence from many taxa that immune defense and reproduction are tightly linked (Lawniczak et al 2007). A parasitic infection can alter host life history strategies such that immunity is traded off against fecundity. Indeed, in many organisms female immune defense is upregulated after mating and females experience a weakened immune response as a result.

Mated females can suffer reduced defense against a bacterial infection compared to virgin females – mated females infected with *Providencia rettgeri* had higher bacterial loads and lower survival compared to virgins (Short et al 2012). In terms of reproductive investment, infection by some species of bacteria can have a negative effect on egg numbers (Brandt & Schneider 2007), suggesting that there is an immediate cost to the infection, whereas an alternative strategy, terminal investment, is to increase reproductive output after infection because of the shortened lifespan that the infection might cause (Adamo 1999; Hanssen 2006).

In our lab, we are examining the effects that infection with two bacteria species, *Escherichia coli* and *Lactococcus lactis*, has on life history strategies in the fruit fly, *Drosophila melanogaster.* In these experiments we let the females mate and then infect those 24 hours later with a fixed dose of bacteria, then we examine fecundity and host resistance to infection. Resistance is defined as a host's ability to limit parasite load, and we measure it as the number of bacterial colony forming units (CFU) inside the fly. A more resistant host has a lower bacterial load than a less resistant host. In the short-term, female flies seem to tolerate the bacterial infections well, laying the same number of eggs as uninfected flies. However, little is known concerning the behavior of diseased female flies and the effects, if any, on fecundity and mating behaviour. There is one example that we are aware of where this has been examined (Khan & Prasad 2013); in this case the authors used a virulent bacteria that causes high mortality (Imroze & Prasad 2011).

However, in this project we will explore the effects of non-lethal, persistent bacterial infections on female behavior. This project will focus on the relationship between infection and reproduction – more specifically, we would like you to design an experiment where you can test whether being sick affects mating behaviour and fecundity.

The main aims would be to test:

1. Whether infection affects:
    a. Aspects of female mating behaviour: the proportion of females that mate, the time it takes for the start of mating (mating latency), and copulation duration.
    b. Fecundity, i.e., the number of eggs laid or egg viability
2. Whether there is a relationship between resistance and:
    a. Aspects of female mating behaviour
    b. Fecundity

**Why flies?**

Insects make excellent model organisms for examining the relationship between immunity and reproduction because we can easily observe mating behaviour, short

generation times mean that we can also quantify fecundity (e.g., number of eggs), as well as experimentally infect them in relatively large numbers. One particularly suitable insect that fulfills all of these criteria is the black bellied dew lover, also known as *Drosophila melanogaster,* and this will be your model organism for this project.

**Methods overview**

To make the flies sick, they can be injected with a known number of bacteria (Figure A). Once they are infected you can watch their mating behaviour (Figure A), and measure the time that it takes for them to start mating and the duration of the mating. Young virgin flies typically mate for around 20 minutes, which is about 10 times longer than the other insect species that we have in our lab, *Tribolium castaneum*.

**Figure A**: The flies are made sick by injecting them with bacteria. You can then observe their mating behaviour. Photos from *Left*: wikibooks.org; *Middle*: Apidianakis & Rahme 2009; *Right*: pk-photography.blogspot.de

Once they have been given time to mate you can measure fecundity by counting the number of eggs that they lay. Virgin flies can also lay eggs, so even if some of the flies don't mate you can still count eggs and see if there is an effect of bacterial infection on the numbers of eggs laid. Resistance can be measured by homogenising the flies and plating them out on a petri dish. The next day the number of CFUs on the dish are counted, which gives you your measure of resistance.

**Literature (recommended reading is marked by *)**


*Adamo (1999) Evidence for adaptive changes in egg laying in crickets exposed to bacteria and parasites. Animal Behaviour. 57:117-124.

*Brandt & Schneider (2007) Bacterial infection of fly ovaries reduces egg production and induced local hemocyte activation. Developmental and Comparative Immunology. 31: 1121-1130.

Hanssen (2006) Costs of an immune challenge and terminal investment in a long-lived bird. Ecology. 87: 2440-2446.

Imroze & Prasad (2011) Sex-specific effect of bacterial infection on components of adult fitness in *Drosophila melanogaster*. Journal of Evolutionary Biology Research 3:79-86.

*Khan & Prasad (2013) Male *Drosophila melanogaster* show adaptive mating bias in response to female infection status. Journal of Insect Physiology 59:1017-1023.

*Lawniczak et al (2007) Mating and immunity in invertebrates. Trends in Ecology and Evolution. 22: 48-55.

*Short et al (2012) Female *Drosophila melanogaster* suffer reduced defense against infection due to seminal fluid components. Journal of Insect Physiology. 58: 1192-1201.

### 5.3.3 Gasterosteus project: analysis of stickleback immune manipulation by excretory products of a tapeworm in cell cultures

Frederik Franke, Madeleine Hamley, Anika Wohlleben, Jörn Peter Scharsack

**Background:** The tapeworm *Schistocephalus solidus* is a parasite with a complex life cycle (Fig. 1) which infects the three-spined stickleback (*Gasterosteus aculeatus*) as the specific second intermediate host.

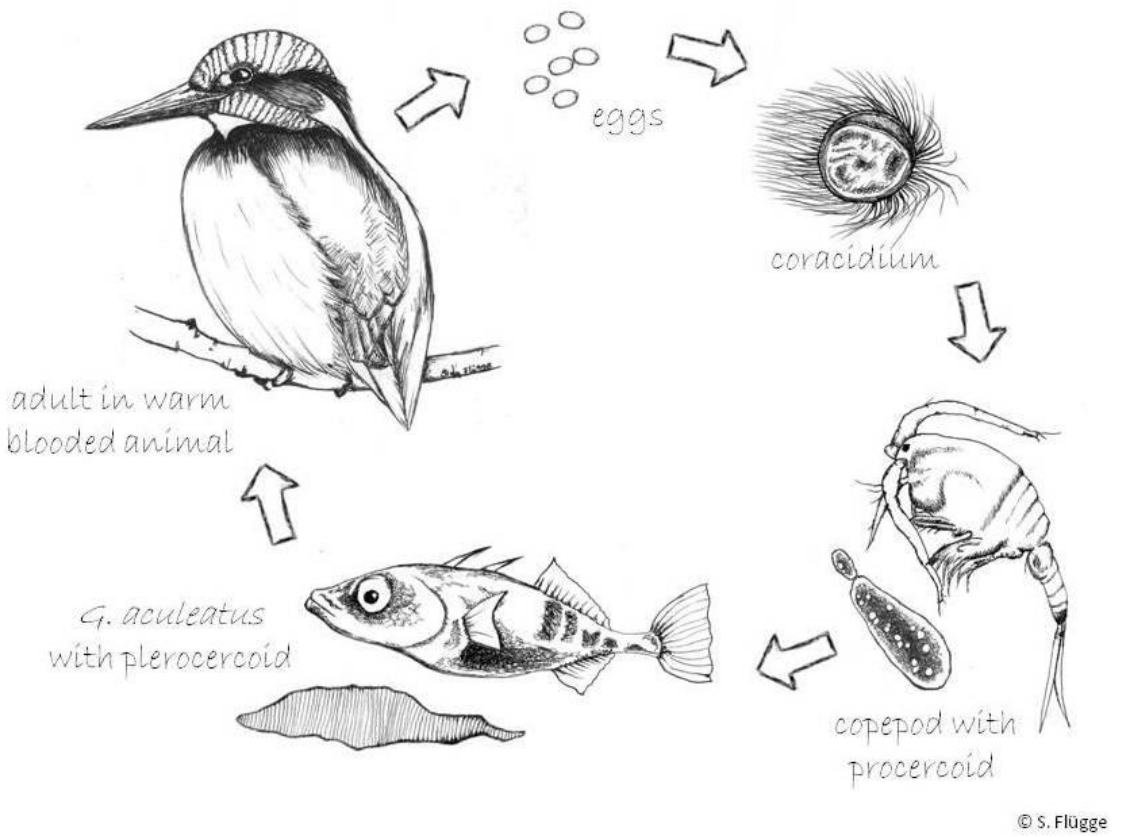



*Fig. 1: The life cycle of S. solidus. The cestode S. solidus is trophically transmitted and has cyclopoid copepods as the first host and the three-spined stickleback (G. aculeatus) as the obligatory and specific second intermediate host. After penetration of the stickleback gut, S. solidus develops in the body cavity. The final host can be any warm-blooded vertebrate, but is most often a fish-eating bird. S. solidus infests the gut of the final host, where it reproduces sexually, either by selfing or outcrossing.*

The specificity of this host parasite pair coincides with a very high level of adaptation. The tapeworm is capable of prominent immune manipulation of its stickleback host, to protect itself from attack by the stickleback's immune system (Barber & Scharsack 2010). Immune manipulation is facilitated by excretory products that were released by the parasite (Scharsack et al. 2013).*In vitro* cell cultures of stickleback leukocytes are an important tool to study cellular immune responses to parasite's excretory products. Although cellular responses of stickleback leukocytes to parasite excretory products in the *in vitro* system were obvious (Scharsack et al. 2013), the exact substances that are excreted by the parasite to manipulate the stickleback's immune system are still unknown.

A first step, to characterise those immune manipulating substances, is to obtain information about their molecular sizes. In a previous study, excretory products of *S. solidus* were collected by incubating parasites in a salt buffer (PBS). The excretory products in the so called parasite conditioned PBS were separated by molecular filtration into different molecular size fractions. The size fractionated excretory products were used to stimulate stickleback leucocytes in the *in vitro* cell culture system. Interestingly only the fraction <10 kD (s3 in Fig. 2) reduced the respiratory burst of cultured stickleback leukocytes as prominent as the not fractionated buffer.
A consequent next step to get information on the parasite derived substances is to analyse conditions under which the activity might be abrogated. One possibility would be to test if the substances are susceptible to heat. If this would be the case one could assume that the substance is still relatively large and complex with a distinct biological function, such as hormones or immuno mediators.

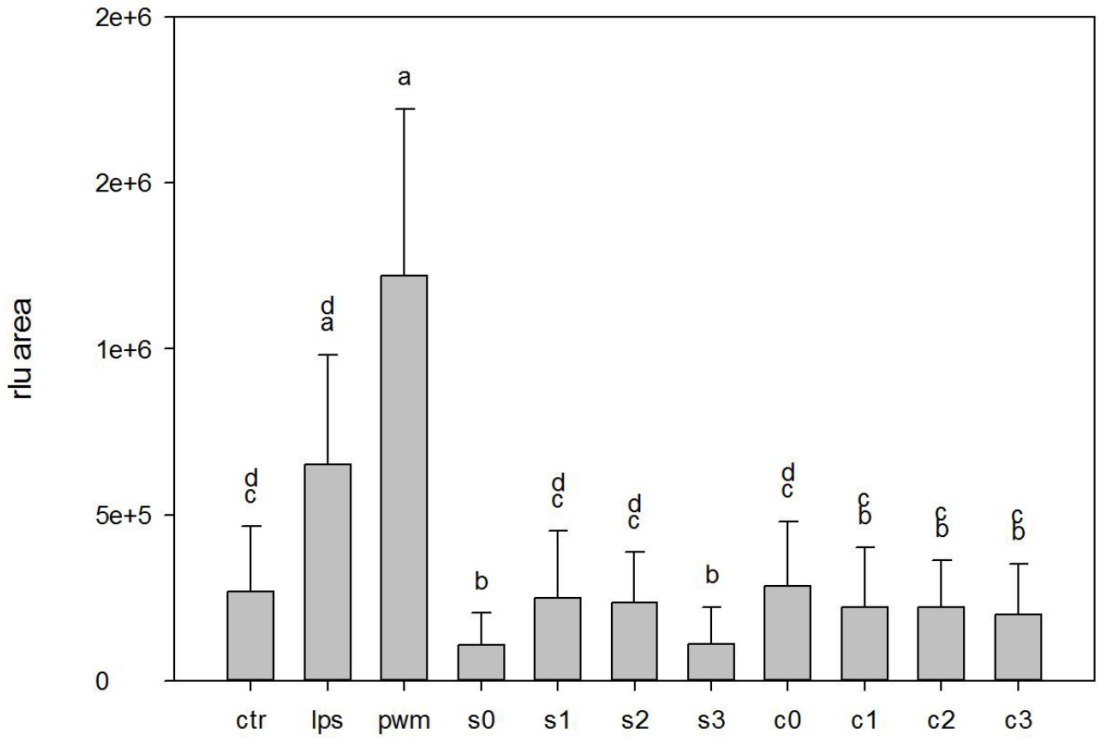



*Fig. 2: Respiratory burst of stickleback leukocytes to size fractionated S. solidus conditioned buffer. After 4 days of cultivation with medium alone (ctr) lipopolysaccharide (lps), poke weed mitogen (pwm), not fractionated (s0) and fractionated (s1 >100 kD, s2 10-100 kD, s3 <10 kD) S. solidus conditioned buffer and corresponding controls with buffer only (c0-c3), the respiratory burst activity (rlu area) was analyzed with a chemiluminescence assay. Different letters abide bars depict significant differences.*

To investigate this idea, heat treated and not heat treated parasite derived substances could be tested on cell cultures with stickleback head kidney leukocytes. After exposure to parasite excretory product samples, stickleback leukocyte cultures will be analysed by means of flow cytometry (FACS, cell viability, morphology) and a luminescence based assay to measure respiratory burst activity will be conducted.

Main goal: Test if the activity of *S. solidus* excretory products is heat labile.

Literature:

Barber I, Scharsack JP (2010) The three-spined stickleback - *Schistocephalus solidus* system: an experimental model for investigating host-parasite interactions in fish. Parasitology, 137: 411-424

Scharsack JP, Gossens A, Franke F, Kurtz J (2013) Excretory products of the cestode, Schistocephalus solidus, modulate in vitro responses of leukocytes from its specific host, the three-spined stickleback (Gasterosteus aculeatus). Fish & Shellfish Immunology, 35: 1779-87

# 6 Protocol collection

## 6.1 *Tribolium castaneum* protocols

### 6.1.1 Tribolium life stages

| Temp. (°C) | egg (d) | hatched (%) | larva (d) | % larval mortality | pupa (d) | total dev. time (d) |
|---|---|---|---|---|---|---|
| 40.0 | 2.7 | 86 | 23.6 | 65 | 4.4 | 31 |
| 37.5 | 2.6 | 77 | 13.7 | 13 | 3.9 | 20 |
| 35.0 | 2.7 | 92 | 12.9 | 4 | 4.5 | 21 |
| 32.5 | 2.9 | 75 | 14.6 | 7 | 4.6 | 22 |
| **30.0** | **3.6** | **88** | **17.2** | **3** | **5.5** | **27** |
| 27.5 | 4.7 | 84 | 24.3 | 13 | 7.5 | 37 |
| 25.0 | 6.8 | 87 | 31.2 | 4 | 10.2 | 48 |
| 22.5 | 9.3 | 88 | 51.0 | 17 | 13.4 | 74 |
| 20.0 | 13.9 | 77 | 109.3 | 60 | 24.4 | 148 |
| 17.5 | ... | 0 | ... | | | |

Larval instars:
- 5-9 larval instars, depending on growth conditions
- Normal conditions: 6 instars

Reproductive onset:
females: - egg production at 25°C: 7d (29°C: 4d) after eclosion (they have to feed on full grain flour before they start laying eggs)
- fertilization immediately after eclosion
males: - mature at 2nd day (29°C)

Fertility:
- single females lay up to 20 eggs/day (29C)
- average egg rate 25°C: 7 eggs in 3 days over 100d, then decline during the next 100 d; high yield only during the first three months
- males may be fertile up to 1 year - number of offspring in a vial depends more on the amount of flour than on the number of females.

Life expectancy:
up to 3 years, average: ca. 200 d
Mean generation time in population conditions: 55d Zone of inhibition for *Tribolium castaneum & Tenebrio molitor*

### 6.1.2 How to work sterile on the bench

1. All media, including plates, liquid media and agar must be autoclaved immediately after its preparation. It is best to prepare media in several small bottles, so you can

open only one at a time. Check the bottle for contamination before you use it by gently swirling it and looking for cloudy material in the center. Always incubate a small amount of broth alone when growing cells overnight as a negative control. Small amount of contamination is not always evident until the media is incubated at 30 °C.

2. Flame inoculating loops and the lips of media bottles before and after pipetting from them. Never leave a media or agar bottle open on the bench and do not take an individually-wrapped pipette out of its protective wrapper until you are ready to use it (i.e. do not walk across the laboratory with an unwrapped pipet). Always use a fresh, sterile pipette or pipette tip when pipetting culture media, and never go back into a media bottle or cell culture with a used pipette.

If you follow this procedure you can always work sterile on the bench with a Bunsen burner and ethanol. If you are working under the hood (laminar flow) you don't need to follow this procedure. It is important to learn how to work sterile and not in the laminar flow, it might be that you will have limited access to the laminar flow in your next lab or even won't have one.

3. When working under the hood (laminar flow), be aware that the surface on which you are working is not sterile if it is not sterilized previously with UW light or cleaned with an antibiotic agent. The "flow" provides only the sterile atmosphere but all the things that are put under the hood remain unsterile and they should be handled adequately.

### 6.1.3 Bacteria overnight cultures in liquid medium

Overnight cultures should be grown only from a single colony on a fresh plate or from a previously-tested glycerol stock that was grown from a single colony. To prepare an overnight culture, from a glycerol stock scrap a small amount of ice from the surface of the culture with a sterile inoculating loop. Sufficient numbers of bacteria are present in the ice so the culture can grow to saturation in 16 hours. Never let the glycerol stock thaw and always make more aliquots.

2. Touch a single colony with the end of an applicator stick and hold it in your primary hand (right hand if right-handed).

3. While holding the culture tube in your other hand, remove the cap of the Falcon tube. Dip the inoculated end of the applicator into the sterile medium and, if necessary, wipe the end onto the side of the tube.

4. Briefly flame the end of the culture tube and replace the cap.

5. Always inoculate a dummy tube with no cells to verify that the medium, tubes, and sticks were sterile. This is your negative control.

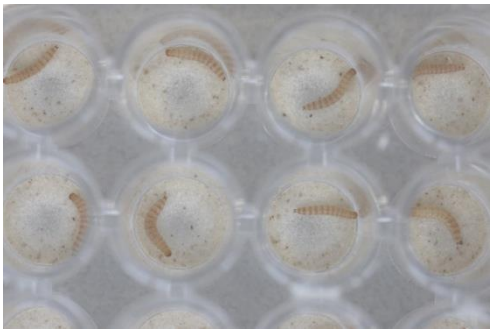
6. Grow culture for 12 to 18 hours. After 24-30 hours, cells have entered stationary phase and should not be used for critical procedures.

### 6.1.4 Spore production protocol for Bacillus thuringiensis for infection

Vegetative cells and spores are cultured at 30 °C.

Plate bacteria from a glycerol stock as described above (aliquots stored at – 80 °C) on and grown them over night (use LB agar plates). Do this freshly before each infection to prevent loss of pathogenicity on the LB agar plates. The following day, inoculate 5 mL of BT medium (0.75 % bacto peptone (Sigma), 0.1 % glucose, 0.34 % $KH_2PO_4$, 0.435 % $K_2HPO_4$) was inoculated with one bacterial colony with the addition of 25 µL of salt solution (2.46 % $MgSO_4$, 0.04 % $MnSO_4$, 0.28 % $ZnSO_4$, and 0.40 % $FeSO_4$) and 6.25 µL of 1M $CaCl_2$ x $2H_2O$ and incubate overnight on a bacterial shaker at 30 °C, 200 rpm. The following day, add the resulting bacteria suspension, 5 mL of salt solution and 250 µL of 1M $CaCl_2$ x $2H_2O$ to 1 L of BT medium, and incubate for a total of seven days in darkness (spores and their toxins are light sensitive!). On day four, add another 5 mL of salt solution and 250 µL of 1M $CaCl_2$ x $2H_2O$ to the suspension. After 7 days centrifuge the suspension at 4000 rpm for 15 minutes at 4 °C, wash once in phosphate buffered saline (PBS) and then resuspend in PBS. Count the spores with Thoma counting chamber. Store such spore preparations together with their crystals (spore-crystal preparations) for a maximum of three days at room temperature and protected from light before you use them in experiments.

### 6.1.5 Oral infection with Bt spores



Adjust the desired spore concentrations by adding PBS to the suspension, and 0.15g of heat-sterilized flour with yeast per ml of spore suspension. Pipette forty µl of the resulting liquid diet (using a multichannel pipette and reservoir) into each well of a 96 well plate in sterile conditions. The diet for the control insects is made in the same way but without the addition of the spores and should be handled separately. Place the open plates in plastic boxes (Tupperware), three in one box. Plug the holes in the container cap (3 cm diameter) with foam stoppers (4.2 cm diameter) to allow the air to circulate. Place the containers in a 50 °C oven overnight to allow the formation of dried spore discs. Once the spore suspension is mixed with flour it should only be used on that same day in order to prevent spore germination and bacteria growth in the media. The next day, place individual larvae on the well dried discs for the infection and seal with transparent adhesive tape. Puncture holes to allow the air to circulate. Place the plates were back into the plastic boxes and keep them in the incubator (at 30 °C and 70 % humidity) during the infection time.

**For surface sterilisation, use only 4% Incidin Active (Ecolab) since in this concentration it is active against the spores!!**

## 6.2 *Drosophila melanogatser* protocols

### 6.2.1 Bacteria culture

1. Remove bacteria from -80 freezer and defrost
2. Remove 5 LB agar plates from refrigerator, bring to room temp and label appropriately (4 for bacteria and 1 control)
3. Prepare 70% EtOH in Falcon tube for flaming
4. Flame spreader and allow to cool
5. Dip spreader into the bacterial solution and streak a few times in one section of the plate. Flame the spreader again and allow to cool. Then move the plate through 90° and pass the spreader once through the bacteria and then continuously backwards and forwards down the plate. The idea is to get single large colonies growing.
6. Put plates upside down into the incubator 30 °C overnight
7. In the morning check you have distinct CFUs growing on the plates and examine the plates for possible contamination under the dissecting microscope
8. If the CFUs have already grown enough, store the plates upside down in the refrigerator until you need them in the evening
9. In the laminar flow, in the the evening, add 100 mL sterile LB media to each Erlenmeyer flask
10. Use the bacteria spreader to take CFUs from the plate and add them to the LB media (4 CFUs, 1 from each plate). Grow at 30 degrees overnight for L. lactis and E. coli; 25 C, 9.5 hours for Bacillus thuringiensis.
11. Remember to use one flask or a bacteria growing tube, as a negative control with only LB-media in it

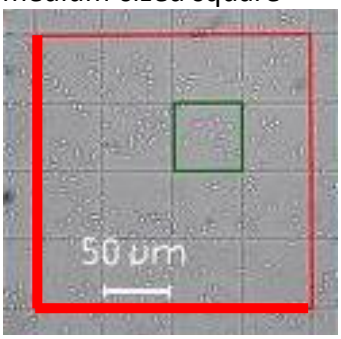
### 6.2.2 Preparing live bacteria

1. The following morning, check the cultures are not contaminated by putting a drop on a slide and checking under microscope
2. Put the bacteria solution into large falcon tubes (50 mL) and centrifuge the bacteria to form a pellet (4000 rpm, 10 mins, 4 ºC)
3. Pour away the supernatant into a waste bottle
4. Wash the bacteria two times in *Drosophila* Ringer's solution (4000 rpm, 10 mins 4 ºC), vortexing thoroughly to remove any lumps in between centrifuge steps
5. Resuspend bacteria in a small amount of Ringer's solution after the final wash (~1.5 - 2 mL for 3 flasks L. lactis)

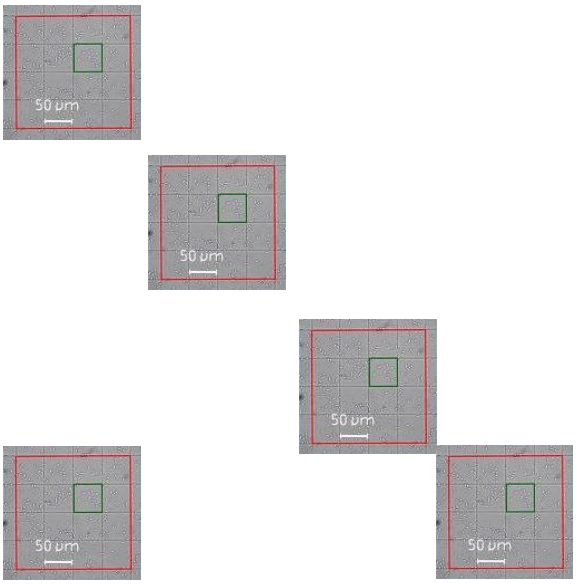
### 6.2.3 Counting the bacteria

1. Label eppendorf tubes. For each bacteria solution, 2 tubes with 1:100, and 2 tubes with 1:1000
2. Vortex the bacteria briefly
3. Calculate the concentration of the bacteria – final concentration required depends upon the bacteria and experiment. Resuspend bacteria thoroughly before pipetting

4. Make two dilutions of the bacteria in Ringer solution:
   2 x 1:100 (add 10 µL bacteria solution to 990 µL *Drosophila* Ringer's solution)
   2 x 1:1000 (take 100 µL of 1:100 dilution and add to 900 µL)
5. To count, add 3 µL of bacterial solution (start with 1:1000, this should usually be around the correct concentration) to each side of the haemocytometer (Thoma, 0.02mm deep, 0.0025 $mm^2$)
6. Under a microscope (x 40 mag) count number of bacteria in 5 medium-sized squares (shown in red below) on each side of the haemocytometer, to gain a total of 10 counts. Each medium-sized square is made up from 16 of the smallest squares (shown in green). Count the bacteria crossing the left and bottom of the red square (shown by a thick red line). Count the four medium-sized squares in a diagonal from top left to bottom right, and then count the square in the bottom left
7. *Repeat for the second dilution so that you can take the mean concentration*
8. You will get a fairly good estimate if you aim for around 20 – 50 cells per medium-sized square




9. Count the four medium-sized squares in a diagonal from top left to bottom right, and then count the square in the bottom left:



10. Calculation from Barabara Hasert to determine the number of bacteria per mL:

Area of small square: 0.0025 $mm^2$
Medium-sized square = 16 small squares
Area of medium-sized squares: 0.04 $mm^2$
Area of 10 medium-sized squares: 0.4 $mm^2$
Depth 0.02 mm
Volume of 1 medium square: 0.0008 $mm^3$
Volume of 10 medium squares: 0.008 $mm^3$

Concentration is calculated per mL

Calculating concentration from different dilutions of bacterial suspensions:

| Dilution | Mult. factor 1 medium square | Mult. factor for 10 medium squares |
|---|---|---|
| 1:1 | $12.5 \times 10^5$ | $12.5 \times 10^4$ |
| 1:10 | $12.5 \times 10^6$ | $12.5 \times 10^5$ |
| 1:100 | $12.5 \times 10^7$ | $12.5 \times 10^6$ |
| 1:1000 | $12.5 \times 10^8$ | $12.5 \times 10^7$ |

11. To determine how much bacteria you need per mL of solution:

E.g., you count 2 x 1:1000 dilution and on average there were a total of 33 bacteria per 1 medium square

$33 \times (12.5 \times 10^8) = 4.125 \times 10^{10}$

If we want $1 \times 10^{10}$ final concentration, then use the following formula (where $C_s$ = Conc. of stock, $V_s$ = Volume of stock, $C_f$ = Conc. of final (diluted), $V_f$ = Volume of final), to figure out the required volume of bacteria in 1 mL

$C_s \times V_s = C_f \times V_f$

$(4.125 \times 10^{10}$ bacteria/mL) $\times V_s = (1 \times 10^{10}) \times (1$ mL)

Rearrange to:

$$V_s = \frac{C_f \times V_f}{C_s}$$

Therefore:

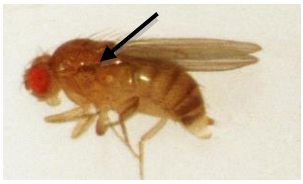
$$V_s = \frac{(1 \times 10^{10}) \times (1 \text{ mL})}{(4.125 \times 10^{10} \text{ bacteria/mL})}$$

$$= 0.242 \text{ mL stock per mL}$$

12. Resuspend the stock in *Drosophila* Ringer's solution

### 6.2.4 Fly infections

1. Dilute bacteria solution to desired concentration and place on ice.
2. Fill glass capillaries with mineral oil for injections. This can be done ahead of time.
3. Anesthetize individual flies and inject 18.4 nl bacteria solution into the thorax.



5. After injections, place flies into individual vials (1 fly per vial) and record time.
6. Repeat for all treatments and blocks

### 6.2.5 Mating assay

1. Set up X single vials (vials will be prepared the day before with single ♂ per vial on wet yeast)
2. Mouth pipette single ☿ into vial with ♂**.** Watch for mating. Record time mating begins and ends on vial. Discard any pairs that mate for less than 5 minutes. Give flies 2 hours to mate.
3. After termination of mating, remove ♂ from vial and discard.
4. Place single females at 25 C O/N until you are ready to infect the next day

### 6.2.6 Egg counts

1. Count total eggs laid in each vial every X hours for X hours. Use a counter to keep track of total eggs.
2. Record number of eggs laid per vial.
3. Transfer flies to fresh vial with wet yeast and place at 25 C.

### 6.2.7 Whole fly homogenates

1. Prepare eppendorf tubes and LB plates (number plates tbd). Make sure you prepare enough plates/ eppendorf tubes for serial dilutions (1:1, 1:10, 1:100, 1:1000) for *L. lactis* and 1:1 and 1:10 for *E. coli.*
2. Under the laminar flow hood, place 1 bead per 1:1 dilution in eppendorf tube with sterile, flamed forceps. Flame forceps before picking up each bead!!
3. Pipette 200 ul sterile LB broth into tubes for 1:1 dilutions, 450 ul LB into 1:10 dilutions, 990 ul LB for 1:100 dilutions and 900 ul LB into 1:1000 dilutions
4. Wash each fly by dipping into 1000 ul 70% EtOH, followed by 1000 ul dd$H_2O$. Blot fly on tissue. Transfer single flies into 200 ul aliquots (1:1) and place on ice. Work in groups of 10-20.
5. Homogenize for 45 seconds, frequency=20
6. Spin down homogenate and place on ice

## 6.3 *Gasterosteus aculeatus* protocols

### 6.3.1 Primary culture of stickleback head kidney leukocytes

#### 6.3.1.1 Buffers and Solutions:

Flow: cytometer fluid (BD FACSFlow Sheath Fluid)
Beads: green fluorescent latex beads in Flow (polyscience, 30000 in 25 µl)
PI: Propidium iodide in Flow (10 µg $ml^{-1}$)

All Buffers, media and solutions for washing and cultivation have to be sterile and refrigerated!

Mitogens: LPS = bacterial lipopolysaccharide
PWM = pokeweed mitogen; lectin
*Schistocephalus solidus* conditioned PBS

L-90 without heparin for washing cells:
L-15 medium (Leibovitz medium) with 10% v/v Millipore $H_2O$ and 10 mM HEPES (add 50 ml sterile Milli-Q water and 5,5 ml sterile 1 M HEPES solution to a new 500 ml L-15 flask)

L-90 with heparin (17 ml) for preparation of cells suspension:
L-90 with heparin in a concentration of 20 U $ml^{-1}$ (add 68 µl heparin (5000 U $ml^{-1}$) to 17 ml L-90).

Culture medium (20 ml) for cultivation of cells:

| | |
|---|---|
| 18 ml | L-90 |
| 1 ml | FCS (fetal calf serum; 5% v/v final conc.) |
| 0,2 ml | Pen/Strep (Penicillin-Streptomycin mix with 10000 U $ml^{-1}$ each) |
| 0,4 ml | carp serum (1:1 in PBS 90 diluted; 1% v/v final conc.) |
| 0,4 ml | L-Glutamin (200 mM; 4 mM final conc.) |

#### 6.3.1.2 Materials:

- “Clean” ice box which can be used under the laminar flow
- Cell strainers (BD Falcon, 40 µm mesh size, Nylon)
- Small Petri dishes (35 × 10 mm)
- sterile plunger
- Petri dishes for fish dissection (60 × 15 or 92 × 16 mm)
- Dissecting instruments (shear and tweezers)
- A bottle of 96 % Ethanol for instrument disinfection
- Bunsen burner or any other fire source for singeing the instruments
- Sterile deep-well plate (2,5 ml) for cell suspensions with a lid
- 50 ml Falcon tubes for the media
- 0,5 and 1,5 ml Eppendorf cups

- sterile pipette tips and clean pipettes (10, 200, 1000 µl tips; 10, 100, 200 and 1000 µl pipette, Eppendorf Multipette with sterile 2,5 ml Combitips)
- 96 well measurement-plate for the FACS
- 96 well half area, flat bottom plate

6.3.1.3 Equipment:

- BD FACS CantoII™
- Coolable $CO_2$ incubator
- Coolable centrifuge for 96 well plates
- Vortex mixer
- Multipette
- Laminar flow

6.3.1.4 The procedure:

Preparations:

- Prepare and decant all media; store refrigerated
- Prepare mitogen work solutions with culture medium:
    - In 100 µl cultures the mitogen work solutions have to be the fourfold of the final concentration

| mitogen | final concentration ($\mu g\ ml^{-1}$) | concentration of work solution ($\mu g\ ml^{-1}$) |
|---|---|---|
| LPS | 20 | 80 |
| PWM | 2 | 8 |
| *S. solidus* PBS | fourfold of work solution | N/A |

- Fill the ice box with fresh ice.
- Place the ice box under the laminar flow. Number the small Petri dishes. For each fish put one cell strainer on a small Petri dish and put the lid on. Avoid touching the sieve or the inside of the Petri dishes!
- Place the dishes with the strainers on ice and add 1 ml of ice cold L-90 with heparin.
- Prepare the dissection instruments by cleaning them carefully with 70 % Ethanol and singeing them with 96 % Ethanol. Place them on a metal rack.
- Check the binocular and prepare some Petri dishes for the dissection.
- Place the ice box with the prepared strainers near the binocular. Before getting it out of the bench, make sure all dishes are covered and all media are closed.
- If everything is prepared, get the fish in a big bucket with enough water (aquarium water).

6.3.1.5 Dissection:

- Start the dissection immediately to avoid stressing the fish.
- Cut of the head of the fish and collect the head kidneys carefully (singe the instruments before dissection and before touching the head kidneys again, but let them cool and make sure all Ethanol was removed!).
- Place the kidneys on the prepared strainer (keep the tissue on ice and in medium all the time).
- After dissection of all fish go on with work under the laminar flow again and force the organ through the strainer with a sterile plunger.
    - Take the Petri dish from the ice; lift up the strainer a bit to avoid crushing of cells on the bottom of the Petri dish and force the cells through the mesh by extensive rubbing. Some interstitial tissue will not pass the mesh, but it is not needed and grinding should be stopped when the red/orange head kidney tissue has passed the mesh. Give 1 ml L-90 with heparin over the strainer to wash the remaining cells from the sieve and collect the drop on the bottom side of it.
- Transfer the 2 ml cell suspension to the sterile deep-well plate on ice.
- Wash the cell suspension:
    - Centrifuge for 5 min at 600 × g and 4 °C.
    - Hit out the supernatant by a strong turn of the wrist into a sink. Do it fast and strong, don't be too careful, otherwise you will loose the cells.
    - Add 1 ml L-90 (without heparin!), resuspend the cells completely (by pipetting up and down) and centrifuge again.
    - Hit out the supernatant again, centrifuge for some seconds to bring the remaining droplets from the sides to the ground of the wells and fill up to 500 µl (add 450 µl) with culture medium. Resuspend the cells.
- During centrifugation start the FACS, connect the High Throughput Sampler, run the "Prime after Tank Refill…", the "Bubble Filter Purge and Degas Flow Cell" and the "Fluidics Startup" (see description at the cytometer) and use a "washing-plate" to wash the FACS with Milli-Q water. Add 250 µl per well of Milli-Q water to 6 wells of the "washing-plate", mark the wells you have used and click "Run Wells".

Measurement of cell number by flow cytometry:

- Prepare the measurement-plate for the FACS inclusive three beads controls on ice
    - 102,5 µl Flow
      30 µl (10 µg $ml^{-1}$) PI

      12,5 µl beads
    - For beads control add 5 µl Flow
    - Add 5 µl cell suspension under sterile condition to all other wells.

- Measure the plate with Diva protocol "stickl head kidney cell count"
    - Open Experiment - new experiment: stickl head kidney cell count
    - Mark the wells you need
    - Start measuring with the beads control

- Create an excel sheet of the data by batch analyses and calculate cell numbers and how much medium has to be added/removed to each tube to adjust a cell concentration of $4\times10^6$ cells $ml^{-1}$:
    1. Cells in 495 µl culture medium: (Events vital cells*30.000/Events beads)*99
    2. ml with $4\times10^6$ cells $ml^{-1}$: cells in 495 µl/$4\times10^6$
    3. plus/minus medium: (2.)-0,495
- Wash the FACS using 6 wells filled with Milli-Q water and run the "Fluidic Shutdown" when you have finished your work on the FACS.
- Adjust cell suspensions to $4\times10^6$ cells $ml^{-1}$ by adding/removing the calculated volume of culture medium.
    - If medium has to be removed, spin cells down (5 min, 600 × g, 4 °C), remove the calculated volume of medium and resuspend the cells again (all wells).

Preparation of the cultures:

- Clean and put under the laminar flow all materials and media you need
    - Culture plates, pipette tips, pipettes, mitogen solutions (on ice), culture medium (on ice), combitips and multi pipette, cell suspensions (on ice).
    - Prepare a sheet with your pipetting scheme
- Pipette in each well:
    1. 50 µl culture medium
    2. 25 µl mitogen solution / culture medium for negative control
    3. 25 µl cell suspension (100.000 cells)
- Incubate the plates in a plastic box with wet tissues at 20 °C, 3 % $CO_2$ for 4 days.

### 6.3.2 *S. solidus* conditioned PBS

#### 6.3.2.1 Materials:

- PBS (autoclaved!)
- 70 % Ethanol
- "Clean" ice box which can be used under the laminar flow
- Small Petri dishes (35 × 10 mm)
- Dissecting instruments (shear and tweezers)
- A bottle of 99 % Ethanol for instrument disinfection
- Bunsen burner or any other fire source for singeing the instruments
- Sterile 24 well flat bottom plate with lid
- Sterile 96 well half area flat bottom plate with lid
- sterile 1.5 ml Eppendorf cups
- sterile pipette tips and clean pipettes (1000 µl, 100 µl)
- Analytical balance
- Laminar flow
- Coolable $CO_2$ incubator
- Inverse microscope

The procedure:

- Start the dissection immediately after getting the fish to avoid stressing them.

- Label an empty Petri dish (fish and worm no.), put it on the balance and tare the scale to zero.
- Cut of the head of the fish, open the abdominal cavity and transfer a single worm to the Petri dish (singe the instruments before dissection and before touching the worm again, but let them cool and make sure all Ethanol was removed!). Note the weight of the worm and place the Petri dish with the worm on ice. Repeat the procedure if you find more than one worm in the fish.
- After dissection of some fish (the worms should not kept too long on ice) go on with work under the laminar flow.
- Add 1 ml sterile PBS to the worm and shake the Petri dish a little bit to remove left fish-tissue or -blood from the worm.
- Take the worm with a singed tweezer, strip off left PBS from the worm into the inside of the lit of the Petri dish and transfer the worm to the sterile 24 well plate.
- Add PBS to the worm in the 24 well plate (10 µl for 1 mg worm).
- Incubate the plate with lid in a plastic box with wet tissues at 20 °C, 3 % $CO_2$ for 24 h.
- After incubation go on with work under the laminar flow again.
- Transfer the worm conditioned PBS in a labelled, sterile 1.5 ml Eppendorf cup and freeze at -80 °C.
- Fix the worm in an Eppendorf cup with 70 % Ethanol. Worm can be stored at RT.

### 6.3.3 Chemoluminescence-Test

Prepare a luminescence plate (white 96 well flat bottom) at room temperature (do not put on ice), add 20 µl of lucigenin to each well of a white luminescence plate, followed by 80 µl of R-90. Use a multichannel pipette to resuspend cells and to transfer 80 µl of cell suspension to each well of the lucigenin plate.

Incubate plate for 30 min in the $CO_2$ incubator at 20 °C (or at RT) in darkness.

Start luminescence reader, computer and program. Adjust measuring time to 3 h. Adjust reader temperature to 20 °C.

In the meanwhile, from each well of the deep-well plate, transfer 200 µl (of remaining cell suspension) to a new deep well plate prepared with 800 µl ice cold ethanol per well. Pipette up and down after transfer for a good fixation of cells. Cover the plate with sealing foil and store at 4 °C until cell cycle measurement.

Continue with luminescense assay:
Add 20 µl of well mixed zymosan to each well at room temperature (do not put on ice). Place the plate into the reader immediately and start the measurement! Here: 3 h!