Drosophila Immune Challenge Experimental Protocol - Larvae

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Adapted from Xie et al. (2023)

I. BACKGROUND SIGNIFICANCE

a. The immune defence of *Drosophila* relies entirely on innate mechanisms. It begins with epithelial barriers, such as the gut and cuticle, which initiate localized immune responses to limit pathogen entry. Once infection occurs, complex internal processes are activated to prevent its spread. For example, melanization and the systemic humoral response led to the production of antimicrobial peptides that modulate signaling pathways countering infections caused by both Gram-negative and Gram-positive bacteria (Lemaitre & Hoffmann, 2007). Together, these pathways ensure an efficient and coordinated defense that protects the organism from a wide range of microbial threats. With the coordinated responses to eliminate pathogens and restore homeostasis, recent evidence shows that the deletion of *nlg3* reprograms the brain transcriptome towards innate immunity by regulating signaling processes that enhance bacteria resistance (Xie et al., 2023).

II. EXPERIMENTAL SIGNIFICANCE

a. It has been confirmed that systemic humoral responses producing antimicrobial peptides (AMPs) are regulated through two major signaling pathways: the IMD pathway, which responds primarily to Gram-negative bacteria, and the Toll pathway, which targets Gram-positive bacteria. When assessing the functionality of *nlg3* knockout in *Drosophila* larvae, these systems appear to play a critical role in survival following *E. coli* infection. A reduction in bacteria growth further supports the presence of a more active and robust innate immune response. These findings emphasize the potential regulatory role of *nlg3* in modulating immune signaling pathways, providing valuable insight into how genetic factors can strengthen host defense mechanisms against bacterial infection.

III. MATERIALS

a. Frozen Stock culture of Bacteria Pellet Gram Negative Bacteria (*Escherichia coli, Erwinia carotovora carotovora 15, Pseudomonas entomopila)*

b. Frozen Stock Culture of Gram-Positive Bacteria (*Micrococcus luteus OR Staphylococcus aureus)*

c. Single ripe banana

d. Bunsen Burner

e. Matches

f. 100 Drosophila Larvae (3rd Instar) (enough per agar plate)

g. 1.5ml sterile Eppendorf Tubes (Microcentrifuge tube)

h. Vial with pre-made food

i. Foam lids for vials
j. 70% Ethanol
k. Luria Agar (LA)
l. Luria-Bertani (LB) broth
m. Glass test tubes and rubber stoppers
n. Paintbrush (an object that could spread larvae onto agar)
o. Wooden stick applicator OR inoculating loop OR glass spreader
p. P1000 pipette
q. 1X PBS (Phosphate Buffer Saline)

IV. PROCEDURE

a. Bacteria Culture for *Eschterchia coli*

i. Sterilize all glassware, tubes, and relevant materials in the Autoclave
ii. Sterilize work bench by wiping it down with ethanol
iii. Turn on the Bunsen Burner and light it for a flame to maintain an aseptic environment during culture
iv. Use sterile wooden stick applicator or a sterilized steel loop to remove a chunk of bacteria from frozen stock culture vial
v. Streak a small ice chunk onto an LB agar plate
vi. Incubate the plate overnight at 37 degrees Celsius for approx. 14-18 hours.
vii. Pour an ~5ml aliquot of LB broth into sterile glass test tubes (or fill tubes to or fill tubes to the top, limit air from entering or escaping)
viii. With a sterile inoculating loop, pick a well isolated bacteria colony from growth plate and inoculate the glass culture
ix. Resuspend the colony in the liquid medium using a P1000 pipette
x. Cap the test tube and incubate at 37 degrees Celsius for another 14-18 hours

b. Oral Bacterial Infection for Larvae

i. Take out culture from incubator and dump broth from tube
ii. Wash pellets with 1X PBS by resuspending using pipette
iii. Transfer into sterile Eppendorf tube
iv. Put into centrifuge for 5 minutes at 5000 RPM
v. Remove supernatant from the top of the bacteria pellet
vi. Repeat Steps (ii - v) if necessary
vii. Crush 400ul of bananas into a new Eppendorf tube and combine 200ul of bacteria pellet into crushed bananas
viii. Immerse Third Instar Larvae into the bacteria mixture
ix. Shake gently to allow bacterial contact and ingestion
x. Insert foam into the tube to ensure that the larvae don't move from mixture but have air supply
xi. For the **initial infection period,** place tubes at room temperature for 45 mins
xii. For **second incubation,** transfer larvae and bacteria into a vial with standard food

xiii. Incubate vial at for 24 hrs at 29 degrees
xiv. After 24-hr incubation, rinse larvae in 500 µL water/1X PBS, then in 70% ethanol (3 times for 5 seconds) for external sterilization
xv. Dry larvae

c. Analyze Immune Response

i. Spread sterilized larvae onto the LA plate using a paintbrush. This will assess the bacteria's growth remaining inside the larvae and incubate for at 25 degrees Celsius.

Drosophila Immune Challenge Experimental Protocol - Adults

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Adapted from Neyen et al. (2014)

I. MATERIALS

a. Frozen Stock culture of Bacteria Pellet Gram Negative Bacteria (*Escherichia coli, Erwinia carotovora carotovora 15, Pseudomonas entomopila) or* Frozen Stock Culture of Gram-Positive Bacteria (*Micrococcus luteus OR Staphylococcus aureus)*
b. ~20-30 2–4-day old adult flies per vial (same sex and genotype per group)
c. Bunsen Burner
d. Matches
e. 22mm Whatman paper disk
f. Vials with pre-made standard fly food (prepared without live yeast on top)
g. Empty vials (no food)
h. Foam lids.plugs for vials
i. 70% Ethanol
j. Control solution (10% sucrose)
k. Luria-Bertani (LB) broth
l. Wooden stick applicator OR inoculating loop OR glass spreader
m. P1000 pipette
n. 1X PBS (Phosphate Buffer Saline)

II. PROCEDURE

a. Bacteria Culture

ii. Sterilize all glassware, tubes, and relevant materials in the Autoclave
iii. Sterilize work bench by wiping it down with ethanol
iv. Turn on the Bunsen Burner and light it for a flame to maintain an aseptic environment during culture
v. Use sterile wooden stick applicator or a sterilized steel loop to remove a chunk of bacteria from frozen stock culture vial
vi. Streak a small ice chunk onto an LB agar plate
vii. Incubate the plate overnight at 37 degrees Celsius for approx. 14-18 hours.
viii. Pour an ~5ml aliquot of LB broth into sterile glass test tubes (or fill tubes to or fill tubes to the top, limit air from entering or escaping)
ix. With a sterile inoculating loop, pick a well isolated bacteria colony from growth plate and inoculate the glass culture
x. Resuspend the colony in the liquid medium using a P1000 pipette

xi. Cap the test tube and incubate at 37 degrees Celsius for another 14-18 hours

b. Oral Bacterial Infection

i. Dehydrate and starve 2–4-day old adults flies by placing them into empty vials with no food and incubating them 2-3 hours at 29 degrees Celsius before exposure to bacteria to ensure synchronous feeding

ii. While flies starve, prepare infection vials consisting of a standard fly food

iii. Place a 22mm Whatman paper disk on top of the food so it completely covers the surface

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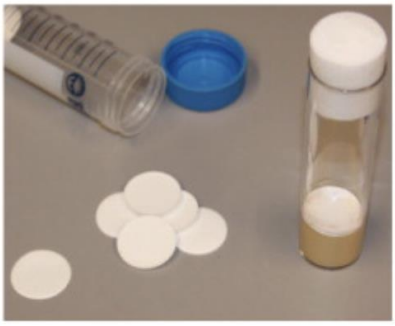

iv.

v. Soak the Whatman paper disk by pipetting 120-150 ul control solution (10% sucrose) or bacterial pellet mixed with control solution.

vi. Ensure the disk is evenly soaked but not flooded.

vii. Flip the starved adult flies into the prepared infection vials

viii. Plug with foam lids and keep vials at the optimal temperature for pathogen growth (refer to Table 1 for commonly used pathogens and conditions)

ix. *Optional Step* – After the initial infection phase (24 hrs.), transfer infected flies into a fresh vial with standard fly food. However, evidence suggests bacteria on the filter disk do not remain viable beyond a couple of hours.

c. Analyze Immune Response (Survivorship)

i. Transfer the infected or control flies from infection vials into standard vials.

ii. Count the number of living or dead flies in each vial every day or as often as required.

iii. Transfer the flies to new vials every 5 days to keep them from getting stuck in food.

iv. Calculate survival over time

Neyen et al. (2014)

Table 1. Commonly used pathogens.

Bacterium	**Gram**	**Culture conditions**	**Dose, route and temperature of infection**
Escherichia coli	Negative, DAP-type	LB, 37 °C	OD_{600} = 400 (S), OD_{600} = 200 (O), 29 °C
Erwinia carotovora carotovora 15	Negative, DAP-type	LB, 29 °C	OD_{600} = 200 (S), OD_{600} = 100 (O), 25–29 °C
Pseudomonas entomophila	Negative, DAP-type	LB, 29 °C	OD_{600} = 200 (O), 29 °C

Note that lower doses should be used when microbes are micro-injected. Infectious doses are indicated as a range; lower concentrations are useful for sublethal challenges. Abbreviations: S, systemic infection; O, oral infection; YPG, yeast peptone glucose; LB, Luria Bertani; BHI, Brain–Heart-Infusion.

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