Journal of Insect Physiology 58 (2012) 342–347

Contents lists available at SciVerse ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

Ontogenetic changes in immunity and susceptibility to fungal infection in Mormon crickets *Anabrus simplex*

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ARTICLE INFO

Article history:
Received 17 October 2011
Received in revised form 8 December 2011
Accepted 12 December 2011
Available online 21 December 2011

Keywords:
Orthoptera
Nymph
Life-history
Trade-off
Microbial control
Defense

ABSTRACT

Insects have innate immunity that may be weakened by resource allocation to growth. I measured enzymatic immunity, encapsulation response, and susceptibility to fungal infection in Mormon crickets of known age. Although the concentrations of circulating spontaneous and total phenoloxidase (PO) increased with age from the most recent molt in late instar nymphs (5th, 6th, and 7th) and 0–5 day old adults, mean values did not differ between stadia, indicating that circulating PO titers are knocked back with each molt. In contrast, encapsulation rate increased throughout nymphal development and adult maturation. No longer required to molt, adult PO titers increased steadily with age. Survivorship also increased with the age at which *Metarhizium acridum* fungus was applied to adults. I conclude that immunity relevant to defense against fungi continues to develop well into the adult stage. With each molt setting the insects back in circulating PO titers, very young adults are much like nymphs in enzymatic immunity.

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1. Introduction

As insects grow from hatchling to adult, their innate immunity develops like any other system (e.g., cockroach: Rheins and Karp, 1985). Although by definition always active, constitutive defenses must change as they develop over time. In many insects, protohemocytes are found in larval stages but not in adults suggesting specialization of hemocyte function with age. Some insects (dragonflies: Rolff, 2001; honeybees: Schmid et al., 2007) have hemocytes in one life stage but have fewer or lack them altogether in another. To make up for the lack of hemocytes, they increase humoral immune functions (Boman and Hultmark, 1987; Rahman et al., 2006), in part by increasing freely circulating phenoloxidase (PO) titers. In addition as exposure to disease and parasites will accumulate with time, the probability of developing induced immunities also increases with age. Induced immunity is often associated with delayed development which suggests that insect immunity compromises growth (Siva-Jothy et al., 2005; Zerofsky et al., 2005; Schmidt et al., 2008). Thus natural selection should act to optimize the timing and magnitude of immune system development relative to allocation of resources to growth and other life history traits that also affect fitness.

Generally PO is an inactive zymogen in the hemolymph (proPO), which is activated when the insect is wounded or infected (recently reviewed by Kanost and Gorman, 2008). PO catabolizes tyrosine to produce toxic quinones that polymerize to form melanin. In many insects, PO and proPO titers are associated with resistance to infections. The PO cascade can also be an important part of cellular encapsulation of foreign bodies, because melanization causes the cell mass to harden and suffocate the intruders (Kanost and Gorman, 2008).

In addition to its immune activities, PO is involved in cuticular tanning and thus utilized in molting of the exoskeleton between larval instars. In some insects different forms of PO are used (e.g., tobacco hornworm *Manduca*, Hiruma and RIddiford, 1993). Cuticular darkness has been shown to correlate with circulating PO titers and immunocompetence (e.g., flour beetles *Tenebrio*, Armitage and Siva-Jothy, 2005). However as a result of the dual functions of PO, its hormonally-mediated function during development might conflict with its immune function (e.g., Rolff and Siva-Jothy, 2002; Cotter et al., 2008).

In the field cricket, higher growth rate as measured by larger body size or faster development time is inversely proportional to immunocompetence (Rantala and Roff, 2005). There may also be trade-offs between reproduction and immunity. PO enzymatic activity and resistance to *Serratia* bacteria increased in teneral adult crickets but declined with maturity in males whereas immune defenses peaked later in mature females (Adamo et al., 2001). A previous study showed that spontaneously active PO and encapsulation were directly proportional to body mass in adult Mormon crickets *Anabrus simplex* (Srygley et al., 2009). If body mass increases with age, then these increases in immunocompetence could reflect ontogenetic changes in circulating PO and the ability to encapsulate foreign bodies.

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0022-1910/$ - see front matter Published by Elsevier Ltd.
doi:10.1016/j.jinsphys.2011.12.005

In this paper, I investigate changes in spontaneous and total PO titers and the rate of encapsulation of a foreign body in late instar nymphs and young, teneral adult Mormon crickets. I also investigate spontaneous and total PO titers and the ability to survive fungal infection from the adult molt to 18 days of age. Because of differences in future allocation to reproduction, I made two predictions concerning sexual differences in allocation to growth and immunity. First, reproductive output is directly proportional to body size in female insects. Thus in order to maximize body size, females should allocate less to immunity in nymphal stages than males. Second, reproductive output is directly proportional to longevity of females but not males that compete for females. Thus in order to maximize longevity, adult females should allocate more to immunity than males.

2. Methods

Mormon cricket nymphs were collected from a Northern Great Plains improved pasture site (45° 20′ 16″N, 107° 16′ 03″W, 1206 m) near Lodge Grass, Montana and transported to the laboratory facility in Sidney, Montana on the same day. Mormon crickets were isolated the following morning in clear plastic, 0.95 l cups turned upside down on a tray. Rearing cups were screened at the top and trays were cleaned daily. The Mormon crickets were raised in designated insect rearing quarters at 30 °C during the day and ambient (minimum ca. 24 °C) nights with fluorescent ceiling lights timed to local photoperiod. They were fed fresh Romaine lettuce daily and given water and ample wheat bran, tropical fish food, and sunflower and mixed bird seeds. In order to assay immunity or treat insects of known age, nymphs were checked for molting twice daily, early morning and late afternoon, and molts were noted on the cup on the day it occurred. Age was measured relative to the most recent molt, with the molt date designated day 0.

2.1. Immunity and protein assays

Immunity was sampled across a range of ages relative to the day of the most recent molt. Hemolymph was drawn from 5th, 6th, 7th instars and adults. In the rearing quarters at this time of year, nymphs remain in 5th instar for approximately 5 days, 6th instar for 6 days, and 7th instar for 8–10 days. To span the first 4–5 days of each instar, hemolymph was drawn from 0, 1, 2, 3, and 4 day old (0–4 day old) 5th instar nymphs, 0–4 day old 6th instars, and 0–5 day old 7th instars (sample sizes depended on availability, Fig. 1). Adult males mature as early as 6 days old, but females do not mature until they are 12–14 days old. To cover the teneral adult period, hemolymph was sampled on 0–3 day olds, inclusive, 5–8 day olds, and 10–13 day olds. No insects were sampled more than once. Mormon crickets were weighed to the nearest mg and then the arthrodial membrane at the base of a hind leg was punctured with a hypodermic needle. In 20 μl capillary tubes, over 15 μl of hemolymph was collected, measured, and diluted 1:50 with phosphate buffered saline (PBS) solution on ice. Diluted hemolymph was frozen for at least 24 h at −20 °C and later thawed to assay spontaneous phenoloxidase (PO), total PO (equal to spontaneous PO plus prophenoloxidase), and total circulating protein concentrations.

Protocols to measure spontaneous and total PO activities are detailed in Srygley and Lorch (2011). Briefly, spontaneous PO activity was measured by combining 5 μl diluted hemolymph with 195 μl 10 mM dopamine solution in a microplate well and measuring the change in optical density at 492 nm with a Biotek temperature-controlled microplate reader at 25 °C. If optical density was linearly related with time between 5 and 15 min from the initial reading, then mean V was calculated as the change in absorbance per min. One unit of PO activity per ml hemolymph is defined as the amount of enzyme resulting in a 0.001 increase in absorbance. Twenty-three of the 123 sampled individuals were excluded because the kinetics of the assay were not linear. Total PO activity was measured by first incubating 20 μl of the diluted hemolymph with 20 μg alpha-chymotrypsin dissolved in 20 μl PBS for 30 min. The incubated hemolymph was combined with dopamine and the kinetics of the reaction measured as that above for spontaneous PO. Kinetics for our assays of total PO never failed to meet the criterion for linearity.

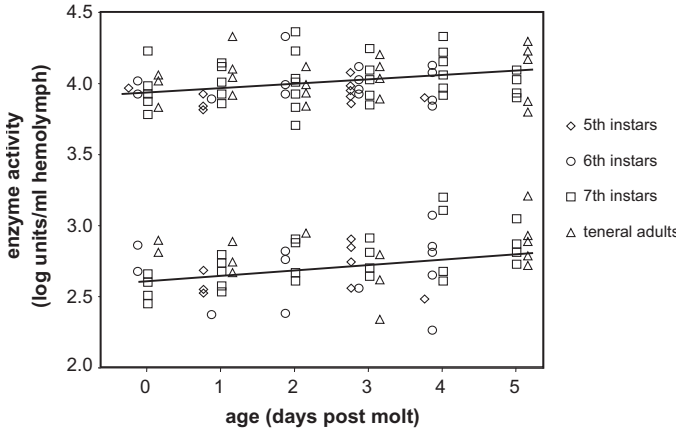

Fig. 1. Spontaneous and total phenoloxidase (PO) titers for 5th, 6th, and 7th instar nymphs and teneral adults. Within each day, data have been moved slightly so that the columns represent different stadia. Below are the data and line representing the ANCOVA modeled regressions of spontaneous PO on age of all stadia combined. Above are the data and line representing the ANCOVA modeled regression of total PO on age of all stadia combined.

Total circulating protein was measured in mg protein per ml hemolymph with a total protein kit (Micro, Sigma), comparing it to a standard serial dilution of human albumin.

To measure the ability of Mormon crickets to encapsulate foreign bodies, two quartz glass rods (2 mm × 1 mm dia.) were inserted dorsally between the first and second abdominal segments. Rods were inserted into two males and two females from each of the following ages and stadia, when available: 0, 1, 2, 3, and 4 days post molt to 6th instar (0–4 day old 6th instars), 0–4 day old 7th instars, 0–3 day old adults, 5–8 day old adults, and 10–13 day old adults. Mormon crickets were kept in the insect rearing quarters on their standard diet for 48 h after the rods were inserted. At 48 h (±8 min), they were frozen to halt encapsulation of the rods. Rods were dissected from the Mormon crickets, dried and weighed to ±0.01 mg with a Mettler analytic balance. To determine encapsulation mass, mass of the cleaned rod was subtracted from that of the encapsulated rod.

2.2. Survivorship from fungal attack

The ability of adults of different age classes to survive infection was assayed with *Metarhizium acridum* strain IMI330189 (Deuteromycotina: Hyphomycetes), an entomopathogenic fungus from Africa that is used in the microbial control of Orthoptera. Eight adult females that were evenly distributed across 0, 1, 2, and 3 days old served as a group for fungal inoculation and eight teneral females distributed across like ages served as a group of sham-inoculated controls. For experimentally treating Mormon crickets in three older age classes (5–8 days, 10–13 days, and 15–18 days), eight females and eight males that were evenly distributed across each group of four days of age were inoculated. Another eight females and eight males of like age in each age class served as controls. Fungal conidia were suspended in sunflower oil at a dose of 1.0×10^8 spores/ml sunflower oil. Two microliters of this suspension was applied topically to the arthrodial membrane at the base