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The effects of juvenile hormone on *Lasius niger* reproduction

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ABSTRACT

Reproduction has been shown to be costly for survival in a wide diversity of taxa. The resulting trade-off, termed the reproduction-survival trade-off, is thought to be one of the most fundamental forces of life-history evolution. In insects the pleiotropic effect of juvenile hormone (JH), antagonistically regulating reproduction and pathogen resistance, is suggested to underlie this phenomenon. In contrast to the majority of insects, reproductive individuals in many eusocial insects defy this trade-off and live both long and prosper. By remodelling the gonadotropic effects of JH in reproductive regulation, the queens of the long-lived black garden ant *Lasius niger* (living up to 27 years), have circumvented the reproduction-survival trade off enabling them to maximize both reproduction and pathogen resistance simultaneously. In this study we measure fertility, vitellogenin gene expression and protein levels after experimental manipulation of hormone levels. We use these measurements to investigate the mechanistic basis of endocrinological role remodelling in reproduction and determine how JH suppresses reproduction in this species, rather then stimulating it, like in the majority of insects. We find that JH likely inhibits three key aspects of reproduction both during vitellogenesis and oogenesis, including two previously unknown mechanisms. In addition, we document that juvenile hormone, as in the majority of insects, has retained some stimulatory function in regulating vitellogenin expression. We discuss the evolutionary consequences of this complex regulatory architecture of reproduction in *L. niger*, which might enable the evolution of similar reproductive phenotypes by alternate regulatory pathways, and the surprising flexibility regulatory role of juvenile hormone in this process.

1. Introduction

For the majority of organisms, reproduction is costly in terms of survival (Reznick, 1985). The resulting trade-off, termed the reproduction-survival (RS) trade-off, has been suggested to be a major driving force governing life-history evolution (Williams, 1966). The presence of the RS trade-off in a wide range of taxa suggests a common underlying mechanism, but despite substantial efforts its mechanistic basis remains elusive (Harshman and Zera, 2007; Partridge et al., 2005). Recently, due to their pleiotropic nature and involvement in diverse fitness-relevant processes, hormones have emerged as prime candidates mediating this antagonistic trait association (Flatt et al., 2005; Harshman and Zera, 2007). In insects juvenile hormone (JH) occupies a central regulatory role and is intimately linked to both reproduction and survival-relevant traits such as immunity (Flatt et al., 2008; Nijhout, 1998). The antagonistic roles of JH, stimulating reproductive output on the one hand while suppressing key insect immune functions on the other, have been suggested as potential mechanisms underlying the RS trade-off in insects (Rolff and Siva-Jothy, 2002).

In contrast to most solitary insects, the reproductive individuals (queens) of many ants and other eusocial insects exhibit both extraordinary reproductive capability (up to 120,000 eggs per month) and unmatched longevity (up to 27 years) rivalling the life span of many vertebrates (Schneirla, 1957; Wilson, 1971). Such exceptional life-history traits, in combination with the demonstrated absence of the RS trade-off in an experimentally tractable ant system (Heinze et al., 2013; Kramer et al., 2015; Schrempf et al., 2005), indicate that ant queens may have somehow circumvented the RS trade-off altogether. In a recent study, it has been demonstrated that the long-lived queens of the black garden ant *Lasius niger* have altered the gonadotropic role of JH in the regulation of reproduction, while maintaining its conserved role as an immune suppressor (Pamminger et al., 2016; Sommer and

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Hölldober, 1995). The alteration of an endocrinological role such as this has been found in a handful of other social and non-social insects and seems to be associated with changes in parental investment care (e.g. Corona et al., 2007; Engel et al., 2016). Although hormones are accepted as key regulators in invertebrate reproduction our factual knowledge of the underlying mechanisms is limited to a handful of model species (Nijhout, 1998). The information available indicates that in many cases these key hormones act pleiotropically influencing various aspects of invertebrate reproduction, including vitellogenesis, oogenesis and egg laying, sometimes directly or indirectly via hormone cascades (e.g. Olive and Bentley, 1980; Laufer et al., 1993; Robinson and Vargo, 1997; Nijhout, 1998; Lindemans et al., 2009). In order to understand reproductive output, and the potential reversal of the endocrinological regulatory role in the process, we need a detailed understanding of the individual mechanisms and regulatory hormones involved (Ronai et al., 2016). Only by taking such an inclusive look at the physiology of reproduction will we be able to pinpoint actual changes causing role reversal and disentangle the possible multiple regulatory pathways generating such a novel regulatory phenotype (Ronai et al., 2016).

In honeybees the most intensively studied example of such endocrinological role reversal JH can suppress reproduction at two key stages namely: 1) the expression of *vitellogenin* (Vg, the primary storage protein in developing eggs), and 2) its uptake into the developing oocytes (Bloch et al., 2009; Robinson and Vargo, 1997). Such role reversals have also been documented in stingless bees (Hartfelder et al., 2002), but not in bumblebees (see Bloch et al., 2009; Robinson and Vargo, 1997 for a review). To date there is mixed evidence on the role of JH in ants and wasps. While in some species JH still acts as a gonadotropin, stimulating Vg expression (Libbrecht et al., 2013; Brent and Vargo, 2003), it has modulatory functions in others species (Pamminger et al., 2016; Sommer and Hölldober, 1995) or has lost the majority of regulatory functions during reproduction (Kelstrup et al., 2015). These findings indicate that JH occupies a wide range of different regulatory roles in different species making it even more important to understand how such flexibility is realized in different systems.

In order to investigate this question, we set up a JH dose–response experiment measuring the role of JH in Vg gene expression, Vg protein levels, and overall impact on reproductive ovarian physiology, focusing on the known stages of hormone regulation of reproduction including oogenesis, vitellogenesis and egg laying. Pinpointing the mechanisms underlying the suppressive role of JH in *L. niger* queens will not only clarify the pathway by which JH regulates the reproductive process in this species, but also enable a deeper understanding of the mechanistic pathway changes generally involved in the evolution of novel regulatory phenotypes.

2. Material & methods

2.1. Ant collection and maintenance

L. niger queens were collected following their mating flight on August 3rd 2015 between 10:00 and 16:00 at the Marina, Brighton, UK (N 50.812785, W −0.098392). All queens used in the experiment had already shed their wings (indicating successful mating) and were immediately transferred into individual glass test tubes. The glass tubes were half filled with water trapped under a plug of cotton wool. To prevent the queens from escaping, the front of the tubes were loosely closed with cotton wool. Ants were kept in their glass vials in a climate chamber at a constant temperature of 23 ± 1 °C and 100% relative humidity in 24 h darkness to mimic their natural claustral behaviour (Sommer and Hölldober, 1995). Ant queens had access to water *ad libitum*, but were not fed because claustral founding queens do not feed until the emergence of the first generation of workers.

3. Experimental procedure

Two weeks after the collection, 90 queens were weighed on a Precisa 125A balance to the closest 0.0001 g and randomly assigned to either one of four treatment groups or to one of the two control groups (acetone or handling), resulting in N = 15 queens per treatment or control group. The treatment groups received 0.01, 0.1, 1 and 10 μg per day of the JH III analogue methoprene (Pestanal® Sigma-Aldrich, Dorset, UK) dissolved in 1 μl molecular grade acetone (Sigma-Aldrich®) as a topical application on the abdomen for seven days. Methoprene and the concentration range was chosen because it produces a comparable reproductive phenotype, has been shown to be physiologically active, not to induce elevated mortality at the concentrations used and keeps the results comparable to previous studies in this system (Pamminger et al., 2016; Sommer and Hölldober, 1995). In the acetone control group, queens were treated with 1 μl molecular grade acetone only. The handling control queens were disturbed once a day by softly tapping them on the gaster with the tip of a pipette simulating the experimental procedure. In all cases the ants were kept inside the glass vials restraining their movement enough to apply the treatment without using a stressful harness procedure.

After the end of the experiment, the queens were anaesthetized at 0 °C, the head (for size measurements) and gaster (for fertility and Vg expression) removed using a sterile razor blade and immediately frozen in liquid nitrogen and stored at −80 °C for subsequent ovary dissection and gene expression analysis. The thorax was transferred to a cooled 0 °C microscope slide and all six legs were carefully cut at the tibia. The secreted (clear) haemolymph was collected using sterile graduated glass capillaries (1–4 μL haemolymph/queen). The thorax was carefully squeezed to promote haemolymph flow, but care was taken that only transparent liquid was collected for the analysis. All haemolymph samples were immediately diluted 1:40 in ice-cold sodium cacodylate buffer (10 mM Na cacodylate, pH 7.2, 5 mM $CaCl_2$) containing protease inhibitor cOmplete® (Roche, Basel, Switzerland) and stored at −80 °C for later protein analysis. The frozen gasters were transferred on a microscope slide cooled to −96 °C on dry ice. Using cooled sterile razorblades the gasters were cut in half along the middle of the dorsal tergit. This procedure was very fast ensuring that the tissue did not defrost during the procedure and guaranteed that one of the two branches of the ovaries was present in each half. One half of the gaster was immediately transferred into Ambion® TRIzol® reagent (Life Technologies™), homogenized using a sterile pastel and stored at 4 °C for later total RNA extraction. The other half of the gaster was transferred on to a new microscope slide and dissected in ddH_2O to assess the ovarian status. In all cases the experimenters were blind to treatment.

3.1. Queen fertility

One day after the start of the experiment the queens were transferred to new test tubes and the number of eggs laid by the queens until the end of the experiment were counted on the last day of the experiment. In this paper we adopt the oocyte classification of Dolezal et al., 2013 (based on Raikhel and Dhadialla, 1992) distinguishing two main classes of small pre-vitellogenic oocytes (PV or developing oocytes) and large vitellogenic oocytes (V mature oocytes). Following this classification both stages are subdivided into three categories ranging from 1 the earliest (smallest) to 3 the oldest (largest), making vitellogenic PV1 the earliest