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SHORT COMMUNICATION

Does juvenile hormone prompt oxidative stress in male damselflies?

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ABSTRACT

In invertebrates, it has recently been reported that secondary sexual characteristics (SSCs) reflect the antioxidant defense of their bearers, but it is not known what physiological link maintains the honesty of those signals. Here, we used the damselfly *Hetaerina americana* to test whether juvenile hormone plays such a role. First, we analyzed whether oxidative damage is a real threat in natural damselfly populations by examining the accumulation of oxidized guanines as a function of age in males. Then, we injected paraquat (a pro-oxidant agent) and added the juvenile hormone analog methoprene (JHa) to the experimental group and the JHa vehicle (acetone) to the control group, to determine whether JHa increases the levels of pro-oxidants and antioxidants. We found that DNA oxidation increased with age, and that levels of hydrogen peroxide and superoxide dismutase, but not catalase or glutathione, were elevated in the JHa group compared with the control group. We propose that juvenile hormone is a mediator of the relationship between SSCs and antioxidant capacity and, based on the literature, we know that JHa suppresses the immune response. We therefore suggest that juvenile hormone is a molecular mediator of the general health of males, which is reflected in their SSCs.

KEY WORDS: Male condition, Handicap principle, Immune response, Antioxidants, DNA damage, *Hetaerina americana*

INTRODUCTION

Researchers have long attempted to explain the evolution of ornaments in vertebrates and invertebrates by assuming that they signal the health of their bearers in terms of immune response and resistance to parasites (Jacobs and Zuk, 2012; Contreras-Garduño and Canales-Lazcano, 2014). However, it is not clear how the signal's receivers (potential mates or competitors) could acquire information based solely on the immunity–ornament relationship (Adamo and Spiteri, 2005, 2009). The most likely situation is that ornaments evolved as signals that correlate with good overall condition (Zahavi, 1975), rather than with immune response specifically (Adamo and Spiteri, 2005, 2009). Along these lines, another key health parameter in animals is antioxidant defense, and it has been shown that males demonstrate their antioxidant resistance mechanisms to females through their ornaments (von Schantz et al., 1999; Monaghan et al., 2009; Garratt and Brooks, 2012).

In *Hetaerina americana*, the red spots at the base of the wings in males are indicative of immune response (Contreras-Garduño et al., 2006, 2007, 2008) as well as antioxidant defense mechanisms (Martínez-Lendech et al., 2018). Juvenile hormone (JH) maintains the honesty of wing spot coloration as a signal of immune response by simultaneously promoting the production of ornaments and repressing the immune response (Rantala et al., 2003; Contreras-Garduño et al., 2009, 2012), but it is not known whether JH also promotes oxidative stress in these males. Answering this question is essential for understanding the mechanisms that maintain the honesty of this signal of condition (Contreras-Garduño and Canales-Lazcano, 2014). It is important to define whether a single mechanism like JH could be responsible for the redundancy of signals of condition (e.g. indicating both immune system and oxidative stress) or whether immune response and antioxidant defense could be driven by independent mechanisms to display an individual's condition.

In this study, by using *H. americana* as a model system, we first explored the existence a real oxidative stress threat to biomolecules in natural conditions by recording levels of DNA damage [e.g. 8-hydroxy-2-deoxyguanosine (8-Oxo-G) lesion; Garinis et al., 2008]. We asked whether this damselfly faced these treats according to male age because it has been demonstrated that they undergo deterioration as a result of territorial fighting (Contreras-Garduño et al., 2006, 2008). Once we corroborated that damselflies faced a real threat in terms of oxidative stress in nature, we then investigated whether the juvenile hormone analog methoprene (JHa) leads to an increase in free radicals and antioxidants in males.

MATERIALS AND METHODS

Insects

Fieldwork was carried out in the Tehuixtla River, central Mexico (18°33′60.7″N, 152 99°16′15.5″W) between May and July, where damselflies, *Hetaerina americana* (Fabricius 1798), were trapped using an aerial insect net. Damselflies that were to be used to quantify oxidative damage on DNA were immediately submerged in liquid nitrogen and later transported to the laboratory. Those used in the experimental test of the effects of JHa on antioxidants and pro-oxidants were individually placed for 5 h after the administration of paraquat in clear plastic containers (4.5×1.4 cm^2) with a piece of wood as a perch and a moist cotton ball to maintain humidity (Contreras-Garduño et al., 2006; Martínez-Lendech et al., 2018). The containers were kept in a dark, cool box in the shade in order to reduce the animals' activity level and energy expenditure (Contreras-Garduño et al., 2006; Martínez-Lendech et al., 2018). After 5 h (see below) the animals were submerged in liquid nitrogen and transported to the laboratory.

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Male age and DNA oxidative damage

Males of three age categories were used: juvenile, middle-aged mature and old mature. They were distinguished by the following characteristics. Juvenile males have shiny wings, the development of body color is incomplete, and they do not defend a territory. Middle-aged mature males have bright body color and highly transparent wings, and they are more likely to be involved in contests for territories. Old mature males have dark or pale coloration, abundant pruinosity, and fragile wings that are less transparent than those of the middle-aged males, and they rarely fight for a territory (Contreras-Garduño et al., 2008).

Pools of five individuals from each age category were ground with a pestle (Motor Mixer, Cole-Parmer, Vernon Hills, IL, USA) after removing the head, intestines, legs and wings. Seven pools of juvenile and middle-aged males and eight pools of old males were used. We quantified DNA damage in the muscles and fat (fat body) because they are used in territorial behavior (Contreras-Garduño et al., 2006), and we speculated that DNA damage would be more evident here than in the legs and head. We used pools of males rather than individuals because we were unable to isolate enough DNA to detect oxidation at the individual level. The DNA was extracted using a Wizard Total DNA isolation kit (Promega, Madison, WI, USA). DNA integrity was assessed in agarose gels stained with ethidium bromide. The concentration of 8-Oxo-G as an indicator of DNA oxidation was measured with 30 µg of total DNA extracted from each pooled sample using a DNA/RNA Oxidative Damage Kit (Cayman Chemical, Ann Arbor, MI, USA), following the manufacturer's instructions. 8-Oxo-G concentration was determined using a standard curve of known concentrations of 8-Oxo-G (0.5, 0.75, 1, 1.5, 2 and 2.5 nmol l^{-1}).

Importance of JHa in oxidative stress

Preparation of JHa (methoprene)

Methoprene is an agonist of JH (Wilson, 2004) and mimics the molecular action of JH in insects in terms of behavior (Campero and Haynes, 1990; Contreras-Garduño et al., 2009, 2011; Wijesekera et al., 2016), development (Wilson, 2004) and reproduction (Schwartz et al., 1985), the three most relevant effects of JH in insects. However, the action of methoprene is stronger, and it is more resistant to degradation than pure JH. Methoprene is considered a pesticide because it is toxic to lobsters (Walker et al., 2005), but in insects it interferes with their life cycle by preventing the larvae from reaching the adult stage, just as JH impedes insect development (US Environmental Protection Agency Office of Pesticide Programs).

Methoprene (CAS-number: 40596-69-8; Sigma-Aldrich, St Louis, MO, USA) (hereafter referred to as JHa) was dissolved in acetone because acetone is the solvent in which methoprene is most soluble (Flatt and Kawecki, 2007). In order to prepare JHa for this experiment, 5 mg of methoprene was dissolved in 1000 µl of acetone (99.5%; Sigma-Aldrich) and 1 µl of that solution was added to 999 µl of acetone, resulting in a final JHa concentration of 5 ng μl^{-1} (Contreras-Garduño et al., 2009). For the control group, we used the acetone solvent alone. Using a 10 µl micropipette (Eppendorf), we applied 1 µl of the methoprene–acetone mixture (5 ng of JHa per organism) on the top of the thorax near the head (close to the corpora allata, the insect organ where JH is released; Contreras-Garduño et al., 2009).

Oxidative stress

Paraquat causes oxidative stress by increasing the production of oxygen free radicals, leading to subsequent peroxidation of tissues and alteration of antioxidant mechanisms (Abdollahi et al., 2004). In this experiment, 146 males were collected and injected with 2 µl of paraquat (10 mmol l^{-1}). These males were immediately assigned to either the control or experimental group. In the experimental group (n=73 males), 1 µl of the 5 ng μl^{-1} JHa solution was applied. The same procedure was carried out in the control group (n=73 males), using 1 µl of the acetone, the vehicle for JHa in the experimental preparation. Males were frozen 5 h later at −70°C for oxidative stress analyses because we have previously shown that paraquat increases oxidative stress at this time (Martínez-Lendech et al., 2018).

The thorax and abdomen of each individual were macerated in 500 µl of phosphate-buffered saline (PBS, Sigma-Aldrich) and centrifuged at 13,000 rpm (Prism R, Labnet International, Cary, NC, USA), and the supernatant was collected (Martínez-Lendech et al., 2018). We used kits to quantify the activity of the antioxidants superoxide dismutase (SOD; BioVision, Milpitas, CA, USA), catalase (BioVision) and glutathione peroxidase (BioVision), as well as the level of hydrogen peroxide (BioVision), which is a free-radical generator (Halliwell and Gutteridge, 2000). All analyses were carried out following the manufacturer's instructions.

We used generalized lineal models to analyze the effect of age on DNA damage. To analyze the role of JHa on oxidative stress, we used a robust generalization with a Welch test because we did not find homogeneity of variance and normality (R program v.3.5.2.). Means±s.e.m. are shown. All data are provided in Tables S1 and S2.

RESULTS AND DISCUSSION

DNA damage

We found differences in the amount of the reactive oxygen species (ROS)-induced DNA lesion 8-OxoG among the three age groups (χ^2=261.08, d.f.=2, P<0.0001; Fig. 1). Middle-aged males had more DNA damage than young males (middle-aged: 17.16±0.06 nmol l^{-1}, n=7, young: 15.61±0.16 nmol l^{-1}, n=7; P<0.0001), and old males had more DNA damage than middle-aged males (old: 17.71±0.03 nmol l^{-1}, n=7; P<0.0001).

As organisms age, body performance deteriorates as a result of the accumulation of ROS-promoted damage in proteins, lipids and DNA (Kirkwood, 2005; Selman et al., 2012). Hence, our results could be explained by age-related deterioration (Finkel and Holbrook, 2000). Damage to DNA is perhaps the most serious of these consequences, as it promotes mutagenesis and imposes irreversible consequences on the DNA sequence (Garinis et al., 2008). Guanine is the most frequently oxidized DNA nucleobase (Bjelland and Seeberg, 2003), giving rise to the highly mutagenic

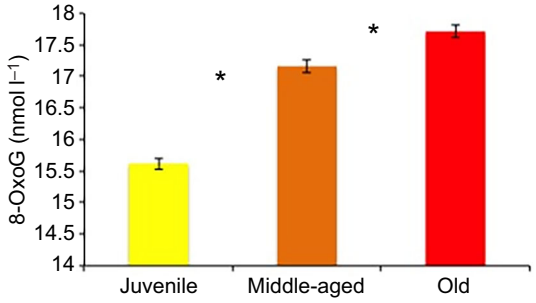

Fig. 1. DNA damage in males of three different age groups. DNA damage level was measured as the concentration of the 8-hydroxy-2-deoxyguanosine (8-Oxo-G) lesion. Seven pools of five juvenile and middle-aged individuals and eight pools of five old individuals were tested and samples of 30 µg total DNA (RNA-free) were analyzed. Asterisks denote significant differences between groups (P<0.05). We used generalized linear models.

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analog 8-Oxo-G (Garinis et al., 2008). We speculate that a potential phenomenon through which males undergo deterioration is territoriality. Territorial aggression has been shown to increase oxidative stress and damage (Guindre-Parker et al., 2013; Sharick et al., 2015), including the elevation of 8-Oxo-G (Margotta et al., 2018). Male odonates spend much of their time fighting for access to females (Córdoba-Aguilar, 1995; De-Marco and Cardoso-Peixoto, 2004; Serrano-Meneses et al., 2007) and in *H. americana* it is more likely that middle-aged males defend their territory better than do young males (Contreras-Garduño et al., 2008) and such behavior could explain the increased DNA damage in middle-aged males. In old males, although they are less likely to be involved in fights than middle-aged males (Contreras-Garduño et al., 2008), their DNA damage could be due to their earlier territorial behavior. Accumulation of 8-Oxo-G is the possible cost that males have to pay for survival, because males that are more likely to survive are territorial rather than non-territorial (Contreras-Garduño et al., 2006, 2007). Therefore, a possible relationship between territorial behavior and 8-Oxo-G should be studied; for example, by comparing territorial and non-territorial males and/or manipulating the territorial time devoted to fighting (Margotta et al., 2018) as well by measurement of JH to find out how the titer of this hormone correlates not only with territoriality (Contreras-Garduño et al., 2009) but also with oxidative stress.

Importance of JH in oxidative stress

The individuals treated with JHa showed increased hydrogen peroxide levels [JHa: 0.1±0.01 nmol, n=73; control: 0.03±0.007 nmol, n=73; familywise error rate (FWE)=7.31, P=0.008; Fig. 2A] and SOD activity (JHa: 69.43±3.13%, n=73; control: 28.76 ±3.15%, n=73; FWE=87.29, P<0.001; Fig. 2B) compared with the control group. However, there were no significant differences in catalase activity (JHa: 0.0318±0.002 nmol min^{-1} ml^{-1}, n=73; control: 0.037±0.003 nmol min^{-1} ml^{-1}, n=73, FWE=0.83, P=0.3) or glutathione peroxidase activity (JHa: 136.99±4.08 ng, n=26; control: 141.99±2.19 ng, n=26, FWE=0.80, P=0.3).

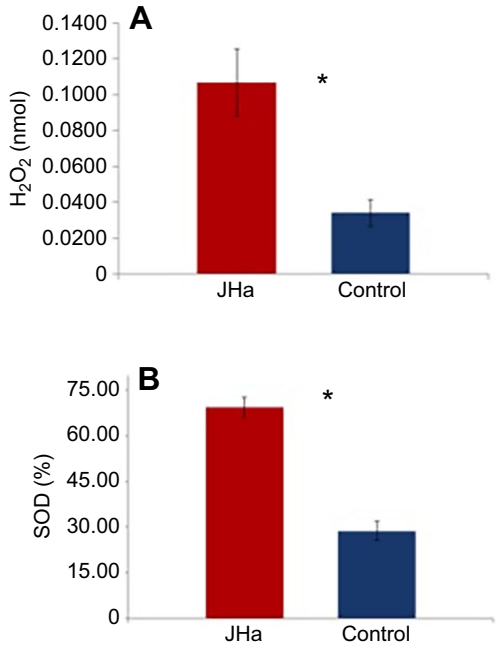

Fig. 2. Effect of juvenile hormone analog methoprene (JHa) on hydrogen peroxide levels and superoxide dismutase activity of male *H. americana*. (A) Hydrogen peroxide (H_2O_2) levels. (B) Superoxide dismutase (SOD) activity. Asterisks denote significant differences from the control group (P<0.05). We used a robust generalization with a Welch test. The sample size is shown in Materials and Methods.

Zahavi's (1975) handicap theory proposes that secondary sexual characteristics (SSCs) are honest communication signals, and this idea is based on the fact that elaborate signals are costly. Hormones could be a mechanism behind the honesty of these signals if they govern trade-offs or have pleiotropic effects on the allocation of resources to SSCs versus other physiological functions such as the immune response (Folstad and Karter, 1992). In insects, JH could fulfill that function because it controls diverse aspects of life history, including, for example, ovary growth, sexual behavior, reproductive diapause, immune response and courtship (Flatt et al., 2005; Tatar et al., 2001; Hernández-Martínez et al., 2007). In addition, JH increases territorial behavior in males; males treated with JHa spend more time defending their territory and they defend it for longer periods than do control males. At the same time, JH represses the immune response (Contreras-Garduño et al., 2009) and decreases survival (González-Tokman et al., 2012). The results of this study show that JHa induces the production of hydrogen peroxide and, in consequence, increases the level of antioxidant enzymes, including SOD, to reduce the ROS-promoted damage to cellular molecules. As such, JHa could be a mechanism that maintains the honesty of wing pigmentation and territoriality as signals of male health in terms of immune response and antioxidant defense. However, our study, like studies of testosterone in vertebrates, assumes that these hormones increase the metabolic rate, which in turn generates ROS that increase oxidative damage. This premise still needs to be carefully tested, taking into account individual differences to determine whether low-quality males (e.g. in *H. americana*, males with smaller wing spots) pay a higher cost than high-quality males (see Garratt and Brooks, 2012).

It must be pointed out that not all of the antioxidants analyzed showed a decrease in activity in the JHa group (here, catalase and glutathione peroxidase). Similarly, in studies of the immune system, some parameters decreased while others did not (Villanueva et al., 2013). It is tempting to speculate that basal levels of catalase and glutathione peroxidase must be maintained in the different stages of *H. americana* to promptly respond to stressful metabolic conditions. Accordingly, future experiments must explore whether disturbed levels of H_2O_2 induce expression of genes encoding SOD but not those encoding catalase and glutathione peroxidase in this organism.

Conclusions

Overall, our results support the idea that diverse parameters should be measured simultaneously to generate a clearer picture of the effect of hormones on physiology, but they also suggest that the state of a diversity of health parameters could be honestly signaled by the same SSCs if they are all linked to a single mechanism, such as JH in the case of insects. Finally, these results are consistent with Garratt and Brooks's (2012) proposal that different mechanisms, (i.e. testosterone in vertebrates and JH in insects) may explain a link between sexual ornaments and oxidative stress.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: N.M.-L., M.O.-B., B.F., M.P.-R., J.C.-G.; Methodology: N.M.-L., M.O.-B., B.F., M.P.-R., A.O., J.C.-G.; Formal analysis: N.M.-L., J.C.-G.; Writing - original draft: M.P.-R., J.C.-G.; Writing - review & editing: N.M.-L., M.O.-B., B.F., A.O., J.C.-G.; Funding acquisition: J.C.-G.

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Supplementary information

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Table S1. DNA Damage

Table S2. Oxidative Stress

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