Vertebrate hormones in insects: effects of Beta-Estradiol on mating, pre-oviposition time, fecundity, and fertility in the female ring-legged earwig, *Euborellia annulipes*

By
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April 3, 2017



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A Senior Comprehensive Project in Partial Fulfillment of the Requirements for a Bachelor of Science Degree from Allegheny College

I hereby recognize and pledge to fulfill my responsibilities as defined in the Honor Code and to maintain the integrity of both myself and the College community as a whole.

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Pledge

Approval by the Senior Thesis Committee:

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Dr. Margaret Nelson

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Abstract

Although Beta-Estradiol (estradiol) is a naturally occurring sex hormone in vertebrates, its physiological role in invertebrates remains unknown, despite its presence in multiple invertebrate species. While estradiol may or may not be naturally synthesized in invertebrates, it alters reproductive structure and function ranging from vitellogenin production and oocyte maturation to larval growth and development (Li et al 1997; Keshan and Ray 1999). The female ring-legged earwig, *Euborellia annulipes*, was used as a model system for observing the potential effects of estradiol in insects. Estradiol was topically applied to adult female earwigs, in either a low dose of 0.05 µg or a high dose of 0.5 µg, on day 0 and again after mating on day 7. Mating times and behavior, pre-oviposition time, number of eggs, and number of hatchlings were examined. In addition, estradiol levels in the adult females were quantified via an ELISA following termination 15 days post-oviposition. Treatment with estradiol did not induce changes in mating behavior, pre-oviposition time, number of eggs, or number of hatchlings, but did alter maternal behavior, such as inducing maternal cannibalism. All groups of females had detectable levels of estradiol, including the untreated control females, suggesting that female *E. annulipes* naturally synthesize estradiol. The presence of estradiol indicates the existence of some metabolic pathway in female *E. annulipes* to regulate and utilize the hormone, supporting the notion that at least some steps of steroid synthesis are conserved in invertebrates and vertebrates.

1. Introduction

Endocrine disruptors (EDs) are compounds that alter hormonal regulation and cause related health effects in the exposed organism or its progeny (Waring et al 2005). EDs may be naturally occurring, such as the antioxidant flavonoids in fruits and vegetables, or environmental contaminants including industrial chemicals (Waring et al 2005). Common EDs include synthetic steroids, pesticides, and chemicals used in plastics and detergents (Kirkbride-Smith et al 2001). These compounds often find their way into the environment through manufacturing processes, agricultural practices, sewage treatment plants, and applying sewage sludge to agricultural land (Kirkbride-Smith et al 2001). Typically, EDs bind agonistically or antagonistically to steroid receptors for estrogens or androgens, therefore affecting reproduction or inducing feminization (Waring et al 2005). EDs also act through other mechanisms such as altering steroid synthesis or steroid metabolism (Waring et al 2005). EDs affect a range of species including mammals, birds, fish, amphibians, aquatic invertebrates, and terrestrial insects.

Most research regarding EDs focuses on vertebrates; less is known about the role and effects of EDs in invertebrates, particularly terrestrial arthropods, including insects (Kirkbride-Smith et al 2001). Invertebrates serve as good models for studying endocrine systems and testing the effects of toxic chemicals because of the ease of using such species, their sensitivity to toxic chemicals, short generation times, and the range of models from which to choose (deFur 2004). In addition, invertebrates are an essential element of the Earth's ecosystems, providing food for other animals, transferring metabolic energy in trophic systems, and decomposing animal and plant material--necessitating their protection from EDs (deFur 2004).

EDs are commonly found in industrial products, and may possess estrogenic properties and/or effects. Frequently examined EDs with estrogenic properties and effects include nonylphenol, methoprene, estrone, and diethylstilbestrol (DES). For example, exposure of the mysid *Neomysis integer* to low levels of nonylphenol induced vitellin production, while the high concentrations of estrone reduced vitellin levels (Ghekiere et al 2005). In contrast, methoprene treatment had no effect on *N. integer* vitellin levels (Ghekiere et al 2005). These results suggest that mysid vitellogenesis can be disrupted by chemical exposure in a chemical-specific and concentration-specific manner (Ghekiere et al 2005). In addition, environmentally relevant concentrations of methoprene and nonylphenol induced testosterone and energy metabolism in *N. integer*, indicating that these processes are affected by endocrine disrupting activity after short-term exposure (Verslycke et al 2003). DES reduces molt frequency and therefore inhibits overall growth of female *Daphnia magna*, in addition to decreasing fecundity in the second generation, supporting a multigenerational effect of endocrine-disrupting chemicals in daphnids (Olmstead et al 2000; Zou & Fingerman 1997; Baldwin et al 1994).

Both vertebrate and invertebrate endocrine systems use a wide variety of hormones to regulate physiological processes (deFur 2004). In insects, juvenile hormones regulate embryogenesis, reproduction, and metamorphosis, while ecdysteroids regulate molting, growth, and reproduction (Kirkbride-Smith et al 2001; Hentze et al 2013). Recently, analogues of such hormones have been used as agricultural insecticides that specifically target and disrupt growth and functioning of insects (Kirkbride-Smith et al 2001). Evidence that the endocrine systems of insects are susceptible to such disruption suggests that they may also be susceptible to other EDs such as vertebrate hormones (Kirkbride-Smith et al 2001). Vertebrate steroid hormonal compounds appear to be one of the most primitive compounds and are most likely shared by

several, if not all, animal phyla (Roy et al 2006). In addition, the molecular mechanisms by which steroids regulate gene expression in arthropods and vertebrates appear to be conserved, despite a significant evolutionary distance (Keshan and Ray 2001).

Invertebrates conserve at least some steps of the vertebrate steroid synthesis pathway (Keshan and Ray 1997). For example, *Drosophila* steroid receptors and receptor-like proteins have structure and function similar to those of vertebrate homologues (Keshan and Ray 2001). In general, an action carried out by a hormone is associated with binding of the hormone to its specific receptors (Keshan and Ray 1997). Surprisingly, estrogenic and androgenic binding substances have been observed in invertebrate systems, including those of insects. For example, a progesterone binding protein occurs in the copulatory organ and spermatheca of the migratory locust (Keshan and Ray 2001). Furthermore, a hemolymph protein in *Sarcophaga bullata* demonstrates specific binding for estradiol and there are binding sites for progesterone and testosterone in *Calliphora vicina* (Keshan and Ray 2001). Testosterone and estradiol have been found in the silkworm, *Bombyx mori*, and testosterone and estrogen-like immunoreactivity have been observed in the moth *Manduca sexta*, the beetle *Tenebrio molitor*, and the fly *S. bullata* (Kirkbride-Smith et al 2001). While these vertebrate hormones have been found in insects, the physiological role, if any, remains unknown.

Estradiol is a vertebrate hormone that has been commonly tested in invertebrates. Research has been conducted to determine the effects of estradiol on invertebrate reproduction by observing the potential effects on vitellogenesis, oviposition, and ovarian development (Li et al 1997; Garcia-Alonso et al 2005; Koskela et al 1991; Ogiso and Ohnishi 1985), in addition to growth and development (Keshan and Ray 1999). In the Pacific oyster, *Crassostrea gigas*, estradiol increased oocyte diameter and vitellin content (Li et al 1997). A similar effect was

found in the sandworm, *Nereis virens* (Garcia-Alonso et al 2005). In contrast, estradiol had no effect on vitellogenin production in the tiger prawn, *Penaeus esculentus*, contrary to the evidence that estradiol is a vitellogenin stimulating hormone in invertebrates (Koskela et al 1991). Estradiol has not been demonstrated to affect vitellogenin protein in *B. mori*; however, an accumulation of vitellogenin-like protein was found in the ovary and hemolymph after estradiol treatment (Keshan and Ray 1997). Surprisingly then, estradiol decreased rate of oviposition by 30% in *B. mori* (Ogiso and Ohnishi 1985). This suggests that estrogens have some biological function in invertebrate ovaries, although oocyte maturation and embryogenesis were not affected (Ogiso and Ohnishi 1985). In addition, estradiol significantly enhanced the cocoon characteristics of *B. mori* larvae and induced several metabolic changes in the fat body and ovary, furthering the evidence that estradiol affects growth and development (Keshan and Ray 1999; Keshan and Ray 1997).

Estradiol has also been utilized in research regarding pheromones and mating behavior, although primarily in mammals. Injection of tritiated estradiol in male mice (*Mus musculus)* and bats (*Eptesicus fuscus*) resulted in radioactivity in the reproductive tissues and brain of cohabitating females (deCatanzaro 2014; deCatanzaro et al 2014). These data suggest that estradiol is transferred from males to females, likely by absorption from males' excretions and potentially also by intravaginal exposure during mating (deCatanzaro et al 2014). Because estradiol is a strong estrogen that regulates mammalian female reproductive physiology and behavior, these data are consistent with the hypothesis that male mammals exposed to estradiol induce sexual receptivity and ovulation in females (deCatanzaro et al 2014). It is unclear if similar results can be observed in insects and if females are able to induce sexual receptivity in

males. However, due to the powerful effects of estradiol in vertebrate systems, there is reason to examine the role estradiol has in mating behaviors of *E. annulipes* following female exposure.

Estradiol is naturally present in the ovaries of some invertebrates, but likely not all (Ohnishi et al 1985). Estradiol has been detected in the ovaries of the Pacific oyster, *C. gigas*, the silkworm, *B. mori,* and the locust, *Locusta migratoria* (Li et al 1997; Ohnishi et al 1985; Das 2016). Estradiol has also been detected in the hemolymph, posterior silk gland, and the larval and pupal fat body of *B. mori* (Das 2016). Because estrogens have been detected in many plant species, it is possible that insects, such as *B. mori* and *L. migratoria*, obtain the estradiol from their food source (Ohnishi et al 1985). However, it is also possible that estradiol is synthesized in these species and is involved in some physiological role of oocyte maturation (Ohnishi et al 1985). The presence of estradiol in ovaries may be widespread among arthropods, but the physiological role of estradiol in insect ovaries is still unknown, including those of *Euborellia annulipes* (Ohnishi et al 1985). However, the presence and reproductive effects of estradiol in other insects and invertebrate species provides reason to study estradiol in earwigs. I hypothesized that application of Beta-Estradiol to adult female *E. annulipes* would decrease time until mating, increase duration of mating, increase pre-oviposition time and number of eggs laid, and decrease number of hatchlings, all in a dose-dependent manner.

2. Materials and Methods

2.1. Test Insects

Zero day female *E. annulipes* were harvested from the colony and reared individually at 28 degrees Celsius in 200 mL plastic containers with water vials and dry cat food ad libitum. All earwigs were initially weighed to ensure equitable sizes across treatment groups.

2.2. Preparation of Estradiol

Estradiol (Sigma) was diluted in 100% acetone to concentrations of 0.025 µg/µL (low dose) and 0.25 µg/µL (high dose). Concentrations were based on previous studies of estradiol (Keshan and Ray 1997; Keshan and Ray 1999), but adjusted to a level appropriate for *E. annulipes*.

2.3. Application of Estradiol

Four treatment groups of 14-15 adult female earwigs per group were treated accordingly: 1) untreated control, 2) acetone control, 3) 0.025 µg/µL of estradiol (low dose), or 4) 0.25 µg/µL of estradiol (high dose). The appropriate solution was topically applied in 2 µL to the abdomen with a Drummond microcap on day zero and additionally on day seven shortly after mating. Mating behaviors, pre-oviposition time, number of eggs laid, and number of eggs hatched were assessed.

2.4. Data collection

2.4.1. Mating time/behavior

Seven-day female *E. annulipes* from each treatment group were mated with five to ten day virgin untreated males during one 15-minute trial. Male and female pairs were placed in 200 mL containers that neither male nor female inhabited previously. Time until mating and duration of mating were monitored. If no mating took place, the female and male pair were housed together in the female's container until oviposition occurred.

2.4.2. Pre-oviposition time, number of eggs laid, and number of hatchlings

Female earwigs were monitored daily for oviposition and pre-oviposition time was recorded. Eggs laid and hatched were counted. If earwigs oviposited a second time, the additional pre-oviposition time was recorded. If earwigs ate their eggs, it was noted.

2.4.3. Quantification of estradiol

Adult female *E. annulipes* were sacrificed 15 days after oviposition, allowing the time needed for larval care. Earwigs were sacrificed by placement in individual Eppendorf tubes at -80 degrees Celsius and stored until ELISA analysis. An estradiol ELISA kit (Cayman Chemical) was used to quantify the concentrations of estradiol naturally synthesized or experimentally introduced in adult female *E. annulipes* from all treatment groups. All earwigs were homogenized in 250 µL of ELISA buffer and centrifuged at 13,000 rpm for 5 minutes. The resulting supernatant was loaded into the plate. For controls and acetone controls, 50 µL of supernatant was assessed for estradiol concentrations. For estradiol treated earwigs, 5 µL and 50 µL of supernatant were assessed. The Estradiol ELISA kit protocol (Cayman Chemical) was followed.

2.5. Statistical analysis

Statistical differences between control and treatment groups were examined using a one-factor analysis of variance (ANOVA) for all effects assessed, except for cannibalism of eggs, which was assessed using a chi square test. JMP12 was used to generate ANOVA and Tukey's post hoc test. Chi square test was generated manually. Significance was defined as $p \leq 0.05$.

3. Results

3.1. Mating time/behavior

Of the females that mated during the trials, there was no obvious change in overall mating behavior as a result of treatment. The acetone treatment group had the longest average time until first mating, followed by the control group, the high estradiol treatment group, and the low estradiol treatment group, respectively (Table 1). These results support my hypothesis that

application of estradiol would decrease time until the first mating, although the trend was not observed in a dose-dependent manner. However, one-way analysis of variance (ANOVA) showed these differences were not significant (p= 0.08). Only a small number of earwigs did not mate during the 15-minute trial. Specifically, 1 control female, 3 low dose estradiol treated females, and 1 high dose estradiol treated female did not mate during the trial.

Females treated with the high dose of estradiol had the longest duration of mating, followed by those treated with acetone, the low dose of estradiol, and the untreated control, respectively (Table 1). These results support my hypothesis that estradiol treatment would increase duration of mating in a dose-dependent manner. However, these data were not statistically different (p= 0.64).

Table 1. Average time until first mating and duration of mating between 7-day female *E. annulipes* and 5-10 day virgin untreated male *E. annulipes*. *p* values determined by one-factor ANOVA.

	Control	Acetone	0.025 µg Estradiol	0.25 µg Estradiol	*p* value
Average time until first mating (s)	106.5 ± 35.4	197.4 ± 35.4	77.5 ± 39.9	84.8 ± 35.4	0.08
Average duration of mating (s)	393.8 ± 50.7	448.8 ± 50.7	403.9 ± 57.2	476.9 ± 50.7	0.64

3.2. Pre-oviposition time, number of eggs laid, and number of hatchlings

Fifty-five of the fifty-eight females oviposited. However, treatment did not affect the pre-oviposition interval (Table 2; p= 0.85). Thus, my prediction that treatment with estradiol would increase pre-oviposition time was not supported.

Similarly, treatment did not alter the number of eggs laid by females of the different treatment groups (Table 2; p =0.42). Thus, my hypothesis that an increased treatment of estradiol would increase number of eggs was not supported. Overall, treated earwigs laid fewer eggs, but there was no direct correlation between treatment and number of eggs laid.

A number of females from each treatment group laid a second clutch of eggs (Table 2). However, the number of females with a second oviposition between treatment groups was statistically insignificant (χ^2= 2.6, d.f.= 3, p= 0.50). Of those with a second oviposition, pre-oviposition time ranged from 21.8 days to 24.0 days between treatment groups (p = 0.27), with the control group having the longest pre-oviposition time and the high estradiol treatment group having the shortest (Table 2). These results do not support my hypothesis that estradiol treatment would increase pre-oviposition time.

The number of eggs from the second oviposition did not vary with treatment (Table 2; p = 0.85). These results do not support my hypothesis that increased estradiol treatment would increase clutch size. Although estradiol treated females had larger clutch sizes in comparison to the control group, it was not in a dose-dependent manner. However, when comparing the first and second clutches among treatment groups, the control group's clutch size did not change, while the acetone and estradiol treatment groups' clutch sizes increased by 13-22 eggs (Table 2).

Table 2. Average first and second pre-oviposition times, presence of second oviposition, and number of eggs laid among treatment groups of female *E. annulipes*. *p* values determined by one-factor ANOVA and chi square.

	Control	Acetone	0.025 µg Estradiol	0.25 µg Estradiol	*p* value
Average pre-oviposition time (days)	12.5 ± 0.9	11.9 ± 0.9	11.7 ± 0.9	12.7 ± 0.8	0.85
Average number of eggs from first oviposition	52.1 ± 6.6	45.3 ± 6.8	36.3 ± 6.8	42.5 ± 6.6	0.42
Number of females with second oviposition (%)	5/15 (33%)	8/14 (57%)	5/14 (36%)	8/15 (53%)	0.50
Average second pre-oviposition time (days)	24.0 ± 1.0	23.8 ± 0.8	23.2 ± 1.0	21.8 ± 0.8	0.27
Average number of eggs from second oviposition	53.0 ± 7.7	61.0 ± 6.1	58.6 ± 7.7	55.6 ± 6.1	0.85

I hypothesized that application of estradiol would decrease the number of eggs hatched. However, my results do fully support my hypothesis. While the low dose of estradiol decreased the number of hatchlings, the high dose of estradiol increased the number of hatchlings compared to the control group (Figure 1). The acetone treatment also decreased the number of hatchlings (Figure 1). These results were not statistically significant (p= 0.15).

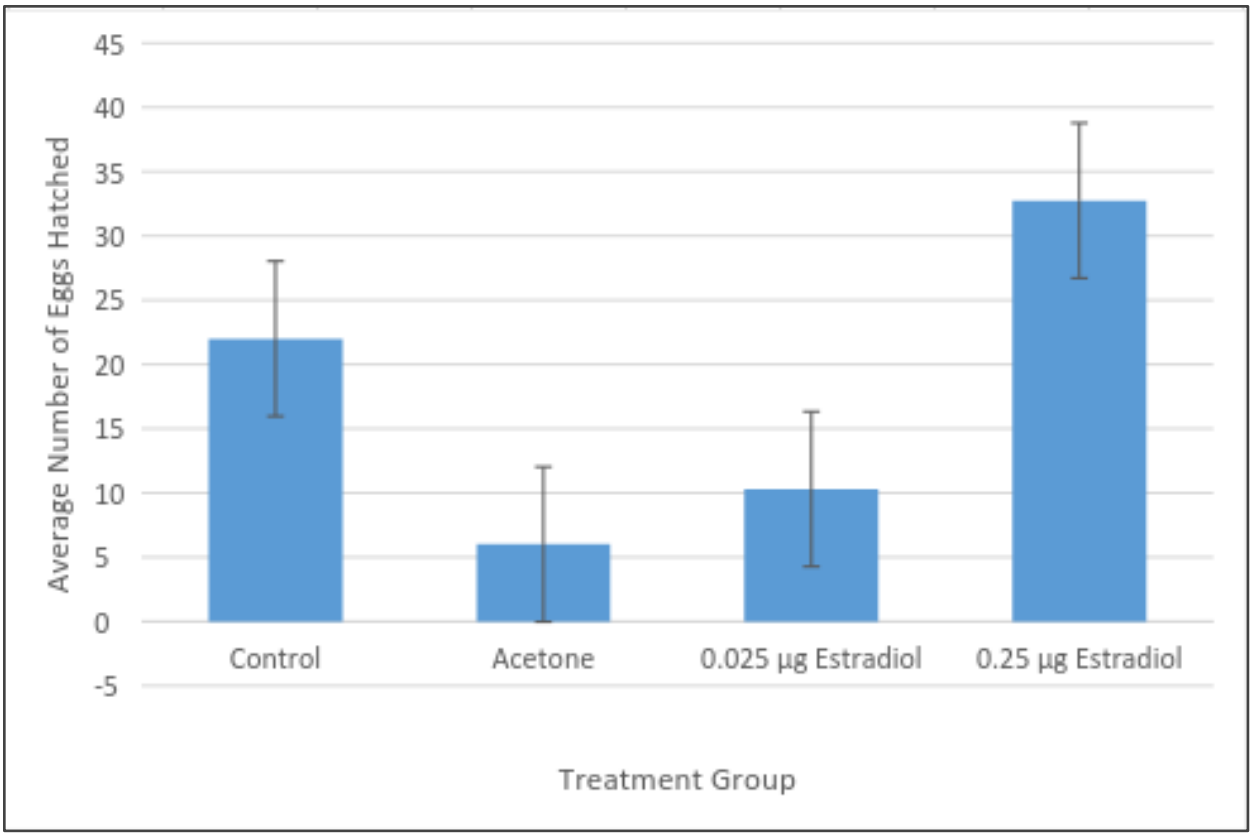

Fig. 1. Average number of eggs hatched from first oviposition among treatment groups of female *E. annulipes*, including only the females that oviposited. $p = 0.15$ determined by one-factor ANOVA. n= 13-15 females per group. Error bars= standard error of the mean (SEM).

Forty of the fifty-eight earwigs ate their eggs. This includes 8 control females, 11 acetone treated females, 10 low dose estradiol treated females, and 11 high dose estradiol treated females. Treatment did not alter the incidence of cannibalism ($\chi^2 = 2.37$, d.f. = 3, p= 0.50). However, of those that ate their eggs, there was a statistically significant difference between the average number of days until the eggs were eaten (p =0.04). The control females maintained offspring for more days than did females treated with the high dose of estradiol (p= 0.03) (Tukey-Kramer HSD test). There was a dose-dependent decrease in the average number of days it took for the females to eat all of their eggs (Figure 2).

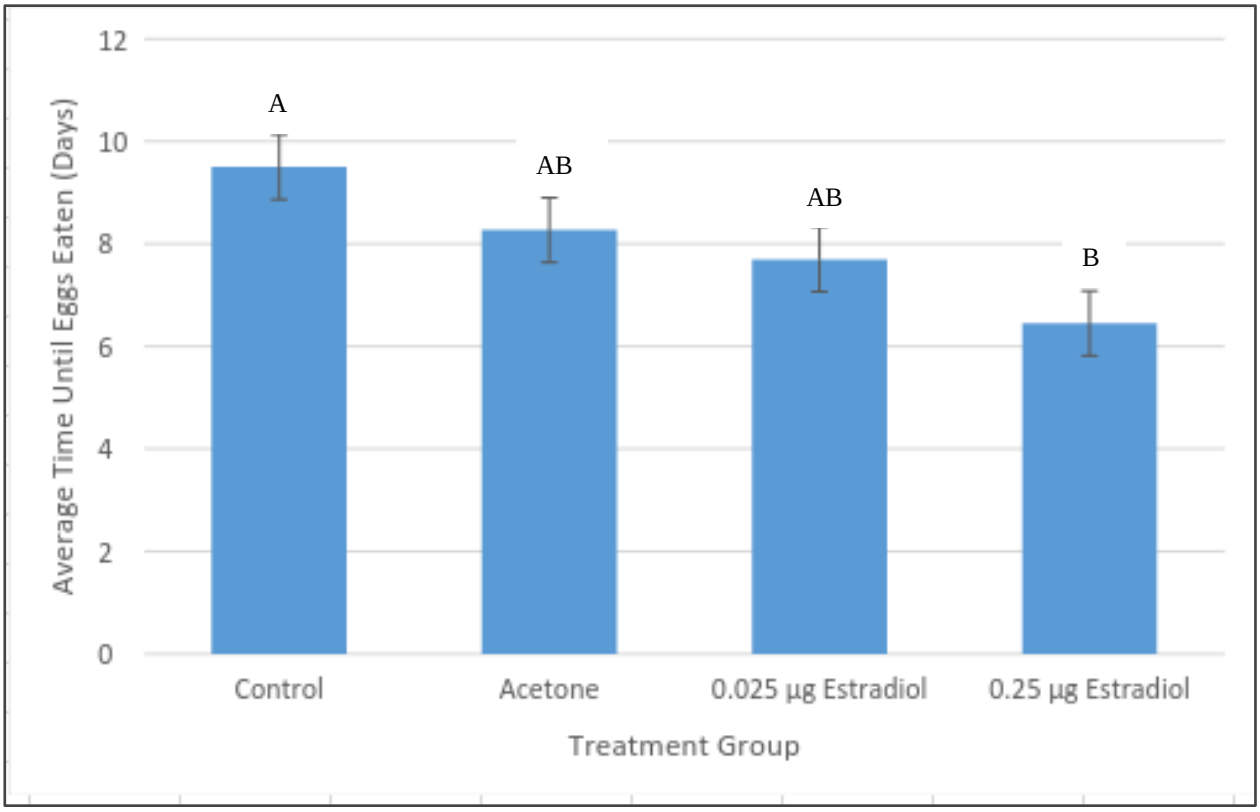

Fig. 2. Average number of days until all eggs were eaten from first oviposition among treatment groups of female *E. annulipes*. $p = 0.04$, determined by one-factor ANOVA. n= 8-11 females per group. Error bars= SEM. Treatment groups designated with different letters (A, B) indicate significant differences.

3.3. Quantification of estradiol

All groups of females had detectable levels of estradiol. Not surprisingly, treatment with estradiol elevated levels detected using ELISA. ELISA analysis yielded the following estradiol concentrations for the control, acetone control, low dose estradiol, and high dose estradiol treatment groups, respectively: 73.33 pg/mL, 925 pg/mL, 1090.83 pg/mL, and 1270 pg/mL (Figure 3; p= 0.05). There were significant differences among the control group and high dose estradiol treatment group (p=0.04), determined by Tukey-Kramer HSD test.

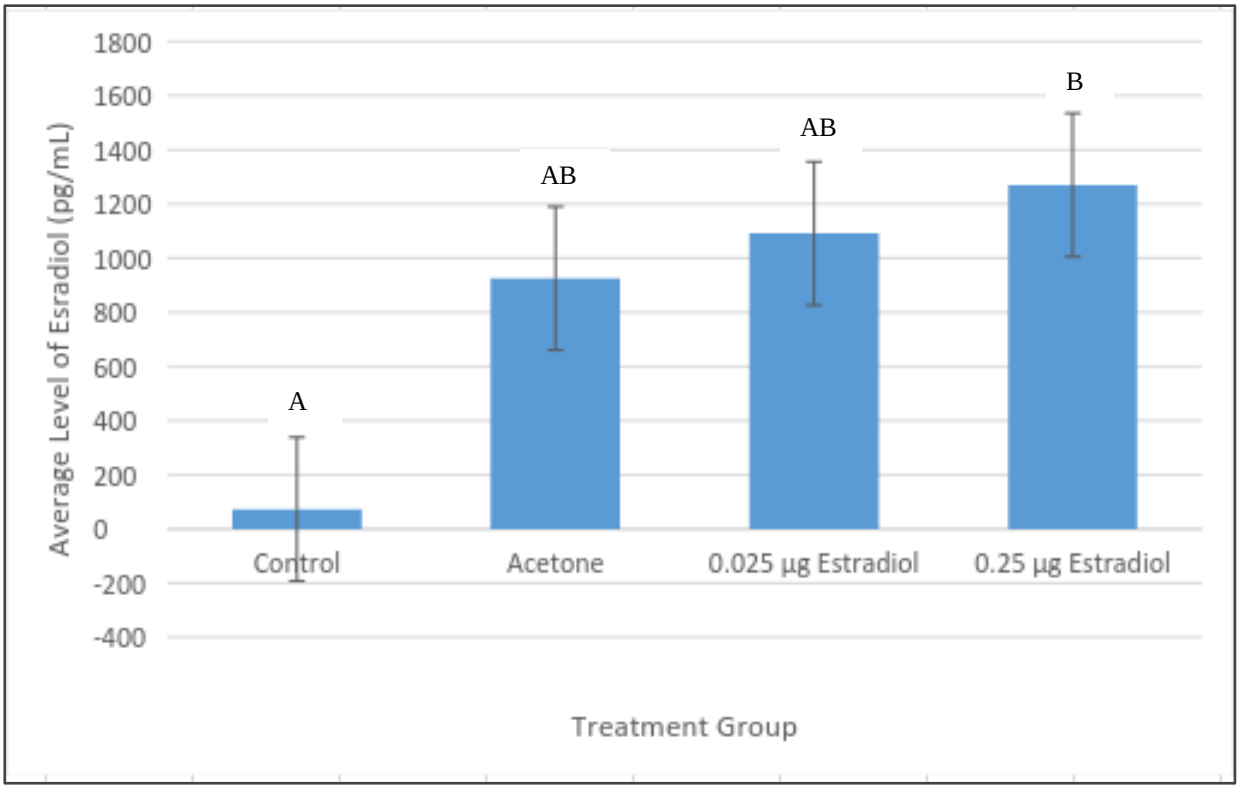

Fig. 3. Average level of estradiol present among treatment groups of female *E. annulipes*. $p = 0.05$, determined by one-factor ANOVA. n= 4-14 females per group. Error bars= SEM. Treatment groups designated with different letters (A,B) indicate significant differences.

4. Discussion

4.1. Mating time/behavior

Treatment had no significant effect on mating behavior. However, treatment with estradiol resulted in a trend of decreased time until first mating and increased duration of mating. Such a significant finding might occur if (a) the estradiol regulated the females' reproductive behavior or (b) the females induced sexual receptivity in the male earwigs. Although we cannot distinguish between these possibilities in earwigs, it is clear that injection of male mice and bats with radiolabeled estradiol resulted in radioactivity in the reproductive tissues of cohabitating females, supporting the hypothesis that male mammals transfer estradiol to females to induce sexual receptivity and ovulation (deCatanzaro 2014; deCatanzaro et al 2014).

4.2. Pre-oviposition time, number of eggs laid, and number of hatchlings

Estradiol treatment did not affect pre-oviposition time, whether it was the first and second oviposition. In contrast, estradiol increases pre-oviposition time in *B. mori*, and estradiol has been detected in the ovaries, hemolymph, posterior silk gland, and the larval and pupal fat body of *B. mori*, suggesting a physiological role in oocyte maturation in this species (Das 2016; Ohnishi et al 1985). The presence of estradiol may be widespread among arthropods, but it has not been previously detected in *E. annulipes.* Furthermore, the physiological role is still unknown and may vary among species (Li et al 1997; Ohnishi et al 1985; Das 2016). Estradiol does not appear to have a physiological role in pre-oviposition time in *E. annulipes*.

Treatment had no effect on the number of eggs laid between groups during the first or second oviposition. The number of eggs laid by the untreated control females remained constant between the first and second oviposition, as expected. However, the number of eggs laid by the estradiol treated females increased from the first to second oviposition-- albeit a trend that did not produce significant differences. Estradiol increased oocyte diameter and vitellin content in *C. gigas* and *N. virens*, but had no effect on vitellogenin levels in *P. esculentus* (Li et al 1997; Garcia-Alonso et al 2005; Koskela et al 1991). We have not yet confirmed the mechanism by which estradiol affects reproduction in *E. annulipes*. Treatment with estradiol may have acted directly by being initially toxic to vitellogenin production, leading to smaller clutch sizes during the first oviposition and larger clutch sizes comparable to the untreated control clutches during the second oviposition. It is also possible that estradiol indirectly affected clutch size by decreasing juvenile hormone or ecdysteroid production, resulting in initially smaller clutches during the first oviposition. Although differences were not significant, it could potentially be inferred that the estradiol remained in the reproductive system long enough to decrease the first

clutch, but not long enough to decrease the second clutch. Therefore, the estradiol may have been partially degraded or sequestered elsewhere in the body by the time the females oviposited a second time, resulting in clutches closer in size to the untreated control.

Treatment with estradiol enhances larval cocoon characteristics and metabolic changes in the fat body and ovary of *B. mori*, supporting that estradiol affects growth and development (Keshan and Ray 1999; Keshan and Ray 1997). However, estradiol treatment in *E. annulipes* did not produce similar results. Treatment with neither the high dose nor the low dose of estradiol resulted in significant differences from the untreated control group. The acetone treatment resulted in the lowest number of hatchlings. Therefore, the acetone may have been responsible for the inhibited larval development. Because the number of hatchlings increased with increasing estradiol treatment, the low dose may have not been strong enough to overcome the effects of the acetone, while the high dose may have been enough to increase the number of hatchlings, although not to a different extent compared to the untreated control.

4.3. Maternal cannibalism

Treatment did not alter the incidence of cannibalism among treatment groups, but treatment with estradiol decreased the time until all eggs were eaten, with significant differences between the untreated control females and high dose of estradiol treated females. However, most research focuses on intraclutch cannibalism rather than maternal cannibalism. Factors that increase the occurrence of intraclutch cannibalism in invertebrates include increased humidity, increased temperature, increased hunger, and deprivation of foraging activity (Ghazy & Amano 2016; Tang et al 2016; Dick 1994). It is unclear if and how hormones affect cannibalism, whether intraclutch or maternal. Maternal cannibalism was evenly distributed among treatment groups; thus, a mutual factor likely caused the cannibalism. All earwigs were maintained at an

appropriate temperature and were given food and water, but were disturbed daily to monitor oviposition and number of eggs and hatchlings. It is possible that these disturbances induced stress or a predation response in the earwigs, resulting in cannibalism.

However, the significant differences in the amount of time until all eggs were eaten between the untreated control females and the high dose of estradiol treated females suggests that estradiol plays a role in maternal behavior. Estradiol induces maternal care and increases oxytocin mRNA levels in female rats, supporting the role of hormones in regulating maternal behavior (Sakai & Yamanouchi 2007; Chung et al 1991). It is unclear how this hormone affects stimuli responses and influences behaviors in invertebrates. Female earwigs may have eaten their eggs out of fear of predation and estradiol might have enhanced a stress response. Conversely, estradiol might have decreased maternal instincts, also resulting in cannibalism.

4.4. Quantification of estradiol

Not surprisingly, treatment with estradiol resulted in elevated levels of estradiol detected by ELISA. However, the untreated control and acetone control females also had detectable estradiol. Estradiol has been detected in the ovaries of *C. gigas*, *B. mori*, and *L. migatoria*, and in the hemolymph, posterior silk gland, and larval and pupal fat body of *B. mori* (Li et al 1997; Ohnishi et al 1985; Das 2016). Estrogens have been detected in many plant species, leading to the hypothesis that insects, such as *B. mori* and *L. migatoria*, obtain estradiol from their food source (Ohnishi et al 1985). It is also possible that these species naturally synthesize estradiol (Ohnishi et al 1985). *E. annulipes* were not exposed to any plant material and the cat food company claims to not include any hormones in the ingredients, leading to the conclusion that the untreated females naturally synthesized the estradiol. This hypothesis is further supported by the presence of vertebrate steroid receptors in invertebrates. For example, the migratory locust

has progesterone binding proteins, while *Sarcophaga bullata* and *Brachinous manjavacas* demonstrate specific binding for estradiol and there are binding sites for progesterone and testosterone in *Calliphora vicina* (Keshan and Ray 2001; Jones et al 2017). Future research would need to be conducted to determine whether vertebrate estrogen receptors occur in *E. annulipes*.

The untreated control females had detectable levels of estradiol, but they were low enough to be reasonable for an earwig to synthesize on its own. The acetone control females had much higher levels of estradiol. If female *E. annulipes* do naturally synthesize estradiol, it is possible that acetone could act as a precursor to estradiol, resulting in the elevated levels of estradiol detected by ELISA analysis. In vertebrates, acetate is involved in cholesterol biosynthesis, which is a precursor to steroid hormones, including testosterone, progesterone, cortisol, vitamin D, and estradiol (Akoh and Min 2008). In addition, acetone is produced as a byproduct during cholesterol synthesis in vertebrates (Akoh and Min 2008). Because the chemical formulas of acetate ($C_2H_3O_2$) and acetone (C_3H_6O) are similar, it is possible that *E. annulipes* utilizes acetone in cholesterol synthesis, which is then converted to estradiol. However, insects are unable to naturally synthesize sterols, such as cholesterol, suggesting that *E. annulipes* should not be able to as well (Nation 2002). The ability to synthesize sterols is mixed among other invertebrates (Nation 2002). Cholesterol synthesis, regardless of the source, is important in insects because it is sequestered from the hemolymph and converted to ecdysone or other related ecdysteroids and therefore helps regulate molting and reproduction (Nation 2002). Because estradiol was detected in untreated earwigs, it is also possible that they naturally synthesize cholesterol, rather than obtaining it from a food source. Further analysis would need to be completed to determine if these mechanisms occur in *E. annulipes*. Although the origin and

precise mechanisms of estradiol in *E. annulipes* are not clear, its involvement in reproductive behaviors seems clear. Future research in this area will hopefully confirm the significance of vertebrate steroids in insects.

Acknowledgements

I am grateful to my research advisor, Dr. Susan Rankin, for not only guiding me in my entire research process, but also for having faith in my research design from the beginning, despite the high level of uncertainties we expected to, and did, face. Throughout the past year Dr. Rankin has challenged me to approach my research with a critical eye and mind, helping me to become the successful Allegheny student I am today. I thank Dr. Rankin for the technical assistance she provided in the laboratory, in addition to the advice and support she provided outside of the laboratory. I thank my second reader, Dr. Margaret Nelson, for providing insight into my research that I did not originally see on my own. I am grateful to the Biology Department for funding my Senior Comprehensive Research Project and for preparing me for life after I leave Allegheny College.

I thank my Allegheny friends-- Sonya Shaikh, Cari Koerner, Ashley Mulryan, Kristen Migliozzi, and Jackie Verrecchia-- for being by my side throughout my entire Allegheny College career, and especially during my senior year. I am grateful for the companionship and support they have provided inside and outside the classroom as I approach the completion of this stage of my life and prepare to transition to the next.

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