The effects of estradiol-17β on the sex reversal, survival, and growth of green sunfish *Lepomis cyanellus*

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Abstract

The feminization of green sunfish *Lepomis cyanellus* could expand their utility as a game fish or aquacultured species by preventing overcrowding and precocious reproduction in stocked systems. Feminization of green sunfish could also help elucidate information on their sex determination system. We report the feminization of green sunfish cohorts via oral administration of estradiol-17β (E2) during early development. A low-dose (100 E2 mg per kg of diet) and a high-dose (150 E2 mg per kg of diet) experimental E2 treatment were fed to juvenile green sunfish from 30 – 90 days post-hatch. Fish were subsequently evaluated for any treatment effect on gonadal development, survival, and growth. Both E2 treatments resulted in 100% feminization, with no morphological or histological differences detected between E2 treated ovaries and those from a control group. The control group was composed mostly of males (82.61%). Overall, there was no effect of E2 on survival ($P = 0.310$) and growth rate data suggested no statistical differences ($P = 0.0805$). However, the growth rate of the high-dose group increased slightly higher after the treatment ended than the other treatments ($P = 0.042$), suggesting that E2 might suppress growth in green sunfish. In addition, the control group did not exhibit a higher survival rate after the treatment period ended ($P = 0.266$), whereas both E2 treated groups did ($P = 0.0003 – 0.0050$). We found that the low dose, 100 E2 mg per kg of diet, was sufficient for fully feminizing green sunfish if administered during development between 30-90 days post-hatch and E2 dosages may result in deleterious effects on green sunfish's health and growth.

Keywords

Neofemales; Exogenous steroids; Feminization

1. Introduction

Green sunfish *Lepomis cyanellus* is a widespread North American Centrarchid species that has been introduced to exotic locales around the world (Lemly, 1985; Dudley and Matter, 2000; Yun-Chang et al., 2008; Fuller et al., 2021). This species belongs to one of the most economically important teleost families, Centrarchidae, which has value in both commercial aquaculture and sport fisheries (Brunson and Robinette, 1986; Wang et al., 2008; Morris and Clayton, 2009; Quinn and Paukert, 2009). However, management of Centrarchids in small water bodies can be difficult due to their proclivity for precocious reproduction resulting in overcrowding and stunting (Goodson 1966; Hackney, 1975; Wang et al., 2008). Green sunfish specifically have a propensity to overpopulate their habitats leading to the suppression of sport fishes and threatened native species (McKechnie and Tharratt, 1966; Moyle, 1976; Werner and Hall, 1977; Dudley and Matter, 2000; Morris et al., 2005). For example, male green sunfish are especially aggressive due to their courtship and nest guarding behaviors (Brunson and Morris, 2000; Teal et al., 2022a) potentially leading to displacement and stunting of more desirable gamefish such as bluegill *Lepomis macrochirus* (Werner and Hall, 1977). The production and stocking of monosex green sunfish via hormonal sex reversal may facilitate stocking green sunfish as sportfish or for commercial aquaculture purposes where reproduction is undesired (Al-Ablani, 1997) and thereby could reduce the problem of overcrowding and assist population management.

Sex reversal methods are useful in aquaculture because they facilitate faster growth curves and the growout of the larger sex (Al-Ablani, 1997; Wang et al., 2008), thus increasing production and profitability. Aquaculture methods for members of the *Lepomis* family are relatively sparse and more research needs to be conducted on the production and economic

feasibility of culturing these species (Brunson and Morris, 2000). Since male green sunfish are larger than females (Hunter, 1963), the production of males for aquaculture purposes could increase profitability. Feminization of males through the administration of estrogen during their sexual development can allow for indirect production of all-male cohorts of fishes (Piferrer, 2001). Feminization is performed by feminizing genetic males to the extent of developing functional ovaries and then selectively spawning these sex-reversed males (neofemales) with wild type males (Piferrer, 2001; Wang et al., 2008). If the fish have a ZZ-male/ZW-female sex determination system then the resulting spawn from a neofemale would be 100% male (Senior et al., 2013), barring any non-chromosomal effects on sex determination (Piferrer, 2001; Shen et al., 2016). If the fish have an XY-male/XX-female sex determination system then YY males from the resulting spawn are selected as broodstock and crossed with wild type females to produce 100% male cohorts (Mair et al., 1997; Piferrer, 2001). The indirect method of producing all-male cohorts is preferential to the hormonal masculinization of cohorts, because stocked or commercially sold fish are never exposed to the exogenous steroid treatment and the possibility of incomplete sex reversal is eliminated (Piferrer, 2001; Wang et al., 2008).

Evaluating feminization methods for green sunfish could be crucial in elucidating their sex determination system (Desprez et al., 1995; Gomelsky et al., 2002). The mechanisms of sex determination and differentiation in green sunfish are unknown. Roberts (1964) did not identify sex chromosomes in green sunfish through karyotyping. Other green sunfish studies found evidence of female genetic markers using amplified fragment length polymorphism (López-Fernández and Bolnick 2007) and restriction-site associated DNA sequencing (Teal et al., 2022b). However, these studies either did not test their markers on larger sample sizes (López-Fernández and Bolnick 2007) or were unable to develop a reliable marker (Teal et al., 2022b).

While these previous studies suggests that females maybe the heterogametic sex, these female specific loci may have been false positives as markers for the sex chromosome due to the small sample sizes and loci discovery methods implemented in their methods. Effective sex reversal treatments could validate the presence of sex chromosomes because sex ratios of progeny from neofemales crossed with wild-type males will be 3:1 male to female or 100% male depending on if the female is the homogametic sex or the heterogametic sex, respectively (Gomelsky et al., 2002; Desprez et al., 1995). This evidence would validate or dispute the preexisting evidence that female green sunfish are heterogametic for the sex determining region or regions of the genome. Uncovering of the sex determination system in green sunfish could provide more insight into the complicated evolution of sex determination systems in Centrarchids (Gamble et al., 2015; Nelson, 2018; Wang et al., 2018).

If sex chromosomes exist in green sunfish, then effective sex reversal treatments could facilitate efforts at controlling invasive populations. Green sunfish are ecologically destructive when introduced outside of their native range (McKechnie and Tharratt 1966; Lemly, 1985; Dudley and Matter 2000). Novel approaches at suppressing and eradicating invasive fish populations, such as the release of Trojan sex chromosome (TSC) carriers, are theoretically effective (Gutierrez and Teem, 2006; Senior et al., 2013; Schill et al., 2017; McCormick et al., 2021) and are already undergoing field trials with brook trout *Salvelinus fontinalis* (Kennedy et al., 2018; Teem et al., 2020). Green sunfish's persistence and fast generation time makes it a desirable candidate for the use of a TSC eradication strategy. The development of TSC carriers requires an effective sex reversal treatment and subsequent selective spawning to develop a broodstock capable of producing large numbers of either YY individuals or ZZ females (Gutierrez and Teem, 2006; Senior et al., 2013; Schill et al., 2016). These TSC carriers would

117 then be released into a nuisance population where they could spawn with wild-type females and
118 shift the sex ratio towards all male, theoretically eradicating the population (Gutierrez and Teem,
119 2006; Senior et al., 2013; Schill et al., 2017; Teem et al., 2020; McCormick et al., 2021). The
120 development of an effective sex reversal treatment would allow for initial investigations into the
121 capability of using a TSC eradication strategy for green sunfish and feminization methods could
122 be useful in uncovering if the basic reproductive biology of this species is conducive to this type
123 of eradication strategy. In a species that is either male or female heterogametic, the first step in
124 producing TSC carriers is the feminization of genetic males (Senior et al., 2013; Schill et al.,
125 2016).

126 Green sunfish, as with all studied Centrarchids (Arslan, 2018), are gonochoristic, with
127 ovaries and testes differentiating directly from undifferentiated gonads (Teal et al., 2022a). Fish
128 are most susceptible to permanent sex reversal via exogenous hormone treatments if the hormone
129 treatments are administered prior to gonadal differentiation and end when gonadal differentiation
130 is first observable through histology (Hackmann and Reinboth, 1974; Piferrer, 2001). This period
131 of gonadal plasticity is referred to as the “labile period” (Piferrer, 2001), the growth period under
132 certain rearing conditions where exposure to endocrine disruptors or exogenous sex hormones
133 can result in permanently altered sex differentiation (Hackmann and Reinboth, 1974; Piferrer,
134 2001). Although the gonadal development of green sunfish has been investigated (Yun-Chang et
135 al., 2008), the timing of the labile period is still generally unknown. We found in a previous
136 study that the labile period is 39 dph up to 99 dph under our rearing conditions (Teal et al.,
137 2022a). However, this information was unavailable to us when designing the featured sex
138 reversal treatments and our onset, duration, and hormone dosages in this study were based on
139 effective male to female sex reversal trials conducted on bluegill (Wang et al., 2008).

Estradiol-17β (E2) is a natural estrogen commonly used in the feminization of male fish. However, E2 treatments have varied in their effectiveness at feminizing certain species. The range for effective E2 dosages for feminization is from 1 mg E2 per kg of diet up to 750 mg E2 per kg of diet depending on the species treated and the duration of the treatment (Piferrer, 2001). Further, E2 treatments can negatively impact the survival and growth rates of fish if an exposure threshold is surpassed (Hunter et al., 1986; George and Pandian, 1996; Piferrer, 2001; Wang et al., 2008). The objective of this study was to examine the effects of two doses of E2 administered via diet on the sex reversal, survival, and growth rates of green sunfish.

2. Methods

2.1 Larval Production

Spawns for the sex reversal treatments were obtained from four 473-L broodstock tanks stocked with two adult males and three adult females. The adult broodstock ($\bar{x}_{\text{total length}}$= 153.6 mm, SD = 47.2 mm) were collected from Parker Canyon Lake, Arizona, USA (GPS coordinates 31°25'37.0" N, 110°27'25.0" W) during the Spring and Summer of 2018 and 2019. Green sunfish rearing methods and feed transitions followed protocols designed by Teal et al. (2022a). Briefly, eggs from each broodstock spawn were given a 30 min 100-ppm formalin treatment before being stocked in 37.9-L plastic tubs each outfitted with a 50-W Jager EHEIM drop in heater (EHEIM GmbH & Co, Deizisau, Germany), air stone, 10 g of activated carbon, and QANVEE Bio Sponge filter (Taian Qanvee Aquarium Equipment Co., Ltd., Shandong, China). Once eggs hatched, larvae were reared in the same tanks with the following water quality parameters: temperature 27-30º C, ammonia < 0.25 ppm, nitrite < 1.0 ppm, and pH 8.0 - 8.4. Upon swim-up stage (3-4 days post-hatch [dph]) larvae were fed with < 24-hour old brine shrimp nauplii four times per day at a rate of ~125 nauplii/L (estimate based on weight of unhatched cysts and ~90%

hatching rate). At 25 dph we continued to feed the green sunfish nauplii four times a day and began feeding Otohime B1 diet (B1: 200-360 μm, 51% crude protein, 11% crude fat) (Pentair Aquatic Eco-Systems, North Carolina, U.S.A.) twice a day. When fish were 30 dph, we fed them nauplii once a day and started feeding B1 diet six times a day using an EHEIM automated fish feeder.

2.2 Experimental Design and E2 Treatments

At 30 dph, when fish were 7.5 mm to 21.0 mm in total length (TL), 50 juveniles from each larval tank, that were progeny from one of four brood stock tanks, were randomly assigned to a treatment tank to create a randomized block design. In our usage of this design, the broodstock tank the juveniles originated from determined their "block". Therefore, each treatment tank was a replicate and contained progeny from one of four broodstock tanks, with a total of four replicates for each treatment. To avoid pseudoreplication, each treatment tank was considered a study unit with each treatment (control, low-dose, high-dose) having four replicates for a total of 600 fish involved in the study. The E2 treatment groups were fed either a 100 mg E2 per kg of diet (low-dose) or a 150 mg E2 per kg of diet (high-dose) from 30 – 90 dph.

Following methods from Wang et al. (2008), treated diets were prepared by dissolving 100 mg E2 or 150 mg of E2 into 400 ml of ethanol. The estradiol-17β was purchased from Sigma-Aldrich (Sigma-Aldrich, Massachusetts, U.S.A). One hundred milliliters of this solution was mixed with 250 grams of the B1 diet in a stand mixer to achieve the 100 mg E2 per kg of diet and the 150 mg E2 per kg of diet concentrations. The treated diet was then spread across a large baking sheet and placed in a fume-hood overnight. The control diet was prepared the same way except without the addition of E2. The tanks used during the treatments had identical configurations as the larval rearing tanks and water quality parameters of these treatment tanks

were maintained at: temperature 15 – 24º C, ammonia < 0.25 ppm, nitrite < 1.0 ppm, and pH 8.0 - 8.4. Each treatment tank was self-contained with its own individual filter and no water was shared between treatment tanks.

Subsets of 10-22 of these 50 randomly selected fish assigned to each treatment tank were measured for TL (mm). Until 37 dph, six daily feedings of E2 treated diet or control diet were supplemented with one daily feeding of nauplii to assist with weaning fish off a live diet. At 37 dph we stopped feeding nauplii and only fed B1 treated diets six times a day. During the treatment period the fish in each tank were fed 5.97 – 11.24% body weight per day. This feed rate converts to 55.00 - 75.60 mg of diet fed to each tank daily. The total amount of E2 distributed to each treatment tank during the treatment period was 0.33 – 0.45 mg. At 91 dph the fish were switched onto an untreated diet and all the fish were measured for TL (mm) and weight (g). At 91 dph a 50% water change was performed to expedite the clearing of any residual hormone from the treatments. Mortalities were recorded daily from the start of the feeding trial at 30 dph to the study conclusion at 495 dph. The treatment tanks were siphoned daily and a 10% water change was performed weekly.

At 285 dph, all surviving fish from each larval rearing tank were measured for TL (mm) and weight (g) before being transferred to one of twelve 757 L round fiberglass tanks that were part of a recirculating aquaculture system (RAS). The RAS was composed of thirty 757 L round fiberglass tanks connected to a filtration system featuring a Lifegard ¾ hp in-line pump, an Emperor 750 W UV sterilizer (Pentair Aquatic Eco-Systems), a DF-6 Polygeyser bead filter (Aquaculture Systems Technologies, Baton Rouge, Louisiana), and a Dayton ½ hp in-line pump (Dayton Electric Mfg. Co., Niles, Illinois 60714 U.S.A.). Aeration was provided to each tank by a blower (WW80 Whitewater, Pentair Aquatic Eco-Systems).

From December 12, 2020 – December 18, 2020, 5-14 green sunfish between 437 – 495 dph were removed from each treatment group replicate and euthanized by immersion for 10 minutes in 100 ppm of MS 222 (Pentair Aquatic Eco-Systems, North Carolina, U.S.A) buffered with 150 ppm sodium bicarbonate. Fish from each replicate were all the same age, but age varied among treatment replicates. We chose this age range for sampling (437 – 495 dph) because we knew green sunfish could reach sexual maturity by seven months (Yun-Chang et al., 2008; Teal et al., 2022a) and we wanted to ensure that all individuals were reproductively mature. The fish were measured for TL (mm) and weight (g). Both gonads were removed from the fish and weighed (g). The sex ratio of each replicate tank was evaluated based on macroscopic inspection of gonads and conducting the gonad squash method on one gonad (Guerrero and Shelton, 1974). The other gonad from 20 green sunfish from each E2 treatment group and the other gonad from 15 green sunfish from the control group were submitted to Fishhead Labs (Stuart, Florida) for routine histological processing and hematoxylin and eosin staining. One histology slide was prepared per submitted fish with two sections of sagitally bisected ovary mounted to each slide. The histology slides were inspected to verify sex ratios obtained from the gonad squash method and to detect intersex individuals. General oocyte developmental stages and structure of the ovaries were compared among the treatment groups, as well as to relevant fish gonad literature (Yun-Chang et al., 2008; Teal et al., 2022a; van der Ven and Wester, no date) to check for any deviation from normal development. We investigated differences in oocyte development by using an AmScope 40x-2000x 3W LED Seidentopf trinocular compound microscope and AmScope 14MP camera (United Scope, LLC, California, U.S.A.) to count previtellogenic, vitellogenic, and atretic oocytes in a randomly selected 1.2 mm^2 section of ovary for all histology samples. Slides were inspected at 100x magnification. Due to the overall uniformity of oocytes

seen among the treatment groups, oocyte developmental stages were classified as previtellogenic, vitellogenic, and atretic. Vitellogenic oocytes were defined as any oocytes with conspicuous yolk granule ("oil droplet") development. We noted numbers of atretic oocytes because exposure to exogenous E2 has been shown to increase atresia and inhibit maturation of oocytes in zebrafish *Danio rerio* (van der Ven and Wester, no date).

2.3 Data Analysis

Data analysis was conducted using Microsoft Excel V 2102 and Program R V 3.6.1 (R Core Team, 2013). We used proportional binomial generalized linear models (GLMs) to compare the mean proportion of fish that were females in the E2 treated groups with the mean proportion of fish that were females in the control group. We used generalized linear mixed models (GLMMs) with a Gaussian error distribution to model the effects of age (dph) and tank treatment (low-dose, high-dose, or control) on the number of previtellogenic oocytes, vitellogenic oocytes, and atretic oocytes. We used random intercepts by 'tank' to control for pseudoreplication among fish from the same tank (Gillies et al., 2006; Bolker et al., 2009; Zuur). We then conducted a Tukey post hoc analysis with the GLMMs using the Kenward-Roger method for calculating degrees of freedom to compare mean number of previtellogenic oocytes, vitellogenic oocytes, and atretic oocytes among the various treatment groups. To isolate the effect that the differences in ages (i.e., days post-hatch) among the replicates might have had on the number of previtellogenic, vitellogenic oocytes, and atretic oocytes we used a GLMM with Gaussian error distribution to test the relationship of age with number of previtellogenic, vitellogenic, and atretic oocytes. We grouped together all sampled fish from the control group to conduct a chi-square test and assess if the sex ratio was significantly divergent from a 1:1 sex ratio. We used $\alpha = 0.05$ for all statistical tests.

We used a beta generalized linear models (BGLM) to compare the mean proportion of fish that survived among the treatment during the treatment period (30-90 dph) and during the post-treatment period (91-285 dph). We then fit additional BGLMs to conduct a post hoc analysis comparing the survival rates for each treatment group during the treatment period (30-90 dph) with their survival rates during the post-treatment period (91-285 dph) and used a Holm-Bonferroni (Holm 1979) correction to adjust *P* values for experiment-wise error.

We tested for differences in TL, weight, and gonadosomatic index among treatment groups using generalized linear mixed models (GLMMs) with Gaussian error distributions and random intercepts by 'tank'. We then conducted a Tukey post hoc analysis with the GLMMs using the Kenward-Roger method for calculating degrees of freedom to compare means among the various treatment groups. One control replicate's mean weight was an outlier that was over one standard deviation (SD) larger than the next largest mean weight. The removal of this one control replicate's mean weight did not change the *P* value enough to affect the significance of the differences among mean weights of the treatment groups so we included this replicate in our analysis. We used a GLMM with a Gaussian error distribution to model the effects of age (dph) and tank treatment (low-dose, high-dose, or control) on TL to test for differences in overall growth rates between the treatment groups during the first 285 dph.

We calculated absolute growth rates (AGRs) to compare growth rates of the different tank treatments during the treatment period (Wang et al. 2008), as well as 195 days after the treatment period ended. AGRs were calculated using the formula $AGR=(TL_2-TL_1)/T\times100$. Where TL_1 and TL_2 are the mean fish total lengths at the start and end of the growth period for each of the treatment tanks, and T is the time between measurements (Teal et al., 2022a). We used a one-way ANOVA to test for differences in AGR among the treatment groups at the end of

the treatment period and 195 days after the end of the treatment. We then used paired t-tests with a Holm-Bonferroni correction to compare differences in mean AGR between the treatment period and post-treatment period for each treatment group.

3. Results

Based on the gonadal squash method and histology results, 100% of fish sampled from the E2 treatment groups were feminized to the extent of developing ovaries absent of spermatogenesis (Table 1). We observed no morphological or histological differences between ovaries in the E2 treatment groups and ovaries in the control group. Oocyte maturation in the E2 treated groups appeared normal when compared to ovaries in the control group and the relevant histology literature (Figure 1). The mean number of previtellogenic, vitellogenic, and atretic oocytes in the treatment groups did not differ significantly (GLMM, $t_{9.38} <$ 0.830, P value > 0.6951; Table 2). We did not observe buildup of eosinophilic staining plasma or evidence of inhibition of ovary maturation that could have resulted from the E2 treatments (van der Ven and Wester, no date). The number of oocytes at various stages of development were not a significant function of age (GLMM, $t_{6.956} <$ -0.943, P value > 0.370).

The mean percentage of green sunfish that were sampled in the control group that were female was 17.39% (SD = 16.64%). The percentages of fish sampled that were male from each control group replicate were 100% (6/6), 83.33% (5/6), 83.33% (5/6), and 60.00% (3/5). The sex ratio of the control group was significantly divergent from a 1:1 sex ratio (Chi-Square Test, df = 1, P value < 0.005). The percentages of green sunfish that were phenotypic females in the E2 treatment groups were significantly greater than the percentage of females in the control group (GLM, $Z >$ 2.83, P value < 0.005).

The mean female GSI of the high-dose group ($\bar{x}$ = 1.62, 95% CI = 1.44 – 1.79) was higher than the mean female GSI of the low-dose group ($\bar{x}$ = 1.34, 95% CI = 1.13 – 1.55) and the control group ($\bar{x}$ = 1.22, 95% CI = 0.88 – 1.55), but the differences in mean GSI among the treatment groups were variable and suggest no statistical significance (GLMM, $t_{36.42}$ = 2.226, *P* value = 0.0802).

Differences in mean survival rates to the end of the treatment (91 dph) were small and not statistically significant among the treatment groups (BGLM, *Z* = 1.015, *P* value = 0.310). There was large variability of survival rates among replicates across the treatment groups (Table 3). Although not statistically significant, E2 did appear to have a deleterious effect on mean survival during the treatment period (Table 1; Figure 2). The control group had a slightly higher survival rate to 91 dph ($\bar{x}$ = 47.50% survived, 95% CI = 23.00 – 72.00 %) than the low-dose treatment group ($\bar{x}$ = 40.00% survived, 95% CI = 22.84- 57.16%) and the low-dose treatment group had a slightly higher survival rate than the high-dose treatment group ($\bar{x}$ = 36.00% survived, 95% CI = 21.07 – 50.93%). The differences in mean survival rates from 91 dph to 285 dph (195 days after end of treatment) among the treatment groups were not significant (BGLM, Z = 0.462, *P* value = 0.644). Mean survival rates increased for all treatment groups during the post-treatment period (Figure 2). This increase in survival rate was significant in the low-dose treatment group (BGLM, *Z* = 3.045, *P* value = 0.004660) and high-dose treatment group (BGLM, *Z* = 3.866, *P* value = 0.000333). The control group did not show a significant increase in mean survival rate during the post-treatment period (BGLM, *Z* = 1.113, *P* value = 0.266).

At the beginning of the treatment period (30 dph) there were no statistical differences (GLMM, $t_{8.94} < 0.986$, *P* value > 0.605) in mean TLs among the control group ($\bar{x}$ = 12.10 mm, 95% CI = 10.09 – 14.10 mm) and the E2 treatment groups (low-dose treatment $\bar{x}$ = 10.90 mm,

95% CI = 8.86 – 12.90; high-dose treatment $\bar{x}$ = 11.80 mm, 95% CI = 9.76 – 13.80). The control group had a slightly longer mean TL ($\bar{x}$ = 26.49 mm, 95% CI = 24.37 – 28.60 mm) than the low-dose treatment ($\bar{x}$ = 23.57 mm, 95% CI = 22.29 – 24.85 mm) and the high-dose treatment ($\bar{x}$ = 23.49 mm, 95% CI = 21.80 – 25.19 mm) at the end of the treatment period (91 dph), but the differences in mean TLs (mm) were not suggestive of being statistically significant (GLMM, $t_{8.85}$ = 2.492, *P* value = 0.0805). The differences in mean weights (g) among treatment groups at the end of the treatment period were not significant (GLMM, $t_{9.72}$ <1.845, *P* value > 0.2065). Overall growth rates (Figure 3), based on mean TLs (mm), did not differ significantly among the treatment groups from the start of the treatment (30 dph) to 285 dph (195 days after end of treatment) (GLMM, $t_{8.506}$ *control* β = 1.645, *P* value = 0.136).

Mean AGR among the treatment groups did not differ significantly during the treatment period (One-way ANOVA, $F_{2,9}$ = 1.916, *P* value = 0.203), and mean AGR among the treatment groups did not differ during the 195 days after the treatment ended (One-way ANOVA, $F_{2,9}$ = 0.074, *P* value = 0.929). Although mean AGR increased for both the low-dose group and the control group, only the high-dose treatment group showed an increase in mean AGR between the treatment period (30-90 dph) and post-treatment period (91-285 dph) that suggested statistical significance (Paired t-test, t_3 = 3.401, *P* value = 0.0424; Figure 4), with the mean AGR during the treatment period being 18.86 (95% CI = 15.08-22.64) and the mean AGR post-treatment being 24.74 (95% CI = 22.83-26.66).

4. Discussion

The treatment duration and E2 dosages we used were highly effective at feminizing green sunfish. We could not discern any morphological or histological differences between the E2 treated groups and the control group. Wang et al. (2008) observed one intersex individual out of

346 20 bluegill (*L. macrochirus*) sampled from their 30-90 dph treatment fed a 100 E2 mg per kg of
347 diet. We did not observe any evidence of incomplete sex reversal in either the low-dose or high-
348 dose treatment. Wang et al. (2008) conducted their treatments in a flow-through system, whereas
349 we used self-contained tanks with filters. Even though we added 10 g of activated carbon to each
350 tank to adsorb any E2 leeching out from the diet, our treated fish may have had some immersion
351 exposure to the E2 since we did not use a flow-through system (Hulak et al., 2008; McGree et
352 al., 2010). Using our treatment tank configurations, it may be possible to fully feminize green
353 sunfish if given a lower E2 dosage than 100 mg E2 per kg of diet from 30-90 dph. Based on the
354 complete cohort feminization we observed, and the 39-99 dph labile period reported by Teal et
355 al. (2022a), we believe the E2 treatment onset and duration were appropriate for this species.
356 However, alternative E2 exposure methods have been attempted and had varied success at
357 feminization in other species (Piferrer, 2001). For example, hormone baths while roughly half of
358 the eggs have hatched from a spawn have proven successful at feminizing cohorts of some
359 Salmonids (Feist, 1996). Therefore, alternate methods for administering E2 and shorter treatment
360 durations may also be effective at feminizing green sunfish. Using the lowest possible E2 dosage
361 and shortest treatment duration is preferential since our results and previous work show that E2
362 can have negative impacts on fish health and growth (Hunter at al., 1986; George and Pandian
363 1996; Piferrer, 2001; Peterson and Davis, 2012). More studies should be conducted with green
364 sunfish to identify the lowest effective E2 treatment for complete feminization.

365 Multiple studies have shown that exogenous E2 exposure can cause inhibition in the
366 progression of oocytes through vitellogenesis which in severe cases can result in sexual sterility
367 (van der Ven and Wester, no date; Komen et al., 1989). Furthermore, other studies on hormonal
368 sex reversal treatments in other fish species often exhibited highly conspicuous effects of E2

on fish gonads such as mixed sex ratios and intersex tissue in gonads of E2 treated fish (Yamazaki, 1983; Komen et al., 1989; Wang et al., 2008; Carvalho et al., 2014). Although infertility due to duct deformities are typically associated with exogenous androgen exposure (Johnstone et al., 1979; Piferrer, 2001), male to female sex reversals from exogenous estrogen exposure or other endocrine disruptors can result in the development of aberrant gonadal ducts (Jobling et al., 2002). We did not investigate occlusions of gonadal ducts or genital pores that could result in sexual dysfunction of our fish, but the overall macroscopic similarity of the ovaries coupled with the absence of any abnormal oocyte development provides strong evidence that the E2 treated fish we examined were sexually viable. We also saw no differences in the number oocytes at various stages of maturation and viability among the treatment groups and no inhibition of vitellogenesis. We therefore have no reason to believe our E2 treated fish are infertile nor sexually dysfunctional (Iwamatsu, 1999). It is possible the E2 treated groups contained larger vitellogenic oocytes than the control group which may explain the marginally significant (*P* value = 0.0802) increase in GSI in the E2 treated groups. However, additional experiments would be needed to explicitly test this hypothesis. In contrast to the trends observed in our study, exogenous estrogens, such as E2, may reduce ovary size, and thus the fish's GSI (Komen, et al., 1989; Piferrer, 2001). We did sample fish from December 12 – 18, which is temporally distant from the typical green sunfish spawning season in southeast Arizona. So. we are uncertain how this slight, and possibly not significant, increase in GSI observed during the winter may translate to GSI or fecundity in the spring spawning season

The highly male-skewed sex ratio (82.61 % male) seen in the control group could be evidence of an environmental influence on the sex determination system of green sunfish. It is

well known that rearing environment can influence sex ratios in many fish species (Piferrer, 2001; Baroiller et al., 2009; Shen et al., 2016). Stress, high temperatures, and high rearing densities during development can result in male-skewed sex ratios in many fish species (Baroiller et al., 1995; Roncarati et al., 1997; Ospina-A´ lvarez and Piferrer, 2008; Mankiewicz, et al., 2013; Hattori et al., 2020). In the closely related bluegill, which in at least some populations are speculated of having an underlying ZW/WW sex determination system, increased temperatures during sexual development can skew sex ratios towards all-male (Wang et al., 2018). Since we saw a sex ratio that is highly divergent from a 1:1 male:female ratio, it is possible that environmental conditions may influence the sex determination or differentiation of green sunfish. Without understanding how various rearing temperatures, rearing densities, and other stressors impact sex ratios, it will be difficult to use sex ratios of progeny from the crosses of neofemales with wild-type males to elucidate if green sunfish have a chromosome-based sex determination system. A chromosome-based sex determination system is necessary for the production of TSC carrying individuals that can be used to control nuisance populations (Senior et al., 2013). Therefore, uncovering the mechanisms that direct sex determination and differentiation is vital in assessing the candidacy of a species for a TSC eradication strategy.

Our heavily male-skewed control group suggests that producing all male cohorts without manipulating a chromosomal-based sex determination system and without the use of exogenous steroids could theoretically be possible for green sunfish (Piferrer, 2001; Angienda et al., 2010). The water temperature we reared our treatment cohorts in was 27-30º C which is within the suitable temperature range for bluegill reproduction (20-30º C; Mischke and Morris 1997) and well below the lethal temperature threshold of 41.2º C green sunfish (Carveth et al., 2006). Therefore, higher rearing temperatures or higher rearing densities could be attempted to

consistently produce all or mostly male green sunfish cohorts that can be stocked for fisheries or aquaculture practices. Environmental manipulation of green sunfish sex determination may also allow aquaculturists to produce high proportions of the larger sex without the need to selectively breed neofemales for indirect masculinization methods, thus avoiding regulatory oversight in the U.S. by the Food and Drug Administration. However, utilizing increased temperatures for producing male-skewed cohorts have been shown to reduce survival and growth rates in tilapia (Baras et al., 2001). Treatments attempting various rearing temperatures and densities with green sunfish should be conducted with a consideration of how these treatments may impact the fish's health.

Overall, the effects of the E2 treatment on the survival of green sunfish were slight. In a concurrent study, *Aeromonas hydrophila* infections were prevalent when green sunfish were being weaned from live nauplii to an artificial diet (Teal et al., 2022a). The increase in infection during this time was likely due to increased organic matter in the form of uneaten artificial diet and concomitant reduction in water quality in the tanks. Other studies have reported a reduced capacity of E2 treated fish to activate their immune response, decreasing their survival rate when challenged with pathogens (Yamaguchi et al., 2001; Wang and Belosevic et al., 1994; Casanova-Nakayama et al., 2011; Wenger et al., 2011). Additional investigations are needed to test the hypothesis that *Aeromonas hydrophila* infection rates are higher in E2 treated groups than the control group and that this contributed to slightly lower survival in the E2 groups. Exogenous E2 can also cause severe liver and kidney damage which can result in organ failure and be lethal (Zaroogian et al., 2001; Costa et al., 2010). The lethal E2 dose varies by species due to fishes' broad range in sensitivity to estrogens (Costa et al., 2010). In the current study it appears we did not cross a lethal threshold with our E2 dosages. However, lower E2 dosages should still be

attempted in green sunfish to mitigate possible damage or increased infection risk derived from E2 induced sex reversals.

The effects of E2 on the growth rate of green sunfish was small and not statistically significant. It is possible that the E2 did cause a slight reduction in mean TL at the end of the treatment period, but other factors such as varying survival and concomitant rearing densities among the treatment replicates might have confounded these effects. Previous research has reported compensatory growth in bluegill (Wang et al., 2008) and brook trout (Schill et al., 2016) after E2 treatments ended that may be attributed to a suppression of growth during the E2 treatments. We observed an increase in mean AGR for all treatment groups during the growth interval after the E2 treatment period, but only in the high-dose group was the increase statistically significant. The increase in AGR of the high-dose treatment group after the treatment period may be evidence of growth suppression caused by E2, but since we also observed a slight increase in AGR in the control group after the treatment period, other factors such as rearing densities may have contributed to this difference.

Fishes often react to exogenous steroids as either growth-promoting agents or as growth suppressors that may cause increased mortality (Pandian and Sheela, 1995; Piferrer, 2001). The deleterious effects of E2 typically only occur if a particular threshold of E2 treatment dosage or duration is surpassed (Hunter et al., 1986; George and Pandian, 1996; Piferrer, 2001; Wang et al., 2008). Although the reduction in survival and AGR we noted among the treatment groups were small and deemed not statistically significant, the high-dose group did exhibit the poorest survival rate and lowest AGR during the treatment period. We also observed that the E2 treated groups exhibited significantly higher survival rates during the post-treatment period than the treatment period while the control group did not show a significant increase in survival rate.

Although fish typically exhibit an increased likelihood of survival up to a certain age (Lorenzen, 1996), only the control group did not differ significantly in survival rates between the treatment period and the post-treatment period which may be due to the E2 treatments increasing mortalities during the treatment period. Overall, the variability in survival and growth rates were high, but our results suggest that exogenous E2 does not act as a growth-promoter in green sunfish and may increase mortality at high doses.

The marginal differences we observed in survival and growth rates of our E2 treated fish further suggest that it is possible to produce and use TSC carrying green sunfish for managing green sunfish populations if sex chromosomes are present in the species. Gutierrez and Teem's (2006) model demonstrated that 3% of the annual reproductive stock of a wild population must be YY females (TSC carrier) in order to eradicate a nuisance population over a matter of decades. The proportion of the wild population that needs to be a TSC carrier to eradicate a population only increases if using YY males instead of YY females or if faster a timeframe for eradication is desired (Schill et al., 2017). Therefore, reliable and efficient production of the TSC carrier broodstock and the TSC carriers is integral to the TSC eradication strategy since continual reintroductions of TSC carriers is necessary for female extirpation (Gutierrez and Teem, 2006), especially if TSC carrier fitness is lower than wild-type fitness (Senior et al., 2013; Schill et al., 2017). If a chromosomal sex determination system is ever discovered within green sunfish, than their fast maturation time and their amenable nature to E2 treatments could alleviate potential TSC carrier production constraints.

5. Conclusions

We developed highly effective male to female sex reversal methods for green sunfish. Using our rearing methods, feeding juvenile green sunfish 100 E2 mg per kg of diet or 150 E2 mg per

kg of diet from 30 – 90 dph resulted in 100% feminization of our treated cohorts with no gonadal abnormalities observed. Although the reductions in AGR and survival we saw for both the low-dose treatment and the high-dose treatment were small and not statistically significant during the treatment period when compared to the control group, we still recommend using a low-dose E2 treatment to prevent potential negative effects on the health and growth of this species. The information presented here could help expand the utility of this species as a game fish or aquacultured species, as well as help elucidate information on the sex determination system of green sunfish. We recommend additional studies evaluate possible environmental variables influencing sex ratios in this species.

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704 **Tables**

Treatment Dose (E2 mg/kg of diet)	*N*	Initial Number of Fish Per Treatment	Total Number of Fish Survived to end of Treatment	Total Number of Fish Sampled from Each Treatment Group for Gonad Assessment	Treatment Duration (dph)	Mean % Female (SD)	95% CI
0	4	200	95	23	30-90	17.39% (16.64%)	0%- 35.90%
100	4	200	83	32	30-90	100% (0%)	0%
150	4	200	72	24	30-90	100% (0%)	0%

705 Table 1.

706

707

Oocyte Stage	Treatment Groups (E2 mg/kg of diet)	Mean Number of Oocytes	SD	95% CI
Previtellogenic	0	91.0	20.8	52.9 – 129.0
	100	95.5	18.80	74.2 – 117.0
	150	93.7	30.67	71.9 – 116.0
Vitellogenic	0	16.2	3.51	4.05 – 28.3
	100	21.2	6.77	14.06 – 28.3
	150	20.7	8.13	13.34 – 28.0
Atretic	0	0.53	1.15	0.00 – 1.61
	100	0.43	0.94	0.00 – 0.96
	150	0.46	0.61	0.0 – 1.00

708 Table 2.

709

Treatment Tank (Replicate)	Treatment Dose (E2 mg/kg of diet)	Number of Fish Survived to End of Treatment	Mean GSI at maturity* (SD)	Mean TL (mm) at End of Treatment (SD)	Mean Weight (g) at End of Treatment (SD)
L24	0	18	NA	25.17 (5.00)	0.23 (0.14)
L28	0	21	1.32 ()	29.71 (5.12)	0.46 (0.24)
L30	0	42	1.09 ()	25.57 (3.89)	0.23 (0.10)
L31	0	14	1.23 (0.12)	25.50 (4.77)	0.24 (0.12)
L19	100	22	1.34 (0.11)	24.00 (4.33)	0.19 (0.12)
L21	100	18	1.19 (0.37)	24.11 (2.61)	0.19 (0.07)
L25	100	32	1.33 (0.29)	24.53 (3.19)	0.24 (0.10)
L27	100	11	1.39 (0.14)	21.64 (4.43)	0.14 (0.10)
L22	150	17	1.51 (0.40)	21.24 (3.40)	0.15 (0.08)
L23	150	14	1.88 (0.64)	24.79 (3.07)	0.20 (0.08)
L26	150	12	1.35 (0.30)	24.92 (1.38)	0.24 (0.04)
L29	150	29	1.66 (0.28)	23.03 (2.63)	0.21 (0.10)

Table 3.

715 **Figures**

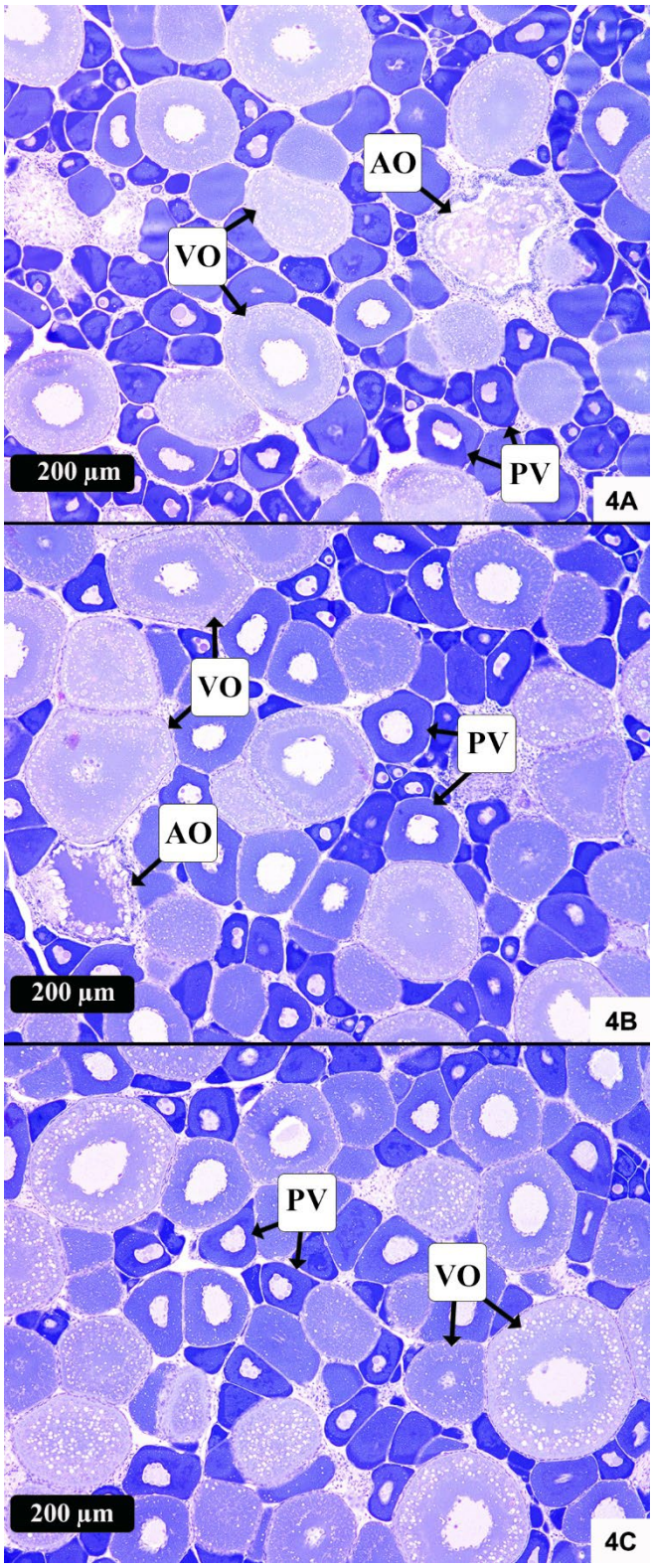

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Figure 1.

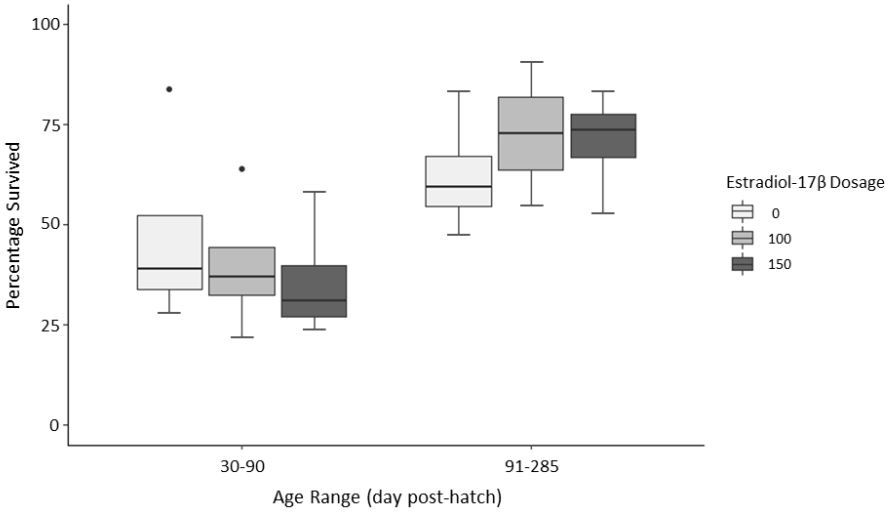
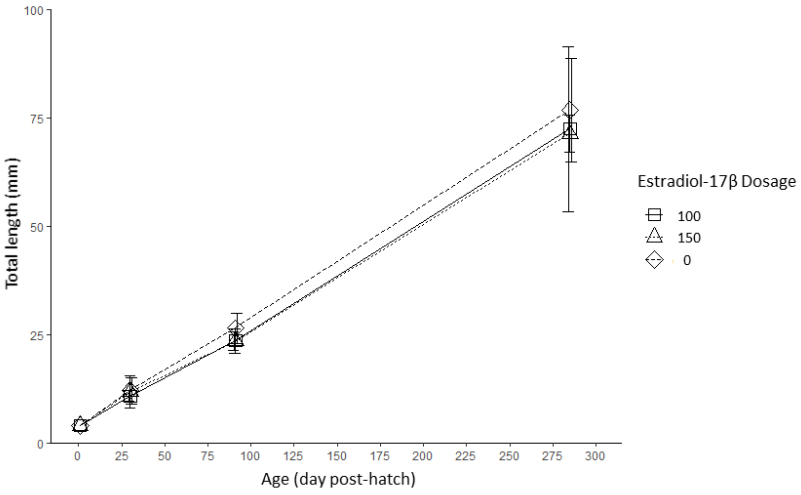
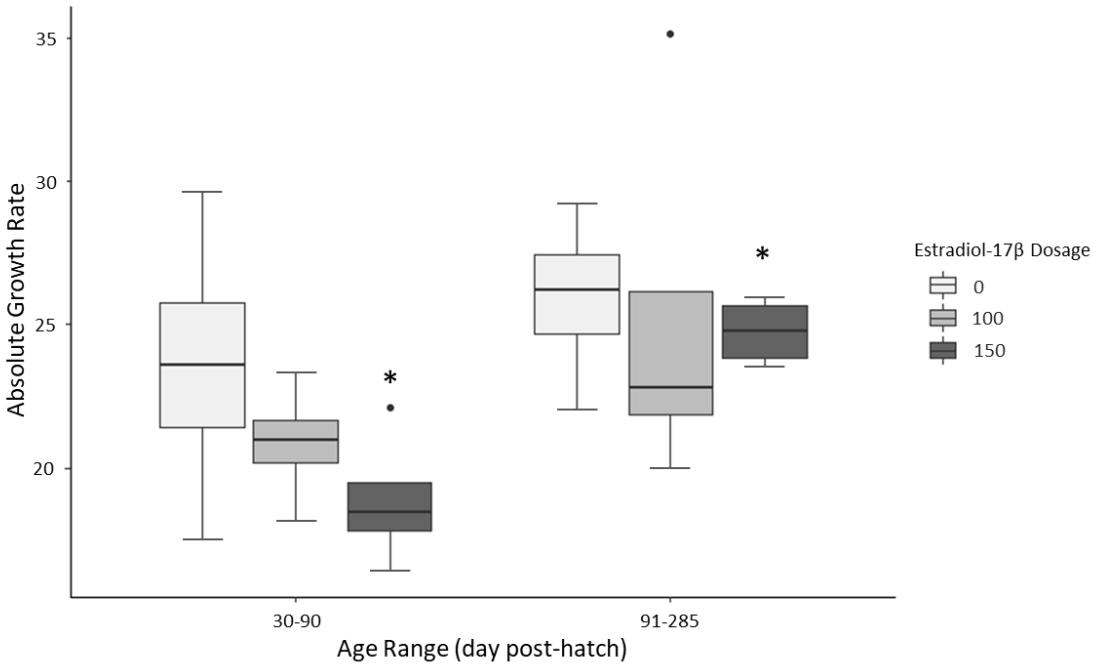


Figure 2.



721 Figure 3.



722

723 Figure 4.

724 **Table and Figure Captions**

725 Table 1. Total fish survived treatment and mean percent female of each estradiol-17β treatment
726 group and control group (0 mg estradiol-17β per kg of diet) of green sunfish.

727 Table 2. Mean number of oocytes at various stages of maturation among the green sunfish
728 treatment groups.

729 Table 3. Summary statistics for each green sunfish treatment tank (replicate). Empty parenthesis
730 for standard deviation (SD) parenthesis in the GSI column are because there was only one

731 female in these replicates. * Denotes that a mean GSI could not be calculated due to there
732 being less than two females sampled from this tank.

733 Figure 1. Ovaries from 431-480 dph green sunfish exposed to 100 mg estradiol-17β per kg of
734 diet (1B) or 150 mg estradiol-17β per kg of diet (1C) exhibited normal development and
735 contained oocytes at various levels of maturation (PV= previtellogenic oocyte, VO =
736 vitellogenic oocyte, AO = atretic oocyte), similar to the ovary in this 437 dph green sunfish
737 female from the control group (1A).

738 Figure 2. Mean percentage of green sunfish survival rate across two groups treated with
739 increased estradiol-17β dosages (100 = 100 mg estradiol-17β per kg of diet; 150 = 150 mg
740 estradiol-17β per kg of diet) and a control group (0 mg estradiol-17β per kg of diet) during
741 the treatment period (30-90 days post-hatch) and during a post-treatment period (91-285
742 days post-hatch).

743 Figure 3. The mean total lengths (mm) among the estradiol-17β treatment groups (100 = 100 mg
744 estradiol-17β per kg of diet, 150 = 150 mg estradiol-17β per kg of diet) and the control
745 group (0 = 0 mg estradiol-17β per kg of diet) of green sunfish up to 285 days post-hatch
746 (error bars represent 95% confidence intervals).

747 Figure 4. Comparison of absolute growth rates (AGR) during the treatment period (30-90 days
748 post-hatch) versus after the treatment period (91-285 days post-hatch) for green sunfish
749 across two estradiol-17β treatment groups (100 = 100 mg estradiol-17β per kg of diet; 150 =
750 150 mg estradiol-17β per kg of diet) and a control group (0 mg estradiol-17β per kg of diet).
751 Significant differences between treatment periods are denoted with asterisks.