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Developmental immunity of the skeletal elements of the Weberian apparatus to the effects of exogenous estrogen (17-β estradiol), a known disruptor of cartliage development

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DEVELOPMENTAL IMMUNITY OF THE SKELETAL ELEMENTS OF THE WEBERIAN APPARATUS TO THE EFFECTS OF EXOGENOUS ESTROGEN (17-β ESTRADIOL), A KNOWN DISRUPTOR OF CARTILAGE DEVELOPMENT

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Date	Dr. Nathan Bird, Honors Thesis Advisor, Biology
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Abstract

Proper vertebrate development is controlled by a tightly regulated sequence of gene activation and repression. Exposure to both endogenous (hormones, gene products) and exogenous (environmental chemicals, toxins, etc.) substances during development can have profound effects on morphology by altering growth, cell fate, cell differentiation, and cell migration. Waterways have long been hot-spots for chemical and toxin accumulation, due to runoff and waste dumping. Growing concern is being paid to endocrine disruptors, like estrogen (17β-estradiol), which are found in increasing levels in rivers and streams. Endocrine disruptors are known to cause a varied number of defects in fish species, but the effect of estrogen on early vertebral development is unknown. In this study, the effect of exogenous estrogen (17β-estradiol) on development of the zebrafish Weberian apparatus is examined. The Weberian apparatus is an evolutionary adaptation of the four anteriormost vertebrae, and is unique to otophysan fishes (minnows, catfishes, characins, and South American electric eels). The function of the Weberian apparatus is to relay and amplify sound pressure changes from the gas bladder to the inner ear through physical coupling, and is hypothesized to be an independent developmental and evolutionary module, separate from ancestral vertebrae. High-dose estrogen proved lethal to larval zebrafish. Lower concentrations and shortened exposure times produced no morphological abnormalities within the cartilage elements of the Weberian apparatus, and overall effect on cranial cartilages was inconclusive. In addition, growth rates were not different across treatment groups. Results suggest that the Weberian apparatus may be immune to low doses of estrogen during early larval development, but given the overall lack of morphological effects across the body, immunity may not be limited to the Weberian apparatus alone.

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INTRODUCTION

Endocrine Disruptors and the Environment

Endocrine Disruption

Endogenous endocrine hormones (estrogen, androgens, growth factors, etc.) are important developmental regulators in vertebrates, and are implicated in proper development, growth, metabolism and reproduction. Precise spatiotemporal control within the body is crucial for proper development and growth in nearly all anatomical systems. A growing concern for all vertebrates is the rapid increase of endocrine disruptors (EDs) present in the environment. Endocrine disruptors have been defined as agents that interfere with the production, release, transport, metabolism, binding action, or elimination of naturally occurring endogenous hormones (Kavlock et al., 1996, reviewed in Pait and Nelson, 2002). A small list of recognizable endocrine disrupting compounds includes estrogens (and estrogen mimics), Bisphenol-A (BPA), Atrazine, DDT, mercury, and lead (Pait and Nelson, 2002). Many of these man-made endocrine disruptors are present in growing concentrations in freshwater systems.

Environmental Impact

EDs have been found in many streams and rivers throughout the United States. Areas of extreme interest are agricultural rich regions (a product of run-off), as well as wastewater treatment plant effluents. A study conducted by Koplin et al. (2002) looked at 139 streams across 30 states, and found 80% of the streams showed measured concentrations of 1 or more of the 95 contaminates investigated. At the time, little data was available on the occurrence of most of the targeted hormones and pharmaceuticals being investigated, so sampling sites focused on areas considered susceptible to contamination from human, industrial and agricultural wastewater (Kolpin et al., 2002). A specific example from the study showed that BPA, a known endocrine disruptor, was found in 41.2% of the streams sampled at μg/L levels (Kolpin et al., 2002). A subsequent study looked into the contamination

of the waters of the Great Lakes and Upper Mississippi River Regions by endocrine disruptors discharged from waste water treatment plants from several large cities, including Akron, Detroit, Indianapolis, Duluth, St. Paul and Chicago (Barber et al., 2015). The 10-year study looked at 16 different chemicals and their concentrations, and found BPA in all waste water treatment plant effluents tested (Barber et al., 2015). Another study evaluated the potential endocrine disruption across 11 Minnesota lakes that did not have waste water treatment plant discharge (Writer et al., 2010). Several endocrine disrupting chemicals, such as bisphenol A and estrogen (17β-estradiol), were found in 90% of the lakes at part per trillion concentrations and endocrine disruption was observed in caged fathead minnows and resident fish in 90% of the lakes as well (Writer et al., 2010). In the study, plasma vitellogenin, an egg-yolk protein normally absent in the plasma of male fish that can be induced by exposure to estrogenic compounds, was detected in minnows after exposure to all lakes studied (Writer et al., 2010). Additionally, testicular feminization was observed and a decrease in the ratio of immature to mature sperm in eutrophic lakes compared to oligotrophic lakes and lakes were classified based on their amount of anthropogenic influences (Writer et al., 2010).

Effects of Exogenous Endocrine Distruptors on Fish Biology

Biological Effects of Estrogen and Other EDs

ED infiltration of streams and lakes has led to profound effects on fish biology across many anatomical systems. Reproductive disruption has been observed in fish found in estrogen-contaminated waters (Jobling et al., 1998) and endocrine disruptor contaminated water (Writer et al., 2010). Effluent from the wastewater treatment plant in the city of Boulder, Colorado induced demasculinization of primary and secondary sex characteristics of male fathead minnows and vitellogenin was maximally elevated within 7 days of exposure (Vajda et al., 2011). The steroidal estrogens 17β-estradiol, estrone, estriol, and 17α-ethynylestradiol, as well as estrogenic alkylphenols and BPA, were also found within the effluent (Vajda et al., 2011).

While disruption of the reproductive system is noted most in the literature, other systems are potentially affected as well. In addition to testicular feminization, a decreased ratio of immature to mature sperm, and increased plasma vitellogenin in males, increased liver hepatocyte vacuolization in urban and urban/agricultural lakes was also found (Writer et al. 2010). Complement activity, peroxidase activity and IgM levels in the gilthead seabream *Sparus aurata* were all altered in the presence of 17β-estradiol (Cuesta et al., 2007) and estrogens and estrogen-like endocrine disruptors appear to affect the balance of proliferation/apoptosis of lymphocytes (Milla et al., 2011). These findings suggest the immune system may also be influenced by endocrine disruption.

ED-Mediated Skeletal Defects

Research surrounding endocrine disrupting chemicals has focused on reproductive disruption, but EDs are also part of a long list of chemicals, genes, and hormones that regulate bone formation and growth. For example, calcitonin (needed for normal bone development), can block bone breakdown by inactivating osteoclasts (Stepnick, 2004). Insulin-like growth factor affects bone growth, including chondrocyte proliferation and hypertrophy (Yakar et al., 2002). Excessive production of Parathyroid hormone (PTH) can lead to bone loss (U.S. DHHS, 2004), and the deletion of the dopamine transporter gene in mice showed a reduced bone mass and strength (Bliziotes et al., 2002). An important ED that shows reproductive disruption and bone regulation is estrogen, which plays a key role in regulating bone mass by controlling activity of osteoblasts and osteoclasts (Kameda et al., 1997; Imai et al., 2009). Estrogen also regulates chondrogenesis (Fushimi et al., 2009; Cohen et al., 2014), and skeletal gene expression (Pashay et al., 2016).

Previous research has focused on estrogen's influence on chondrogenesis and skeletal gene expression during larval head development, specifically earlier than 7 days post-fertilization (Fushimi et al., 2009; Cohen et al., 2014; Pashay et al., 2016). Research showed that zebrafish exposed to aqueous 17β-estradiol at larval stages showed cartilage malformation leading to fish with shorter and flatter

faces (Fushimi et al., 2009; Cohen et al., 2014; Figure 1), and in general skeletal gene expression during larval head development decreased when exposed to 17β-estradiol (Pashay et al., 2016).

Vertebrate bones can form through two different developmental modes: through a cartilage precursor or by direct ossification from a mesenchymal condensation, also called membrane bone or dermal bone (Bird and Mabee, 2003; Hall, 2015). In the vertebral column, both types of bone are derived from cells of the somitic mesoderm (Gilbert, 2003), and the migration of these cells from segmented mesoderm (somites) leads to a resegmentation and results in the classical pattern of vertebrae (van Eeden et al., 1998; Morin-Kensicki et al., 2002). Alteration of the development and segmentation of the mesoderm into somites, migration of the precursor cells, or signals directing their fate often lead to abnormalities in both chondrogenesis and osteogenesis within the axial skeleton (van Eeden et al., 1996).

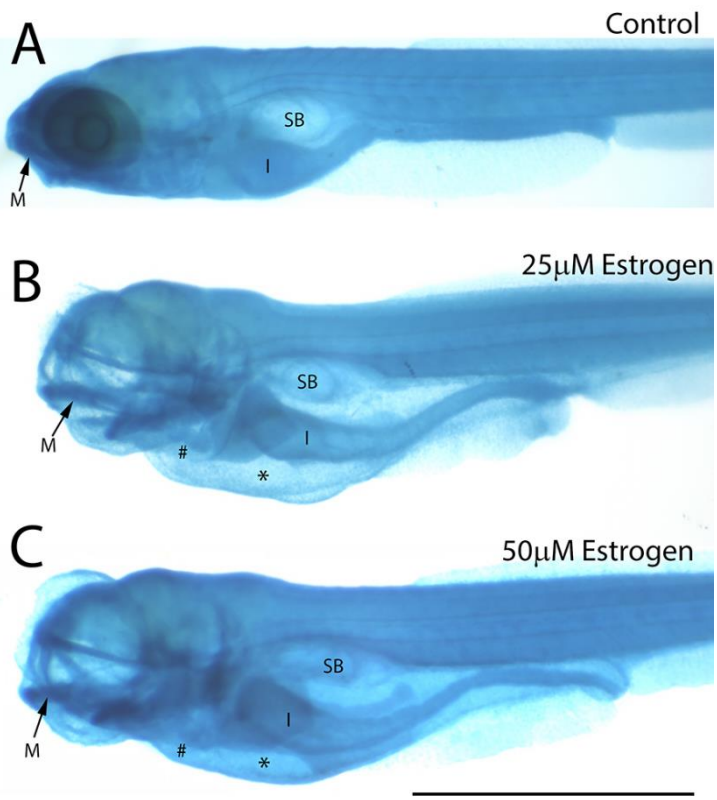

Figure 1. Generalized effects of high-dose estrogen on 9-days post-fertilization larval zebrafish (treatment from 7-9 dpf). Normal morphology seen in A (control) versus 25 (B) and 50 μM (C) estrogen. Effects include reduction of swim bladder (SB) and intestinal thickness (I), pericardial (#) and abdominal (*) edema, and shortening and improper projection of Meckel's cartilage (M). Scale bar = 1mm.

Studies in the zebrafish, *Danio rerio*, have identified several genes important at various stages of vertebral development (van Eeden et al., 1998). Previous work has identified a group of mutants, known as segmentation/*Fss*-type mutants, which lead to abnormal vertebrae via breakdown of proper

mesodermal segmentation during embryogenesis (van Eeden et al., 1996; van Eeden et al., 1998). These include the mutants *fused somites* (*fss*), *beamter* (*bea*), *deadly seven* (*des*), *after eight* (*aei*), and *white tail* (*wit*), which all exhibit breakdown in paraxial segmentation by disrupting the formation of the anterior-posterior somite boundaries (van Eeden et al., 1996; van Eeden et al., 1998). All of these mutants are adult viable, and show dramatic defects in the vertebral column. While defects are extensive in posterior vertebrae, one region, the Weberian apparatus, is unaffected in these mutants (Bird, unpublished), suggesting potential immunity to the loss of these genes and potential developmental independence from the rest of the vertebral column.

Weberian Apparatus and Vertebral Column

The Weberian apparatus is a complex evolutionary innovation of the four anteriormost vertebrae, swim bladder, and inner ear (Figure 2). The Weberian apparatus captures far-field sound via the swim bladder (Figure 2, green), transduces it into a near-field source, amplifies the sound, and relays it from the swim bladder to the inner ear (Figure 2, blue) through physical coupling of modified vertebral elements (Figure 2, red) known as the Weberian ossicles (Ladich and Popper, 2004). Fish without the Weberian apparatus or another auditory adaptation generally hear only near-field sound inputs, *i.e.* those close enough to cause shearing of the otoliths against the sensory epithelium (Fay,

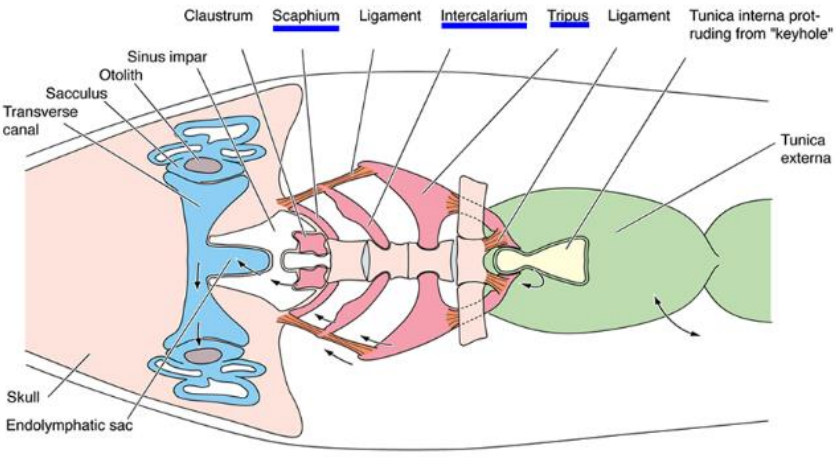

Figure 2. Cartoon representation of the integration and function of the Weberian apparatus. Sound captured by the swim bladder (green) is transduced, amplified, and relayed to the inner ear (blue) by the Weberian ossicles (red), which are connected via ligaments. The critical ossicles (scaphium, intercalarium, tripus) are underlined. Modified from Liem et al. 2001.

1999; Ladich and Popper, 2004), while fish with the Weberian apparatus allows for detection of sound over much wider frequency ranges, and requires less amplitude (loudness) to be detected (Fay, 1999). The function of the Weberian apparatus is analogous to the middle ear of mammals, which also uses coupling of three bones (malleus, incus, stapes) to transfer and amplify sound from the receiving unit (tympanum) to the sensory receptive cells in the cochlea. In both systems, sound causes vibration of a membrane that interacts with air (swim bladder/tympanum), which is physically linked to bony elements, thereby causing the bony elements to "rock". The last element in the series of bones (scaphium/stapes) is coupled to a fluid-filled chamber containing the sensory epithelia, and it's rocking causes fluid motion over the sensory cells in the inner ear, the hair cells (Schellart and Popper, 1992).

The vertebral modifications of the Weberian apparatus are subdivided into two regions, the pars auditum (the relay component) and the pars sustentaculum (the support component; Bird and Mabee, 2003; Bird and Hernandez, 2007). The pars auditum (Figure 3) is composed of the series of bilaterally paired ossicles that are directly involved in hearing, and include the scaphium, intercalarium, and tripus (from anterior to posterior respectively; Bird and Mabee, 2003; Bird and Hernandez, 2007). Two other ossicles, the claustrum and os suspensorium, are classified with the main three bones, but their role in sound

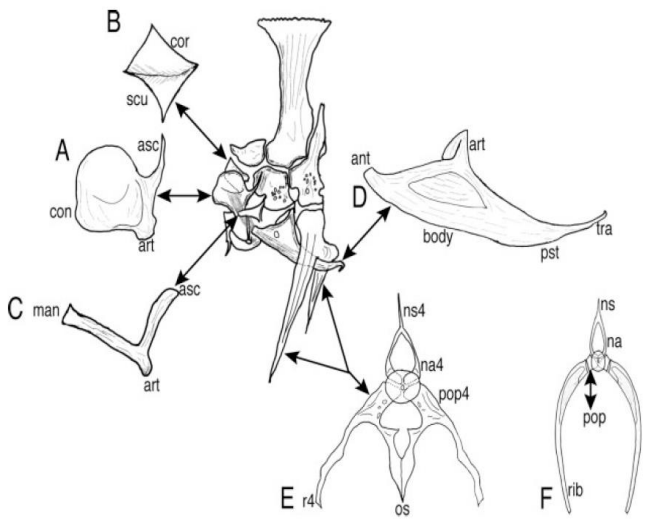

Figure 3. Morphology of the Weberian ossicles. The primary transducing elements are the scaphium (A), intercalarium (C), and tripus (D). Supplementary ossicles are the claustrum (B) and the os suspensorium (E). F shows a typical rib-bearing vertebra, from which the Weberian ossicles are derived. Modified from Bird and Hernandez (2007).

transmission is unknown (Bird and Hernandez, 2007). The scaphium, intercalarium, and tripus are connected to one another via a strong ligament (interossicular ligament), and these bones directly transduce and amplify the sound captured by the swim bladder (Ladich and Wysocki, 2003; Ladich and Popper, 2004).

The pars sustentaculum is composed of modified elements of the first four vertebrae, but are not directly associated with hearing. It is hypothesized that these elements instead support and protect the ossicles, and serve as the anchor for the extensive ligamentous web found in the region (Bird and Mabee 2003). While the sound transmitting elements are remarkably conserved in shape across cypriniforms, the elements of the pars sustentaculum undergo substantial morphological change across cypriniform fishes that is likely tied with environment (Bird and Hernandez, 2007).

Elements of the pars auditum have been shown to exhibit remarkable conservation in shape across very different species of fishes, likely due to the constraint on maintaining their function (Bird and Hernandez 2007). The first ossicle, the scaphium (Figure 3A), is associated with centrum 1 (body of the first vertebra) and has three characteristic features: the articulating process which meets with centrum 1, the ascending process which projects dorsally for ligamentous attachments, and the concha scaphium, a large cup-shaped feature (Bird and Mabee, 2003) which makes up the main body of the scaphium. The articulating process ossifies from the basidorsal, the original cartilage precursor of the scaphium, the ascending process develops as an extension from the same basidorsal, and the concha develops from direct mesenchymal condensation (Bird and Mabee, 2003). The second ossicle is the intercalarium (Figure 3C), and is associated with centrum 2. The intercalarium is composed of an articulating process which meets with centrum 2, an ascending process for ligamentous attachment, and the manubrium (Bird and Mabee, 2003). The manubrium is a process of the intercalarium that projects laterally and is imbedded in the interossicular ligament, a dense ligament that connects the ossicle chain. Similar to the scaphium and claustrum, the intercalarium appears as cartilage but further

development is through membrane bone (Bird and Mabee, 2003). The tripus (Figure 3D) is the last ossicle of the direct auditory chain, and is associated with centrum 3. It has an articular process that meets with centrum 3, an anterior process that attaches to the interossicular ligament, a large ax-shaped body, and a transformator process that is embedded between the layers of the swim bladder. The main body of the tripus begins development from basiventral cartilage found on centrum 3 via cartilage condensation. From the main body the anterior and transformator processes are formed from membranous ossification rather than any cartilage precursor like the main body and articular process of the tripus (Bird and Mabee, 2003). The other defining feature of the tripus is the anterior process which is slowest to develop and ossify. In early developmental stages the anterior process extends only laterally from the articulating process. As development continues the anterior process begins to project laterally and anteriorly where it connects with the interossicular ligament (Figure 2). Although all otophysan fishes (10,000+ species) have the Weberian apparatus, little is known about the variation outside of one subgroup, Cypriniformes, where a large amount of interspecies variation is present with distinct morphologies appearing to be correlated with specific niche (Bird and Hernandez, 2007).

As mentioned previously, the scaphium, intercalarium, and tripus are directly involved in sound detection while the claustrum and os suspensorium are only indirectly related due to their close association with the swim bladder and ligamentous attachem to the tripus. The claustrum is usually small and diamond shaped, and sits dorsally and medially to the scaphium. The ventral side of the claustrum caps the top of the scaphium creating the bony posterior walls of a fluid filled cavity called the sinus impar, which projects into the inner ear and leads to activation of the hair cells (Chardon and Vandewalle, 1997). The claustrum initially appears as cartilage but further development is via membrane bone (Bird and Mabee, 2003). Lastly, the os suspensorium rests on the anterior surface of the swim bladder and articulates with the tripus. The os suspensorium develops from direct mesenchymal condensation outgrowth from the basiventral cartilage of the fourth vertebra (Bird and

Mabee, 2003). The ossicle extends anteriorly and arches ventrally over the swim bladder. It is important to note the os suspensorium does not form directly from the fourth rib or vertebra, but from the cartilage of parapophysis 4 (Bird and Mabee, 2003). In zebrafish, all parapophyses develop from clusters of chondrocytes on the lateral side of centra (Bird and Mabee, 2003). This is important as any effect on chondrogenesis due to exogenous estrogen exposure as seen in previous research could potentially alter parapophysis development and thus alter the positioning of the os suspensorium indirectly. Such potential has led to the current investigation of exogenous estrogen's effect on Webarian apparatus development.

Current Study and Hypothesis

Estrogen and ED-contaminated water has biological effects on fish in the wild (Jobling et al., 1998; Writer et al., 2010; Vajda et al., 2011) and lab studies have shown that estrogen also affects cartilage and bone growth through multiple pathways (Kameda et al., 1997; Fushimi et al., 2009; Imai et al., 2009; Cohen et al., 2014; Pashay et al., 2016). While research regarding the effects of estrogen on chondrogenesis has focused primarily on the head and early larval development (< 5dpf), little research has been done specifically on the vertebral column, including the Weberian apparatus. Developmental regulation of the Weberian apparatus is independent of the segmentation gene network that controls development of other vertebral regions, and may be immune to other regulators of mesodermal-based bone and cartilage development.

Hypothesis: Although evolutionarily tied to the axial skeleton, the development of the Weberian apparatus will be buffered from estrogen-based defects that affect other regions of the axial skeleton, as well as cartilage development in non-axial regions

Null hypothesis: The Weberian apparatus will not be immune to estrogen-based defects, and will show cartilage-based defects in growth and morphology similar to those predicted in other skeleton regions, including the fins and skull.

MATERIALS AND METHODS

Fish Husbandry and Breeding

Adult zebrafish were obtained from Pet Solutions (Beavercreek, OH). Adult and juvenile zebrafish were maintained at 28.5 +/- 0.5°C on a 12:12 light cycle following standard protocol (Westerfield 2000). Adults were fed twice daily with live brine shrimp (Brine Shrimp Direct, Ogden, UT), or commercial zebrafish pellet (Pentair Aquatic Eco-Systems, Apopka, FL). Embryos and larval zebrafish were maintained in incubators at 28°C until 5 days post-fertilization, then moved to permanent housing in a Z-Hab Mini zebrafish system (Pentair) and fed a mixture of newly hatched brine shrimp, powdered spirulina, and powdered larval food (Pentair).

For breeding, adults were placed in breeding hotels (Pentair) the night before collection, with males and females separated by a clear plastic divider, allowing them to see each other, but not interact. The next morning, breeding groups were moved to fresh water within five minutes of first-light to stimulate breeding, and barriers removed. Embryos were collected every 30 minutes. Once collected, embryos were transferred to a petri dish filled with embryo medium (Westerfield, 2000), then placed in an incubator at 28°C. At 10 hours past fertilization, embryos were screened, and dead, unfertilized, or deformed embryos were removed. Remaining embryos were then placed in corresponding treatment and control groups of fifty individuals per petri dish (40mls of embryo medium per dish).

Experimental Methods

Estrogen Treatment. Estrogen (17β-estradiol, Sigma #E8875) was diluted to a 10mM stock solution (in 100% Ethanol) and stored at 4°C in a foil-wrapped vial to prevent degradation. Viable embryos were separated into groups of 50 embryos as above. Experiments always consisted of two control groups (control = embryo medium, control + ethanol = embryo medium with ethanol equal to ethanol found at the highest estrogen concentration) and combinations of several different working estrogen concentrations (2.5μM, 5μM, 10μM, 15μM, 25μM, or 50μM 17β-estradiol diluted in embryo

medium). Larvae were treated in combinations ranging from seven to eleven days past fertilization (dpf). This age range was selected as it is the size range when cartilage elements of the Weberian apparatus have begun development (Bird and Mabee 2003). During the treatment period, larvae were housed in a darkened incubator, to prevent degradation of the estrogen.

Fixation, Clearing, Staining, and Observation

In order to determine the effect of exogenous 17β-estradiol on overall chondrogenesis and formation of the Weberian apparatus, zebrafish were enzyme cleared and double-stained for skeletal analysis using standard methods (Dingerkus and Uhler 1977, Potthoff 1984, Bird and Mabee, 2003). Larvae were anesthetized using buffered 0.04% MS-222 (Tricaine, Fisher #AC118000500), then fixed in chilled 10% buffered formalin (Fisher #F79P) for a minimum of 24h at 4°C. After fixation, specimens were processed through a dehydration series to 100% Ethanol, then stained for 6-16 hours in a 0.02% Alcian blue solution (20% glacial acetic acid in absolute ethanol), which selectively stains cartilage. Next, cartilage staining was differentiated using 100% ethanol (2-12h), and then specimens were rehydrated through a descending ethanol series and transferred into an aqueous saturated sodium borate solution overnight to neutralize any remaining acid. Next, specimens were bleached in a 0.5% KOH + H_2O_2 solution for up to 3 hours (under light) to remove surface pigmentation, followed by muscle digestion using 1% trypsin solution (30% dH_20, 70% saturated sodium borate). Trypsin digestion lasted from 6-48h. Once sufficient muscle mass had been cleared and elements of the Weberian apparatus were visible, specimens were rinsed to remove the trypsin solution and then stained overnight with a 0.025% Alizarin red solution (in 0.5% KOH) to stain bone. Lastly, specimens were placed in an increasing glycerol series (3:1 KOH: glycerol, 1:1 KOH: glycerol, 3:1 glycerol: H_2O; each step overnight) to finish the clearing process. Specimens were stored in 3:1 glycerol: H_2O for skeletal analysis. Observations were made using a VanGuard 1272ZL dissecting microscope outfitted with a VanGuard IS500 camera. Images were collected using IS Capture on a Dell Optiplex 960.

RESULTS

Treatment Mortality (Table 1, Figure 4)

The initial experimental design (using brood Dr-Wt-0011uni) was planned to run from days 7-11, with a solution change mid-way through the course, and food available at all times as larvae normally are feeding at this age. Concentrations were set at the high end (25 and 50μM) of previous reported usage (Fushimi et al., 2009). The initial cohort, including both control groups, were all dead by the morning of Day 9. A second group from the brood was then started at Day 9 to run through Day 11, but all individuals were dead late on Day 10.

	Dr-Wt-0011uni		Dr-Wt-0012uni		Dr-Wt-0013uni		Dr-Wt-0017uni		Dr-Wt-0018uni	
	7-11d	9-11d	7-9d	9-11d	7-9d	9-11d	7-9d	9-11d	7-9d	9-11d
Control	0/50	0/50	50/50 (14)	50/50 (*)	50/50 (25)	50/50 (17)	50/50 (16)	50/50 (0)	50/50 (8)	50/50 (36)
Control + Ethanol	0/50	0/50	50/50 (12)	50/50 (*)	50/50 (22)	50/50 (4)	50/50 (10)	50/50 (0)	50/50 (11)	50/50 (10)
2.5μM Estrogen	-	-	-	-	-	-	-	-	50/50 (16)	50/50 (9)
5μM Estrogen	-	-	48/50 (17)	0/50	50/50 (21)	50/50 (1)	50/50 (0)	50/50 (0)	50/50 (4)	50/50 (15)
10μM Estrogen	-	-	32/50 (3)	0/50	50/50 (9)	50/50 (0)	?/50 (15)	50/50 (0)	50/50 (10)	50/50 (18)
15μM Estrogen	-	-	-	-	-	-	-	-	50/50 (3)	50/50 (5)
20μM Estrogen	-	-	-	-	-	-	-	-	40/50 (#)	31/50 (#)
25μM Estrogen	0/50	0/50	-	-	-	-	-	-	-	-
50μM Estrogen	0/50	0/50	-	-	-	-	-	-	-	-
Number Surviving Treatment/Number At Start of Treatment (Number Surviving to Fixation)										
* = Culled due to lack of experimental group survival										
# = Survivors fixed immediately after conclusion of treatment protocol										

Table 1. Mortality in control and experimental groups during treatment and post-treatment growth. All control and treatment groups started with 50 viable larvae.

Due to the total mortality seen in the first round, several modifications were made for subsequent treatment trials. First, algae was not given during treatment, as it was determined that the reaction of the estrogen with the algae lead to water fouling. Second, concentrations were lowered significantly, down to 5 and 10μM from 25 and 50μM. Third, the initial treatment duration of 5 days was reduced to two days (7-9 and 9-11), such that individuals were placed into treatment on the afternoon of Day 7, and removed from treatment in the morning of Day 9. The reduction in treatment duration was an attempt to mitigate the effects of fasting. The second round of treatment (with Dr-Wt-0012uni)

proved more successful (Table 1), with all control individuals making it through treatment, however mortality was seen in both the 5 and 10μM group during 7-9 day treatment, and total mortality was seen in the experimental groups during 9-11 day treatment (Table 1). After inspection, it was determined that during the transfer, algae remained in the dishes of the 9-11 day treatment group (they were being fed normally from Day 5 to Day 9), which is the likely source of the mortality. Two additional experimental groups (Dr-Wt-0013uni and Dr-Wt-0017uni) proved even more successful, with all individuals making it through the treatment protocol (Table 1), however, they had limited survival during the growth period. A final experimental group (Dr-Wt-0018uni) composed of several different treatment concentrations (2.5, 5, 10, 15, and 20μM) was run for a more defined analysis on skeletal changes. Overall, the survival was excellent across all groups except the 20μM treatment group (Table 1

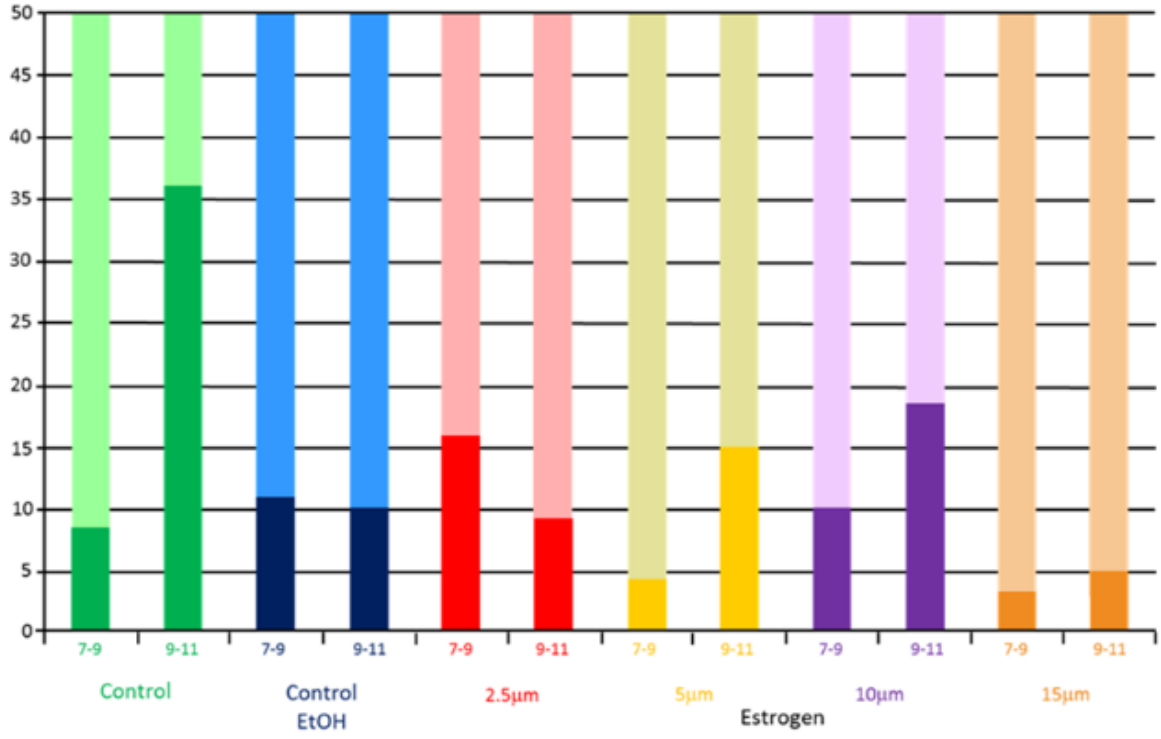

Figure 4. Graphical representation of mortality during and post-treatment in brood Dr-Wt-0018uni. All groups started with 50 individuals. Light color represents the number of individuals surviving treatment, dark color represents the number of individuals surviving the growth period until fixation.

and Figure 3), which upon post-treatment observation exhibited significant developmental defects across several organ systems, and individuals were fixed immediately. All other experimental concentrations had several individuals make it through the growth period for analysis (Figure 4).

Effects on Growth (Table 2, Supplementary Figure 1)

Survival and growth of individuals varied greatly across experimental groups. In the final experimental group (Dr-Wt-0018uni), no significant difference in mean size was observed within either the 7-9 dpf or 9-11 dpf treatment times (Table 2).

Brood 18 7-9dpf Treatment						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	11.56726	5	2.313452	1.471635	0.217474	2.417356
Within Groups	72.31332	46	1.572029			
Total	83.88058	51				
Brood 18 9-11dpf Treatment						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	5.352285	5	1.070457	0.89204	0.49014	2.319277
Within Groups	104.4008	87	1.20001			
Total	109.7531	92				

Table 2. ANOVA analysis of mean growth among control and experimental groups within treatment times.

Similarly, t-tests conducted across treatment time (7-9 dpf vs 9-11 dpf) provided no significant difference between control or experimental groups (Supplementary Table 1, Supplementary Figure 1). The sole exception was the comparison of at 5μM (p=3.066E-5). However, given the very low sample number for the 7-9dpf treatment group, the confidence that this result is a true difference between experimental treatment time periods is low.

Effects on Formation and Development of the Weberian Apparatus (Figures 5, S2, S3)

Elements of the Weberian apparatus begin development as a series of cartilaginous condensations, and portions of the ossicles (articular processes, Figure 3) remain as cartilage during ontogeny. Therefore, any effects on the initial cartilage elements are predicted to manifest as defects in size or shape of the adult ossified elements.

A total of 332 individuals were collected after sufficient growth to easily visualize elements of the Weberian apparatus. All individuals were screened for morphological defects. A subset of 24 individuals (2 per treatment time and control/experimental group) were then selected for in-depth analysis (see Appendix). Individuals were size matched across all groups to minimize the effects of size on variability in Weberian ossicle morphology.

Analysis of size and shape of the Weberian ossicles found no clear or repeated morphological changes. All control and experimental groups displayed normal size and shape of the scaphium, intercalarium, and tripus, relative to body length and compared to previously documented wildtype morphology (Bird and Mabee, 2003). Similar normal developmental patterns were also seen in the non-sensory portions of the Weberian apparatus, with only two fish exhibiting minor alterations in the first lateral process. Ligamentous connectivity also showed no deviance from wildtype when comparing

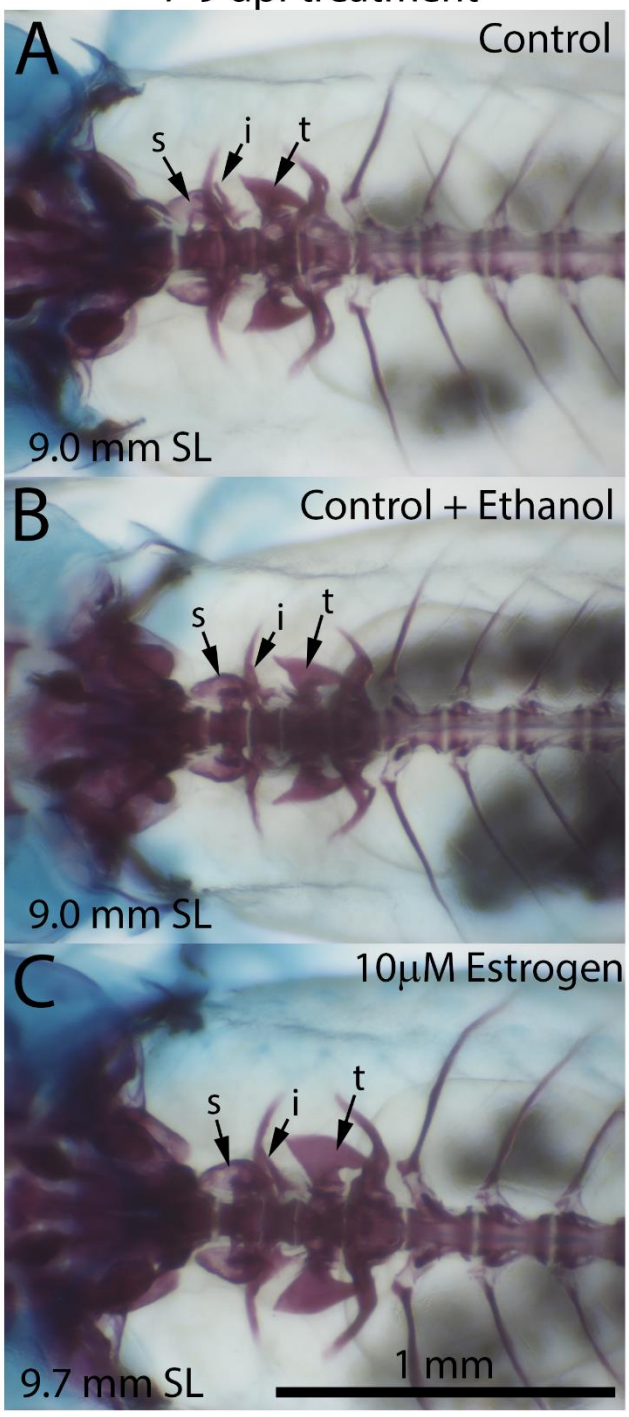

Figure 5. Representative morphology in control and treatment groups. Cleared and stained Weberian ossicles (scaphium = s, intercalarium = I, tripus = t) showed no gross morphological abnormalities in any of the lower estrogen concentrations.

treatment to control. No other deviations from wildtype morphology were noted in the Weberian apparatus of control and experimental individuals at the estrogen concentrations tested, including size and shape of the swim bladder and size and position of the otoliths, suggesting that, generally, the Weberian apparatus appears immune to low levels of estrogen early in development at the stages examined in this study.

DISCUSSION

Fine Tuning the Experimental Design

Previous experiments examining the effect of exogenous estrogen on skeletal development in zebrafish have focused only on embryonic and early larval stages of development (<5 dpf; Fushimi et al., 2009). Using young larvae at these stages avoids the need for exogenous feeding, which begins at Day 5. As the elements of the Weberian apparatus don't begin developing until Day 7 (Bird and Mabee 2003), dealing with feeding was a substantial obstacle. After it was determined that the estrogen and algae were incompatible, the larvae were forced to fast for the duration of the treatment period, which even at 2 days proved problematic. An unforeseen problem was lethal effects of high estrogen concentrations on other anatomical regions (Figure 1), which were unanticipated due to earlier studies claiming treatment after development has begun did not affect cartilage development in the skull (Fushimi et al., 2009), a finding disputed by this study. This forced the reduction of estrogen to much lower concentrations and shortened treatment durations, which may place treatment below the threshold of morphological effect at the stages examined.

Mortality and Survival Post-treatment

A previous study looked at the mortality rates following 17β-estradiol treatments at 5-50μM concentrations over a 1 to 5 dpf, and resulted in mortality rates of as low as 1% at 5μM and 100% at 50μM (Fushimi et al., 2009). This study looked at 17β-estradiol exposure over a shorter time period (7-9 dpf and 9-11 dpf) with mortality rates immediately following exposure at 0% (total survival) for both

treatment times at concentrations ranging from 2.5-15 μM, indicating a lowered sensitivity to estrogen at these later stages. However, a threshold was reached at 20μM concentrations, with mortality of 20.0% and 38.0% for 7-9 dpf, and 9-11 dpf respectively, and 100% mortality at 25μM and 50μM. These results indicate that, while larval zebrafish have a strong capacity to tolerate low levels of estrogen, higher levels overwhelm developmental regulatory systems in a similar fashion as seen in very early larvae.

The unknown effects of fasting during the treatment periods complicate the analysis of estrogen effects during the treatment period. It was impossible to quantify the effects of fasting, but given the lower than expected survival (50%) of control groups (Figure 4 and Table 1), it is likely that fasting also played a role in the mortality seen across all groups. Paired with the effects of high-dose estrogen on cranial cartilages, in direct contradiction to previous studies (Fushimi et al., 2009), feeding could have also been reduced due to lower jaw and branchial arch defects.

Effects on Growth

Previous work that looked at the effects of exogenous estrogen focused on chondrogenesis or gene expression in the head, and immediately fixed following treatment exposure which did not exceed 7 dpf for any study (Fushimi et al., 2009; Cohen et al., 2014; Pashay et al., 2016). In this manner, the current research is the first of its kind. Late stage growth and morphogenesis is often overlooked in zebrafish studies due to the complexity of raising exogenous feeding larvae, but necessary to see how early developmental challenges manifest in adult morphology. Elements of the Weberian apparatus undergo a dramatic and complex morphogenesis that continues from early larval stages to early adult (Bird and Mabee, 2003).

Analysis via ANOVA produced no significant difference in the growth rates of surviving individuals among control and experimental groups in either the 7-9 or 9-11 treatment groups of Dr-Wt-0018uni. Similar to the concerns regarding mortality, the inability to see significant differences could be

due to using low estrogen concentrations and limited exposure times. However, given the larger survival rates for Dr-Wt-0018uni, these results are well supported.

Effects on Morphology

In the Weberian apparatus, no large morphological changes were found. All groups showed similar development patterns in connectivity and chondrogenesis (Figures 6, S2, S3). Overall shape of the Weberian ossicles, as well as supporting structures, was unaffected at low estrogen concentrations (2.5-15μM). These results suggest that the Weberian apparatus is immune to the effects of low-dose estrogen at the stages examined. However, the novelty of this immunity is tenuous, as overall only limited effects were seen in the cartilages of other anatomical regions. No defects were seen in the cartilages of the median and paired fins. Potential minimal effects were seen in the otic region of the chondrocranium (reduction), as well as the 4^{th} and 5^{th} branchial arches (reduction). However, morphometric analysis of these structures is necessary to elucidate the potential effects, which was beyond the scope of the study.

Estrogen in Context

The goal of this research was two-fold: 1) to determine the effect of estrogen on the development of the Weberian apparatus and 2) to determine whether the immunity of the Weberian apparatus to segmentation perturbation extends to chondrogenesis perturbations. In light of the results, the hypothesis of Weberian apparatus immunity cannot be rejected, but only for a narrow analysis at low estrogen concentrations. No effects on morphology were seen at all of the lower concentrations (2.5-15μM), supporting the claim that elements of the Weberian apparatus can compensate to the challenge, but whether the Weberian apparatus is unique in its immunity depends on the morphometric comparisons on other regions, which were only minimal at best. The challenges of feeding, high-dose toxicity, and unanticipated retroactive effects on large cranial cartilages made a large-scale analysis of robust estrogen challenges impossible under the current experimental design.

Future Directions

Several unforeseen challenges made execution of the original experimental design difficult. These included problems with feeding, concentrations, exposure times, and cranial defects. The presence of cranial defects suggests that estrogen's effect is substantial on even large cartilages well after formation has begun. This may allow the treatment time to be moved to later in ontogeny, perhaps to a stage when the larvae are less susceptible to fasting stress. With regards to feeding, the elimination of fasting would be optimal, and the identification of a food-type that is neutral to estrogen exposure would negate the need for fasting. This likely requires testing manufactured non-live feed. It may also require daily water changes and brief times in light to allow for locating food (zebrafish are visual feeders). Moving the treatment time to later in development may also result in reduced effects on cranial and visceral systems (intestine, heart), potentially allowing for increased concentrations and exposure times to be utilized. It is clear that at concentrations over 20μM, estrogen has a marked effect on cartilage formation, so testing at these levels will be critical to truly address the hypothesis presented in this project.

While the effects of estrogen on the developmental morphology and the immunity of the Weberian apparatus has provided some insight into its independence from ancestral units, continued investigation is needed. Furthermore, estrogen is one chemical in a list that influences chondrogenesis and bone formation. Many other chemicals and genes can be used to investigate the proposed developmental buffering of the Weberian apparatus. Two potential candidates to use in further investigation would be insulin like growth factor 1 (IGF-1) and transforming growth factor-β1 (TGF- β1) which affect articular cartilage growth (Balcom et al., 2012). Continued identification of bone and cartilage modulators and testing their impact of Weberian apparatus and vertebral column development is necessary to fully test the immunity of the Weberian apparatus.

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APPENDIX

Data sheet for in depth morphological analysis.

Brood #_____ Loc_____ CLEARED AND STAINED Date data collected_____
Age_____ Length_____ DATA COLLECTION SHEET Initials_____

Region	Structure	Present?	Cell Type	Comments
V1	Centrum 1			
	Scaphium: Basidorsal 1			
	Scaphium: Ascending Process			
	Saphium: Concha Scaphium			
	Claustrum: Corpus Claustri			
	Claustrum: Scutulum Claustri			
	Lateral Process 1			
V2	Centrum 2			
	Intercalarium: Articular Process			
	Intercalarium: Ascending Process			
	Intercalarium: Manubrium			
	Lateral Process 2			
V3	Centrum 3			
	Tripus: Articular Process			
	Tripus: Body			
	Tripus: Anterior Process			
	Tripus: Transformator Process			
	Neural Arch 3			
V4	Centrum 4			
	Neural Arch 4			
	Neural Spine 4			
	Parapophysis 4			
	Rib 4			
	Os suspensorium			
Other	Supraneural 2			
	Supraneural 3			
	Roofing Cartilage			

C=Cartilage, B=Bone PCh=Perichordal E=Endochondral
ND=No difference from control PCo=Perichondral M=Mesenchymal

Supplementary Table 1.

Pairwise comparisons of Dr-Wt-0018uni treatment groups (control, control + ethanol, 2.5μM, 5μM, 10μM, and 15μM estrogen) between treatment exposure times.

Pairwise t-Test Between Treatment Groups		
	C 7-9d	*C 9-11d*
Mean	7.45	7.019444444
Variance	1.24	0.993039683
Observations	8	36
df	10	
t Stat	1.007621257	
P(T<=t) two-tail	0.337395211	
t Critical two-tail	2.228138852	
	CE 7-9d	*CE 9-11d*
Mean	7.572727273	6.84
Variance	1.472181818	1.013777778
Observations	11	10
df	19	
t Stat	1.510818062	
P(T<=t) two-tail	0.147287915	
t Critical two-tail	2.093024054	
	7-9d 2.5uM	*9-11d 2.5uM*
Mean	7.0875	6.8
Variance	1.821166667	2.5225
Observations	16	9
df	15	
t Stat	0.457967068	
P(T<=t) two-tail	0.6535403	
t Critical two-tail	2.131449546	
	7-9d 5uM	*9-11d 5uM*
Mean	8.55	6.46
Variance	0.096666667	1.712571429
Observations	4	15
df	17	
t Stat	5.61921165	
P(T<=t) two-tail	3.06564E-05	
t Critical two-tail	2.109815578	
	7-9d 10uM	*9-11d 10uM*
Mean	7.24	6.555555556
Variance	1.604888889	0.695555556
Observations	10	18
df	13	
t Stat	1.533800636	
P(T<=t) two-tail	0.149049258	
t Critical two-tail	2.160368656	
	7-9d 15uM	*9-11d 15uM*
Mean	6.2	7.2
Variance	3.43	1.135
Observations	3	5
df	3	
t Stat	-0.85425374	
P(T<=t) two-tail	0.455753177	
t Critical two-tail	3.182446305	

Supplementary Figures

Figure S1. Box-and-whisker plot showing size range of surviving individuals of brood Dr-Wt-0018uni, comparing treatment groups across treatment times (7-9 vs 9-11). See Supplementary Table 1 for results of pairwise comparisons.

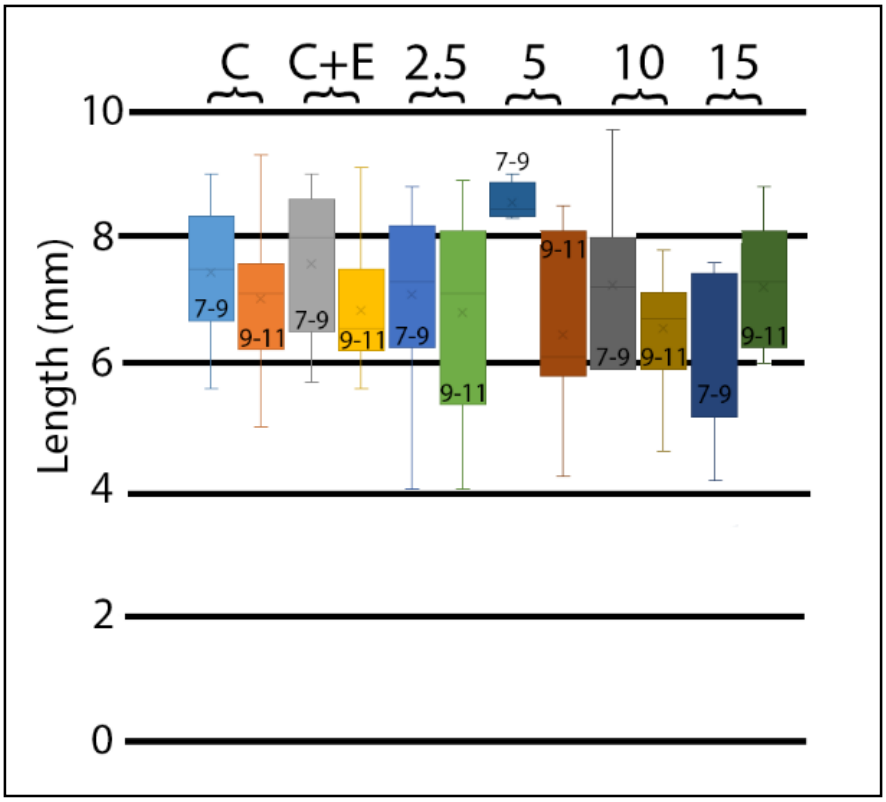

Figure S2. Cleared and stained zebrafish showing the Weberian apparatus in the largest individual from all treatment groups and treatment times in dorsal view (anterior to the left). No significant differences in the Weberian ossicles (scaphium = s, intercalarium = i, tripus = t) were found within 7-9 (A-F) and 9-11 (G-L) treatment times, or between control (A,G), control plus ethanol (B,H), 2.5μM estrogen (C,I), 5μM estrogen (D,J), 10μM estrogen (E,K), or 15μM estrogen (F,L) at different treatment times. Scale bar = 1 mm.

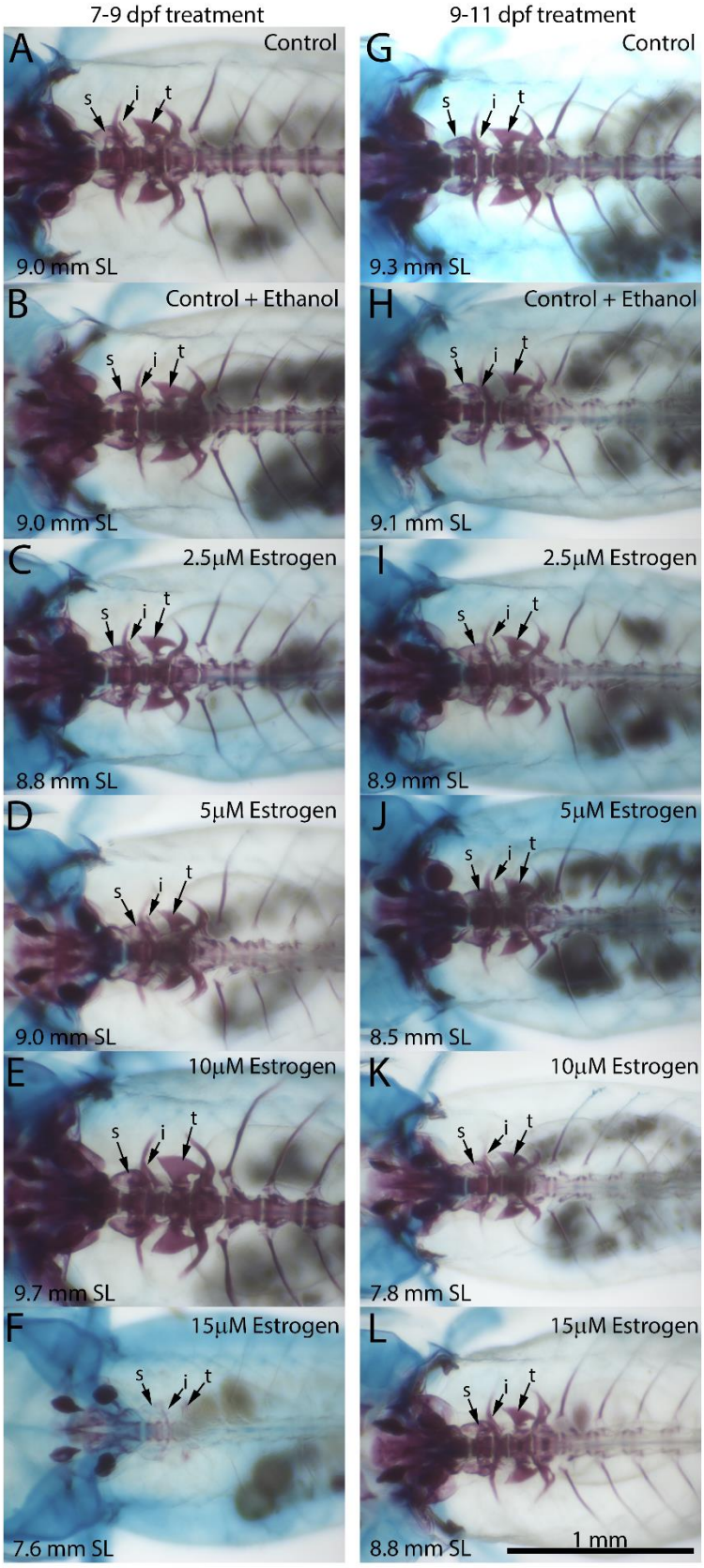

Figure S3. Cleared and stained zebrafish showing the Weberian apparatus in the largest individual from all treatment groups and treatment times in lateral view (anterior to the left, dorsal to the top). No significant differences in the Weberian ossicles (scaphium = s, intercalarium = i, tripus = t) were found within 7-9 (A-F) and 9-11 (G-L) treatment times, or between control (A,G), control plus ethanol (B,H), 2.5µM estrogen (C,I), 5µM estrogen (D,J), 10µM estrogen (E,K), or 15µM estrogen (F,L) at different treatment times. Scale bar = 1 mm.