In vivo assessment of gonad status, secondary sex characteristics and spawning in transparent *Casper* zebrafish

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ABSTRACT

Important aspects of vertebrate reproduction, such as gametogenesis, involve changes in organs found deep internally and thus not easily studied directly in most living vertebrates due to obscuring pigment and overlying tissues. Transparent lines of zebrafish, especially the *Casper* double mutant, allow direct observation and analysis of reproductive events in the gonads in vivo. The natural production of fertilized eggs in zebrafish is a complex process involving oogenesis, spermatogenesis, mating behavior, endocrine and neurological processes with inputs from the environment including light, temperature and nutrition. While these factors play important roles, the hypothalamic-pituitary-gonadal axis (HPGA) is central in the regulation of embryo output. Endocrine disrupting compounds (EDCs) include a variety of pollutants often present in the environment. EDCs may have direct effects on the HPGA or indirect effects through toxic action on supporting organs such as the liver or kidney. Estrogenic compounds such as diethylstilbestrol (DES) have been reported to affect reproduction in a variety of species including man. In this study, the effects of DES on reproduction were determined in a novel way by using transparent *Casper* zebrafish that allow direct visualization of gonad status over time. Changes in gonad status with DES treatment were correlated with effects on secondary sex characteristics (i.e., genital vent size and breeding tubercles) spawning and embryo production. The results suggest that the *Casper* zebrafish is a useful model for studying dynamics of reproductive events in vertebrate gonads in vivo and for determining effects of EDCs on zebrafish reproduction.

1. Introduction

Vertebrate reproduction and gonad function have fundamental importance yet, since gonads are generally internal or otherwise cryptic, their direct visualization and study in the living intact vertebrate is usually not readily possible. However, with the development of transparent lines of fish including the "see-through medaka" (Wakamatsu et al., 2001) and *Casper* zebrafish (White et al., 2008), it is now possible to directly visualize gonad dynamics. Our lab is developing assays for studying effectors of reproduction utilizing transparent *Casper* that allows direct in vivo observation of the reproductive organs and the effects of various treatments on the gonads. This new use of *Casper* will allow for visualization of reproductive changes over time in a living vertebrate animal; thus, providing a direct assay of treatment effects on reproduction, especially any gonadal effects. Prior assays relied primarily on dissection and histological processing of gonads requiring invasive biopsy or sacrifice of the study specimens.

Endocrine disrupting chemicals (EDCs) are substances found ubiquitously in the environment which are capable of disturbing endocrine events in organisms, such as reproduction (Yang et al., 2008; Yang et al., 2015). In this paper we have used an EDC, diethylstilbestrol (DES), to test the *Casper* model in assays of reproduction. EDCs have been detected in aquatic environments and in many fish species (Campinho and Power, 2013; Król et al., 2014; Yang et al., 2008; Yang et al., 2015). DES is a synthetic estrogen mimic; paradoxically, it has progestogen activity in eliciting oocyte maturation in zebrafish (Tokumoto et al., 2005; Tokumoto et al., 2007) and was shown to interact with the progesterone receptor found in the zebrafish oocyte membrane (mPR) (Tokumoto et al., 2012). Thus DES may have both estrogenic and progestogenic effects in zebrafish.

DES is transgenerational in its effect on humans causing increased reproductive tract cancers in females carried by DES-treated pregnant women (Nilsson and Skinner, 2015). Reports have indicated that this EDC can be found in aquatic environments with concentrations ranging to high µg/L (Campinho and Power, 2013; Yang et al., 2008). DES concentrations of 10 µg/mL have been shown to modify the testis of male silver carp (Yang et al., 2008). It is lipid soluble and is readily absorbed into the body (Reed and Fenton, 2013) and often enters aquatic environments via run off from the chemical and medical industry (Yang et al., 2008). Since DES is water insoluble, it tends to accumulate in nonpolar areas at high concentrations in the aquatic environment (Yang et al., 2008). Channel catfish ovary cells (CCO) and Chinese hamster ovary cells (CHO) showed increased cell division after 72 hr treatment with 100–500 µg/L DES and had an Inhibitory Concentration for 50% (IC50) of test subjects of 4.5–6.5 mg/L indicating that in vitro vertebrate cells showed similar cytotoxicity but at relatively elevated concentrations (Radošević et al., 2011).

Our lab is currently developing assays useful for studying the effects of EDCs and other compounds utilizing the vertebrate model, the transparent *Casper* zebrafish. The transparent *Casper* allows direct in vivo visualization of the reproductive organs and thus, effects of DES or other EDCs on the gonad are observable in vivo. This novel use of *Casper* will allow for visualization of reproductive changes over time (i.e., longitudinal study design) in a living vertebrate animal; thus, providing a direct assay of EDCs effects and other treatments on reproduction, especially the gonadal effects. Prior assays relied on dissection and histological processing of gonads requiring invasive biopsy or sacrifice of the study specimen; use of *Casper* avoids these disadvantages and allows longitudinal or repeated observational studies.

2. Results

2.1. Comparison of in vivo measurements with those of dissected ovary

To verify that the follicles measured in vivo represented the actual ovarian follicle number, two *Casper* females imaged in vivo were determined by ImageJ to contain 46 and 34 large follicles, respectively. After sacrifice and ovarian dissection, there were 43 and 38 large follicles, respectively, by dissection. Furthermore the in vivo size measurements were 582 and 566 µm while the in vitro dissected follicles measured 599 and 496 µm, respectively (Table 1). These results indicate the validity of the estimates obtained by in vivo imaging.

2.2. Effect of 24 hr DES treatment on follicle size dynamics in live females

Six female *Casper* zebrafish, approximately one year old, weighing 0.35–0.45 g, were imaged for gonad status the week prior to experimental treatment to establish the gonad state of each animal. The females, housed individually, received 0.5 µg/mL (1.86 µM) DES in the

Table 1
Comparison of in vivo *Casper* ovarian follicle diameter measurements versus dissected follicles in vitro.

	Female 1				Female 2			
	In vivo	In vitro	F-test	*t*-test	In vivo	In vitro	F-test	*t*-test
Oocyte #	46	43			34	38		
Average	562	599	ns	ns	566	496	ns	ns
sd	91	85			91	81		

Oocyte (follicle) number data include both right and left views for in vivo counts, total large follicles for in vitro dissected ovaries and mean follicle diameters ± standard deviation (sd) for both in vivo and in vitro. Counts and measurements were done blind by separate investigators. F-tests indicated normality of distribution and the Student's *t*-test indicated no significant (ns) difference at p = 0.05 between in vivo and in vitro data for a given female.

tank water (500 mL) for 24 h. The animals were monitored for 35 days post application. A matched control group treated with steroid vehicle for 24 h was also followed for comparison. Reproductive status was assessed by physical appearance of the ovary, in vivo, by ImageJ analysis of images captured every 3–4 days with a camera-mounted dissecting scope. Ovarian follicle sizes were determined by manual measurement using the calibrated line tool in ImageJ (Fig. 1). Only follicles containing opaque yolk were measured if the outlines were visible. Usually small previtellogenic follicles did not meet these criteria. The follicle size data were plotted along with oocyte number (derived from follicle measurement data). The overall data from the 24 hr DES treatments are shown in Fig. 2; strikingly, the mean oocyte diameter decreased significantly between 10 and 14 days post DES. Approximately a week later oocyte number increased significantly returning to baseline approximately 10 days later (Fig. 2). Throughout the experiment, body weight remained relatively constant (data not shown), suggesting the animals maintained normal feeding and overall health.

2.3. Effect of DES on post pubescent males

Fifteen sibling *Casper* zebrafish males 17 WPF were randomly divided into 3 groups and were imaged 1 day prior to treatment; all had breeding tubercles, opaque white testes and small vents. The next day, two treatment groups received either 0.1 or 0.01 ng/mL DES and the control group received steroid vehicle. DES was applied directly to tank water and fresh DES was added every other day with a water change. Effect of DES was determined by imaging the testes, vent, and breeding tubercles at weekly intervals. The 0.1 ng/mL concentration of DES induced complete loss of breeding tubercles (Fig. 3H) and an increase in the vent size by the end of 3rd week (Fig. 4). This higher DES concentration induced a noticeable regression of the testis, with decreased white opacity (Fig. 5). At 0.01 ng/mL DES breeding tubercle size decreased noticeably, producing more rounded, less prominent tubercles (Fig. 3E). This lower DES concentration induced less regression of the testis compared to the higher dose; there was no change in breeding tubercles of the control males (Fig. 3B). DES treatment caused a statistically significant increase in the size of the vent in both DES treatment groups, but not in the control group (Fig. 4). Imaging of tubercles at 23 WPF, 2 weeks after DES withdrawal, revealed a re-emergence of breeding tubercles in the 0.1 ng/mL concentration (Fig. 3I) and a reduction to pre-treatment vent size (Fig. 6).

2.4. Effect of DES on spawning

Five pairs of 4-month-old *Casper* post-pubescent adults were placed pairwise in separate spawning tanks and the embryo output was assessed daily. The females averaged 2.1 cm in length and produced an average of 29.8 ± 6.0 (SEM, N = 5 pairs) embryos at the first spawning event. The pairs spawned an average of 7.8 ± 0.9 times in the 8 weeks before treatment (Table 2). Treatment with 1.0 ng/mL DES began at 24 WPF until 28 WPF. DES was applied directly to 1 L tank water and fresh DES was added daily with the tank water change. Effects of DES on male *Casper* were determined by weekly imaging of the testes, vent, and pectoral fin breeding tubercles; female *Casper* ovary and vent were also imaged weekly. For pairs, spawning frequency and number of embryos produced were tracked. Spawning stopped completely in all DES-treated pairs at 2 days after DES treatment began, however control pairs continued to spawn normally. Spawning resumed 3 weeks after DES was stopped when one pair of animals spawned. However, four of ten DES-treated animals died during treatment from apparent hemorrhage and only two of five were original spawning pairs; these were followed until 35 WPF when pre-DES treatment spawning frequency was restored.

There was a dramatic loss of the visible gonad in vivo for all DES animals of both sexes during treatment. Female animals no longer had an obvious ovary 14 days after DES treatment began and the ovaries
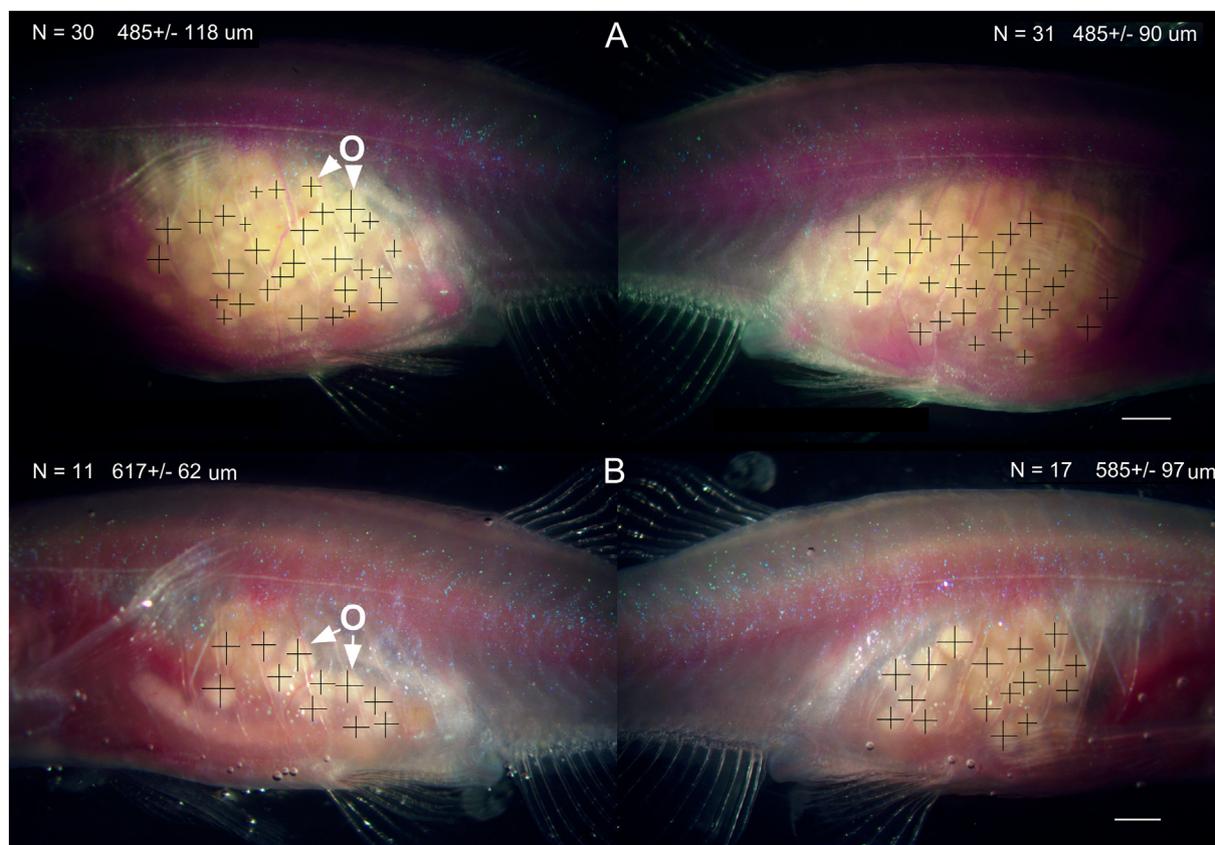
Fig. 1. Method for in vivo ovary measurement in *Casper* zebrafish. The calibrated line tool in ImageJ was used to measure the diameter (height and width cross hairs) of larger, vitellogenic ovarian follicles with opaque yolk (indicated by O with arrowheads). Panel A: Left and right ovaries of a moderately gravid female *Casper* zebrafish. Data represent mean ± SD of larger follicle size class and N = number of the larger follicles, bar equals 1 mm. Panel B: Left and right ovaries of a less gravid female *Casper* zebrafish. Data represent mean ± SD of larger follicle size class and N = number of the larger follicles, bar equals 1 mm.

remained cryptic until the end of DES treatment (Fig. 7). There was also a significant increase in the vent size in DES-treated males and females (Fig. 8). The DES-treated males had vent sizes significantly larger than pre-DES male vents and equaling the pre-DES female vents, while the DES-treated females had significantly larger vents than pre-DES female vents (Fig. 8). The first male lost breeding tubercles 14 days after DES treatment began, another male lost breeding tubercles at 21 days, and the remaining males lost their breeding tubercles by 28 days. Breeding tubercles were not observed in any female during this study. For all males, the testes were no longer opaque and milky-white 28 days after DES treatment began, however, by 3 weeks post-DES, the surviving 3 males had regained tubercles, vent size reduction and testes opacity.

3. Discussion

3.1. Effects of 24 hr diethylstilbestrol treatment

Sexually mature female *Casper* zebrafish were placed in 0.5 μg/mL (1.86 μM) DES in tank water for 24 h to determine the effects on ovarian morphology, and to test the *Casper* model as a potential system to study gonad dynamics. DES has well documented effects on reproduction in vertebrates including man and the development of a *Casper* reproductive bioassay to assess this and other potential EDCs has possible environmental testing value. High DES concentrations have been found in some aquatic environments (Yang et al., 2008).

A 24 hr pulse of 0.5 μg/mL DES had no obvious, short term effect on ovarian morphology. However, when examined on a longer-term time frame, a significant decrease in follicle size was observed followed by a significant increase in oocyte number (Fig. 6). This suggests that DES may provoke a longer-term effect on reproduction measured in weeks

rather than hours or days. The precipitous decrease in large follicles is similar to atresia which normally occurs in vertebrate ovaries to various degrees depending on the species.

Exogenous estrogenic compounds including DES generally elicit ovarian regression and oocyte resorption in many teleost species; adult goldfish immersed in 1 nM estradiol-17β (E2) or 0.3 nM 17α-ethinylestradiol (EE2) resulted in gonad regression after 3–24 hr treatments (Marlatt et al., 2010). Adult female fathead minnows treated with 0.47–3.92 ng/L EE2 had reduced egg production after 21 days (Armstrong et al., 2016). Adult female zebrafish exposed to 25 or 100 ng/L E2 for 3 weeks had reduced egg production (Brion et al., 2004), and a different study using 10 ng/L EE2 or mixtures of megestrol acetate were found to also reduce ovarian output (Hua et al., 2016), both results are similar to the DES findings reported here. Bisphenol A (BPA) another estrogenic compound has been reported to elicit ovarian follicle atresia in adult zebrafish (Migliaccio et al., 2018).

Interestingly, the Tokumoto group found that 0.1 μM (26.835 ng/mL) of DES induced germinal vesicle breakdown (maturation) over the course of 3 h (Tokumoto et al., 2005). The data presented here involved doses of DES either higher (i.e., 0.5 μg/mL) or lower (i.e., 0.01–1 ng/mL) than used by Tokumoto's lab and may indicate a dose-sensitive effect. Various hormone concentrations can produce opposite or different responses (vom Saal et al., 1997). Alternatively DES may act both as a progestogen depending on the cell type (Tokumoto et al., 2012; Tokumoto et al., 2011; Tokumoto et al., 2005; Tokumoto et al., 2007; Tokumoto et al., 2004) or as an estrogen eliciting ovarian atresia and male breeding tubercle loss (Hoffmann et al., 2006; McMillan et al., 2013; Orn et al., 2006; Pang and Thomas, 2009; Van den Belt et al., 2001; Van den Belt et al., 2004; van der Ven et al., 2007).
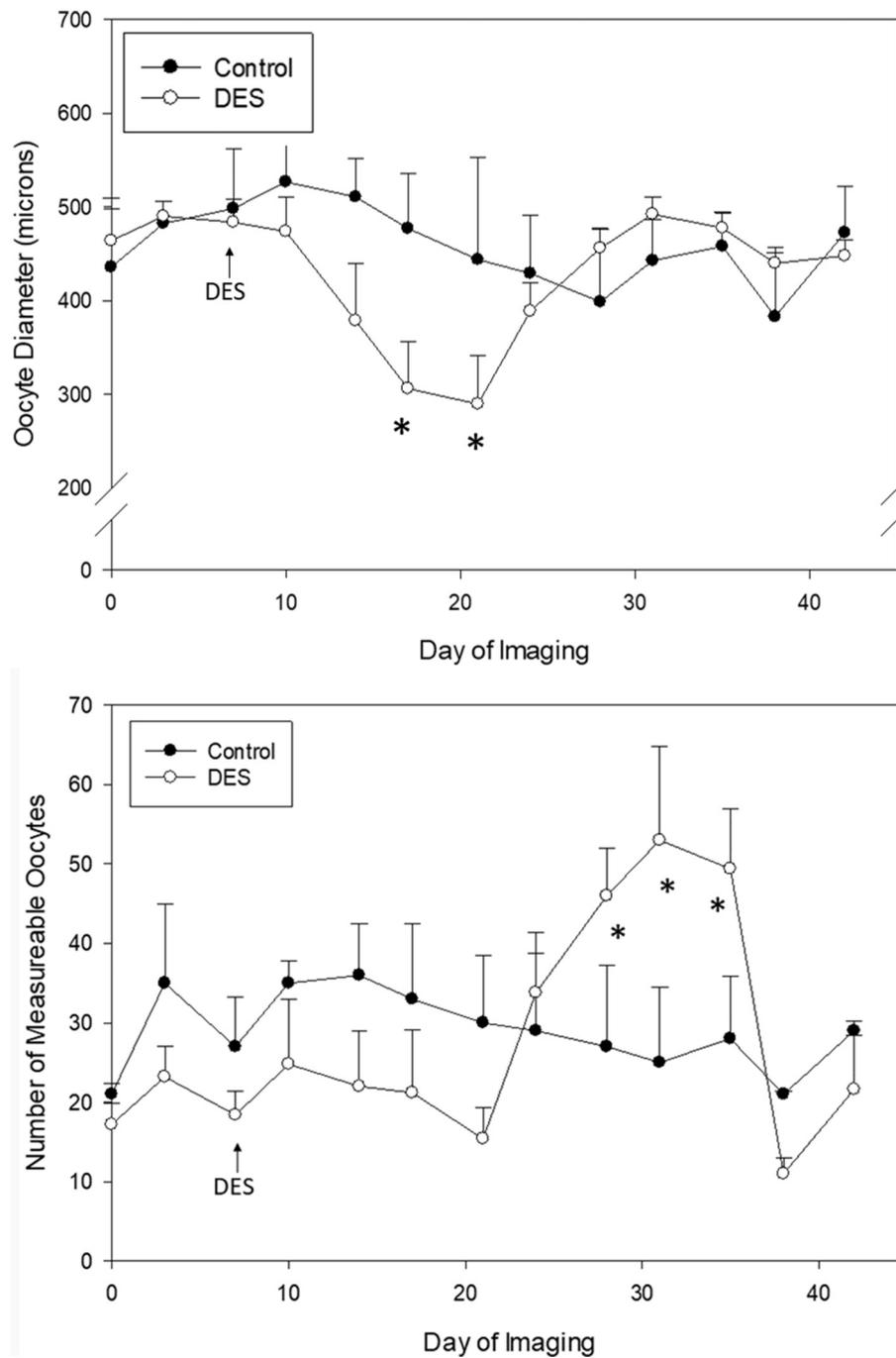
Fig. 2. Effect of 24 hr diethylstilbestrol (DES) treatment on diameter and number of larger ovarian follicles in vivo of female *Casper* zebrafish Over 42 Days. The upper panel: Ovarian oocyte diameter versus day of imaging. The lower panel; Number of measurable oocytes versus day of imaging. Graphs in both panels represent treatments of 0.5 μg/mL DES (open circles, arrow denotes 24 hr treatment) and vehicle control (dark circles), data are mean ± SEM, n = 6 females, asterisks denote significant differences at p < 0.05 versus values at initial timepoint.

3.2. Effects of diethylstilbestrol on post pubescent male Casper juveniles

We used adult *Casper* zebrafish males to determine the effects of DES on breeding tubercles, testis and vent size. Sexually mature males were treated for 4 weeks (28 days) with DES. DES induced a dramatic loss of breeding tubercles, regressive changes in testes (Fig. 8) along with increased vent size. We hypothesized that DES would induce regression of breeding tubercles and testes in sexually mature males based on previous studies using E2 (McMillan et al., 2013; McMillan et al., 2015). According to a DES study in silver carp, a related species, 10 ng/mL is required to induce complete loss of testes (Yang et al., 2008). A

reported inhibitory mechanism may involve DES competitively binding receptors which would induce estrogenic activity and reduce testosterone levels (Fitzgerald et al., 2015; Marlatt et al., 2010; Pang and Thomas, 2010). The larger genital vent has been reported as a method for sexing female zebrafish, while males have noticeably smaller vents (Yossa et al., 2013). Male vent size was expected to increase with DES since it has been shown that certain steroids, such as progesterone, can induce dramatic vent size increase in other fish species (Asahina et al., 1985). In fact, in the rose bitterling, tests of 19 steroids showed highest vent (ovipositor) growth due to progestogens and not estrogens (Asahina et al., 1985). DES has also been shown to bind to membrane-
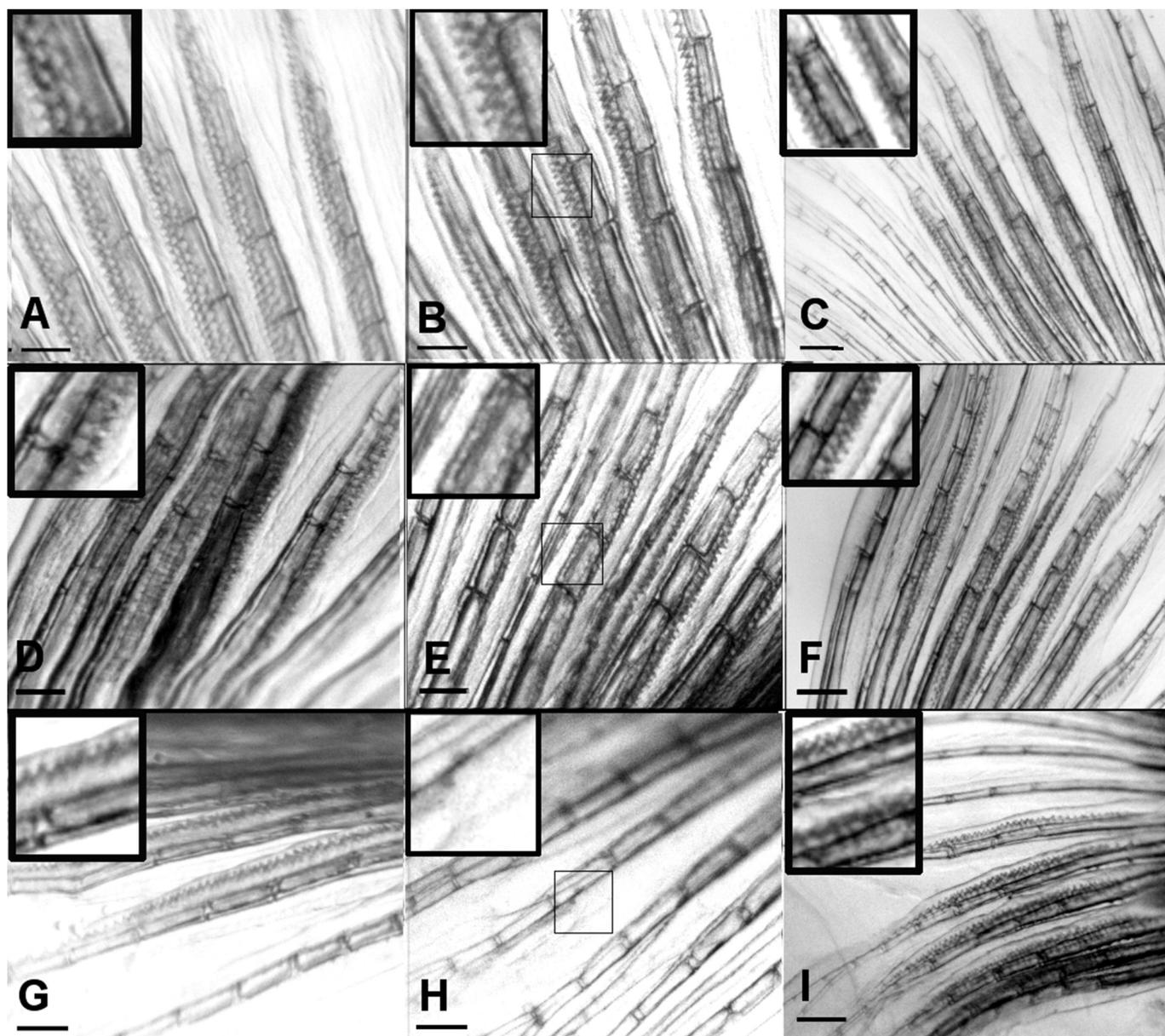
Fig. 3. Effects of diethylstilbestrol (DES) on breeding tubercles of adult male *Casper* zebrafish. Panels A – C show pectoral fins of same control treated male. Panel A: control male at 17 WPF. Panel B: control male at 20 WPF. Panel C: control male at 22 WPF. Panels A – C show no reduction in breeding tubercles over time. Panels D – F show pectoral fins of same 0.01 μg/L DES treated male. Panel D: DES male at 17 WPF, the day of treatment, before 0.01 μg/L DES was applied. Panel E: DES male at 20 WPF after DES treatment for 21 days. Panel F: DES male at 22 WPF, 14 days after DES withdrawal. Panels D – F show a modest reduction in the height and pointedness of the breeding tubercles after treatment. Panels G – I show pectoral fins of same male treated with 0.1 μg/L DES. Panel G: DES male at 17 WPF, the day of treatment, before 0.1 μg/L DES was applied. Panel H: DES male at 20 WPF after 21 days of DES treatment. Panel I: DES male at 23 WPF, 21 days after DES withdrawal. Images show a clear reduction in breeding tubercles after treatment and reformation with DES withdrawal. Images were taken at $32\times$ magnification; all scale bars represent 400 μm, except inset which are $64\times$ and scale bar represents 200 μm.

bound progesterone receptors in zebrafish oocytes to induce maturation and could have progesterone-like effects on the male vent (Tokumoto et al., 2004). In another study, adult male zebrafish exposed to 25 or 100 ng/L E2 for 3 weeks had significant genital vent growth in males, similar to the DES findings reported here (Brion et al., 2004).

3.3. Effects of diethylstilbestrol on spawning

Pairs of mature, mated *Casper* zebrafish were used to determine the effects of DES on spawning activity, gonad morphology, breeding tubercles, and vent size. Constant exposure of 1 ng/mL of DES for 28 days led to a complete loss of embryo output. The last spawning event occurred on the second day of treatment. DES exposure also led to a loss of

a visible testes and breeding tubercles in all males. DES exposure led to a loss of a visible ovary in females (Fig. 7). DES also induced a statistically significant increase in vent sizes in both males and females (Fig. 8). This increase in vent size could be caused by DES acting as a progestogen or estrogen, on this putative steroid sensitive organ (Asahina et al., 1985). The increase could also be caused by swelling or peritoneal pressure, leading to protrusion of the vent. The exact nature of the vent changes and the causal mechanism remains to be explored.

3.4. Nonspecific effects of diethylstilbestrol

DES causes disruption of thyrocytes in zebrafish at similar concentrations as used here (Campinho and Power, 2013). DES treatment
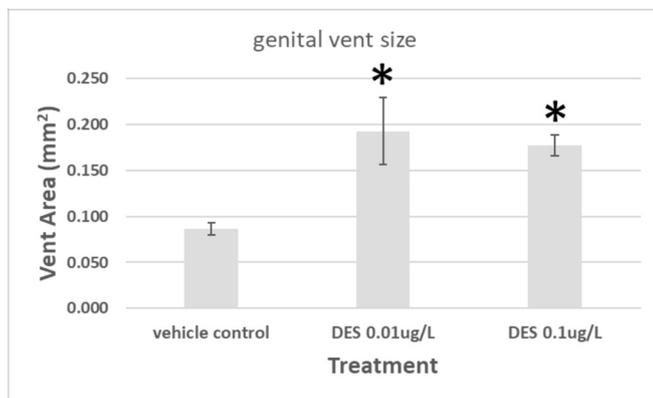
Fig. 4. Effects of Diethylstilbestrol (DES) on Vent Size in Adult Male *Casper* Zebrafish. Fifteen male Casper zebrafish were divided at random into 3 groups and treated with either vehicle, 0.01 µg/L DES or 0.1 µg/L DES for 28 days. The data are presented as mean vent area ± SEM mm^2 of the five males per group at the end of the incubation period. The asterisks represent means that differ from the vehicle control by $p < 0.05$ with the Student's *t*-test.

of European catfish yielded enlarged liver and abdominal swelling but no mortality at the concentrations used (Król et al., 2014). In humans, it has been shown that DES compromises the ability of blood platelets to clot properly (Dobrydneva et al., 2003; Dobrydneva et al., 2010). DES is a calcium channel blocker in blood platelets and acts by blocking calcium channels which leads to t3he inability of platelet activation and without activation, the platelet cannot function properly (i.e. clot) (Dobrydneva et al., 2010; Dobrydneva et al., 2003). These reported effects on blood platelets potentially explain the DES viability effects and hemorrhaging seen in *Casper* zebrafish.

4. Experimental procedures

4.1. Zebrafish

A *Casper* zebrafish ($mpv17^{-/-};mitfa^{-/-}$) line was generously donated by Ryan Gebert at St. Jude Children's Research Hospital, Memphis, TN from stock established by Michael R. Taylor. *Casper* is named for its ghost-like appearance because it lacks functional melanocytes and iridiophores and thus is virtually transparent (White et al., 2008). *Casper* is a double mutant line maintained on an AB background; the first ($mitfa^{-/-}$), lacks pigmented melanocytes due to a *mitfa* gene mutation (Lister et al., 1999). The second ($mpv17^{-/-}$) zebrafish lacks functional iridiophores due to a mutation in Mpv17 mitochondrial protein (D'Agati et al., 2017; White et al., 2008). Crosses of these two mutants yield a transparent animal with the gonad clearly visible in vivo.

Fish were reared at 28 °C on a 14 hour photoperiod cycle, and fed a combination mixture of tropical fish flake (Aquaticeco.com), Artemia (freeze-dried brine shrimp, Aquaticeco.com), and earthworm flakes daily (Aquaticeco.com) (Lawrence, 2007). Tank water was changed every other day. For spawning studies, spawning tanks were checked daily and embryos were collected. Anesthetized fish were blotted quickly with a paper towel and weighed to the nearest 0.01 g on a digital balance prior to imaging. Animals were housed individually in uniquely labeled 1 L tanks for the repeated measures study of gonad dynamics and utilized under an approved IACUC protocol.

4.2. Bright field transmitted and reflected light microscopy

Animals were anesthetized in 0.04% tricaine methanesulfonate (TMS), (0.1 g in 250 mL) in egg water (reverse osmosis purified water: dechlorinated tap water (1:1) containing 3 drops 1% methylene blue per 4 L) and weighed to nearest 10 mg after blotting briefly on filter paper. Early in the study, individual fish were placed in a petri dish filled with TMS and imaged using a Zeiss Stemi SR microscope with an Amscope 9MP eyepiece camera at 8× magnification. After images of the right and left sides of the mid-trunk containing the gonad were captured using transmitted and reflective modes, the animals were placed in continuously aerated fresh water for recovery from the anesthesia. Subsequently, we developed a simpler procedure using lightly anesthetized fish in TMS water-filled viewing bags imaged with a camera-stereoscope. Fish were netted and placed into a beaker with 10 mL of TMS water then immediately transferred to a clear zip-lock
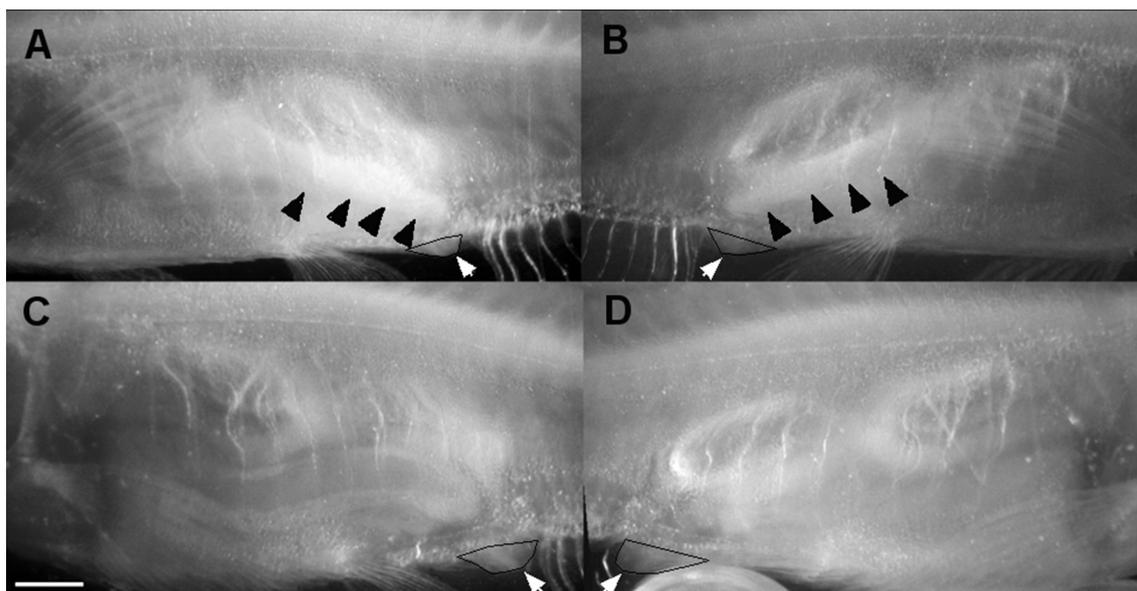

Fig. 5. Effects of diethylstilbestrol (DES) on testes and vent of *Casper* male zebrafish. Panels A and B: Image of left and right testes, respectively, and vent of a sexually mature male (17 WPF) before DES treatment. Black arrowheads point to opaque lower area of testes connecting to vent (white arrows). Panels C and D: Image of left and right testes, respectively, and vent of sexually mature male (21 WPF) after 4 weeks of 0.1 µg/L DES treatment. Testes are notably more transparent without obvious connection to vent. Images show reduction in testes opacity and increase in vent size after treatment. Images were taken at 12× magnification. Scale bar represents 1 mm.
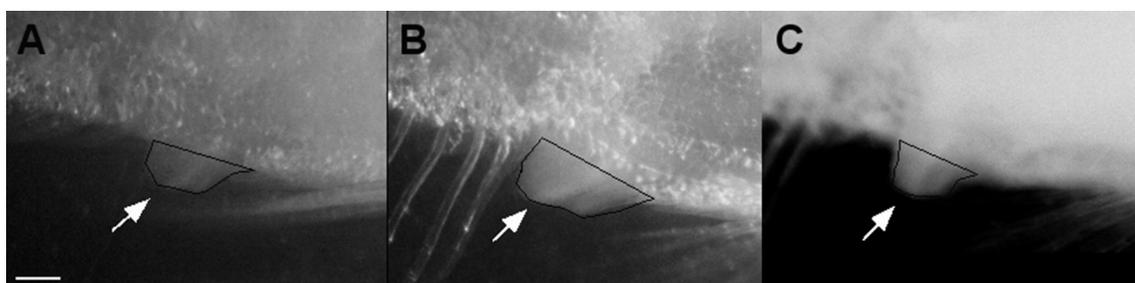
Fig. 6. Representative male vent changes with diethylstilbestrol (DES) treatment and withdrawal. Adult male *Casper* zebrafish before treatment (A), after 21 days of 0.1 μg/L DES (B) and after 14 days of DES withdrawal (C). The vent (white arrows) has been outlined in black with the imageJ line tool for determining area in each image. The scale bar represents 1 mm.

bag (7.5 × 14 cm, Amazon.com) and imaged. Total time for procedure of about 2 min thus fish were not deeply immobilized but swam readily when transferred to fresh water. This procedure works well for routine imaging of trunk and eliminates or reduces anesthesia recovery times, but is not suitable for imaging breeding tubercles (see below).

4.3. Analysis methods

Bright field digital images taken at 8× magnification were downloaded from the Amscope digital camera as .jpg files. These files were analyzed using ImageJ analysis software. ImageJ was downloaded from http://rsb.info.nih.gov/ij/. For quantification of ovarian follicle diameters, a stage micrometer image was captured and used for calibration in ImageJ.

4.4. Measurement of ovarian follicle diameters

Using bright field dissecting microscopy images, ovarian follicle diameters were determined in ImageJ. In these images, all visible ovarian follicles were measured for the left and right side images of the animal. To better illustrate the measurement technique, two *Casper* females, in different ovarian states, were imaged and the larger follicle size class was measured in each as depicted in Fig. 1a and b. The right and left ovary each contained different numbers and sizes of ovarian follicles, Generally, oocytes smaller than 200 um in diameter had very low contrast and could not be reliably measured, thus the focus was usually on the increasingly opaque, growing vitellogenic oocytes. The swim bladder and gut optically separate the ovaries so left and right ovaries may be measured. In Image J, a calibrated line tool was used to calculate the height and width of the individual follicles (Lessman et al., 2010; Lessman and Carver, 2014). Line lengths were recorded in Microsoft Excel as ovarian follicle diameters

4.5. Secondary sex structure assessment

For measurements of vent (i.e., genitalia) sizes, bright field microscopy images were used and vent areas were determined using ImageJ. Using a calibrated line tool, the vent perimeters were traced and recorded using the "calculate area" setting.

For imaging breeding tubercles, anesthetized animals were placed ventral side up in a shallow depression in an agarose-lined petri dish filled with TMS water. Pectoral fins were displayed on top of the agarose and forceps used to spread the fin to clearly display the tubercles if present (McMillan et al., 2013; McMillan et al., 2015).

4.6. Diethylstilbestrol treatment

In this study, the effect of exogenous DES on sexual maturation, gonad morphology and spawning was determined. DES was purchased from Sigma Aldrich. DES was added directly to tank water at 0.01, 0.1, 1.0 ng/mL, or, for shorter incubations, 0.5 μg/mL ($3.73E^{-5}$μM, $3.73E^{-4}$μM, $3.73E^{-2}$μM, or 1.86 μM, respectively) from concentrated stocks dissolved in steroid vehicle (EtOH:propylene glycol; 1:1); this binary vehicle reduces the concentration of either solvent exposure to fish (< 0.00001%) and provides excellent drug solvent properties. DES treatment lasted for either 1, 21, or 28 days depending on the experiment. Tank water was changed daily, or every other day and fresh DES added. Animals were imaged before and after DES treatment to assess the effects in vivo.

4.7. Statistical analysis

In this study, statistical analysis was performed in SigmaPlot 14 or Microsoft Excel. Generally, a one-way ANOVA was run on data sets to determine distribution normality followed by pairwise analysis to determine statistical significance between treatments and incubation
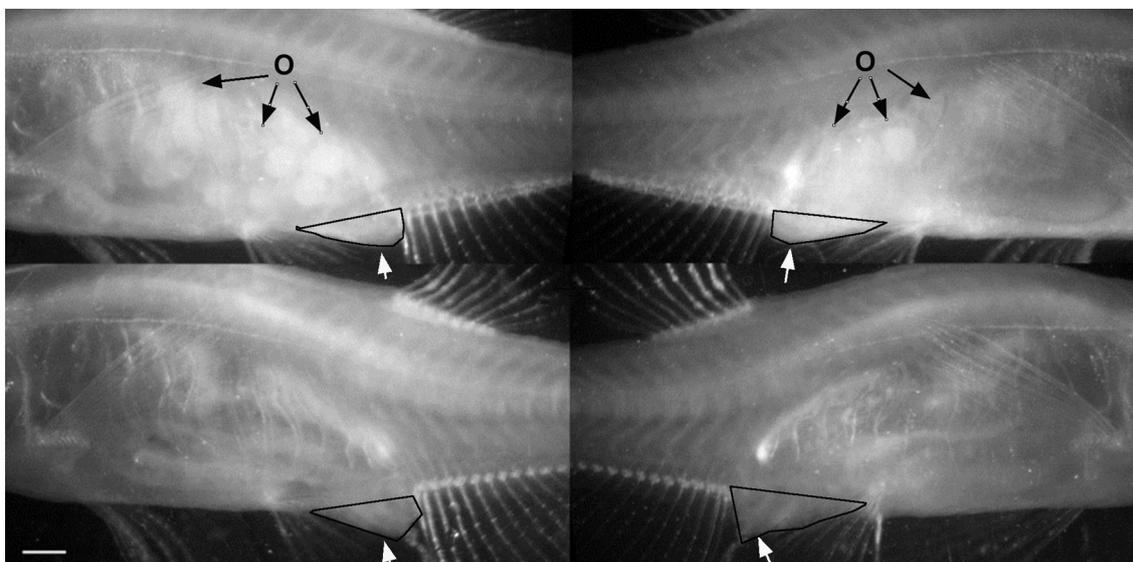
Table 2
Effect of diethylstilbestrol on spawning pairs of *Casper* zebrafish.

	Before diethylstilbestrol treatment				During 1 mg/L DES treatment				After 3 weeks post DES			
	(8 weeks)				(4 weeks)				(3 weeks)			
Female	#emb	#sp	freq	emb/day	#emb	#sp	freq	emb/day	#emb	#sp	freq	emb/day
C56	40	9	5.7	7.1	0	0	0	0	Dead	–	–	–
C58	30	7	7.3	4.1	10	1	28	0.4	2.3	3	6.7	0.3
C62	17	8	6.4	2.7	0	0	0	0	5.7	7	2.9	2.0
C63	16	5	10.2	1.6	0	0	0	0	Dead	–	–	–
C64	46	10	5.1	9.0	0	0	0	0	12	1	20	0.6
Mean	29.8	7.8	6.9	4.9	2	0.2	N/A	0	6.7	3.7	9.8	1.0
SD	13.4	1.9	2.0	3.1	4.5	–	–	–	4.9	3.1	9.0	0.9

#emb is the average number of embryos per spawn, #sp. is the number of spawns during the time indicated in weeks for interval, freq is the days between spawns or the spawning frequency and emb/day is the average number of embryos produced per day during the interval. N/A indicates an indeterminate number and – indicated missing or indeterminate data.

Fig. 7. Effects of diethylstilbestrol (DES) on female spawning: ovarian morphology. Panels A and B: Left and right sides of female C62 before DES treatment at 25.5 WPF (O with black arrows indicate large follicles, black outlines with white arrows denote vents). Panels C and D: Left and right sides of female C62 after 28 days of 1 μg/L DES exposure, 29.5 WPF, note the atretic ovaries and increased vent size (black outline and white arrow). All DES females in the study exhibited regression of the ovary. Images were taken at 8× magnification. Scale bar represents 1 mm.
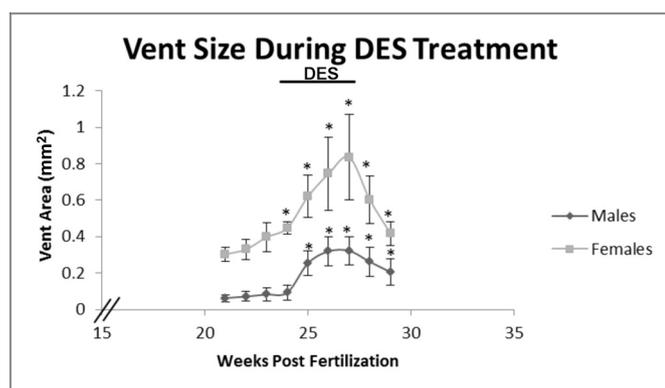

Fig. 8. Vent size (area) during DES treatment of adult zebrafish. Four males and four females were treated with DES for 28 days (24–28 WPF) and vent size was measured before, during, and after this period in weekly increments. Mean ± SD male and female vent sizes were plotted over time. Bar indicates treatment period. Asterisk $p < 0.05$ compared to same sex vent at start of study, i.e., 21 WPF.

time. A one-tail student *t*-test was used to determine the *p* value of measurements derived from DES and non-DES treated animals. Two arrays were selected to compare two groups of data (e.g., DES females versus non-DES females). The Kruskal-Wallis one-way ANOVA on ranks was used for some data sets followed by Dunn's test for pairwise analysis. A $p = 0.05$ or less was criterion for significant differences.

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Declaration of competing interest

The authors have no conflicts of interest regarding this work.

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