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## Accepted Manuscript

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**Surflan<sup>TM</sup> and Oryzalin Impair Reproduction in the Teleost Medaka (*Oryzias latipes*)****Hall, L.C.,<sup>1</sup> Okihiro, M.,<sup>2</sup> Johnson, M.L.,<sup>3</sup> and Teh, S.J.<sup>3</sup>**

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**Abstract**

We conducted studies to determine if the xenoestrogens Surflan<sup>TM</sup> and its active ingredient oryzalin, affect indices of reproductive fitness in medaka (*Oryzias latipes*). Oryzalin (0.5, 0.25 mg/l) or Surflan<sup>TM</sup> (2.0 µl/l) and oryzalin (0.5 mg/l) significantly increased the mean number of non-fertilized eggs produced by treated females paired with untreated males, or by untreated females paired with treated males. Oryzalin (1.0, 0.5, 0.25 mg/l) and Surflan<sup>TM</sup> (3.8, 2.0, 1.0 µl/l) significantly affected the time to hatch of eggs from treated females paired with untreated males, and from untreated females paired with treated males. Surflan<sup>TM</sup> (3.8, 2.0, 1.0 µl/l) induced intersex lesions in 80-100% of males. Oryzalin-exposed males exhibited a significant increase in the incidence of necrotic spermatids and necrotic spermatogonia, while oryzalin-exposed females had significantly fewer immature oocytes and an increase in the occurrence of hyperplastic ovaries. Our results indicate that Surflan<sup>TM</sup> and oryzalin affect both reproduction and gonadal histology in male and female medaka.

**Keywords** endocrine-disruptor, medaka, herbicides, ecotoxicology, toxicity, Surflan, oryzalin

**Introduction**

Surflan<sup>TM</sup> is a proprietary herbicide emulsion formulated and marketed by Dow Elanco that contains 40.4% oryzalin (3,5-dinitro-N4, N4-dipropylsulfanilamide). We have identified both Surflan<sup>TM</sup> and oryzalin as xenoestrogens – compounds that may be capable of eliciting many of the biological responses of endogenous estradiol (Hall, Rogers, Denison & Johnson, 2004).

In teleosts, endogenous estrogens (primarily 17β-estradiol) have a multitude of functions in normal physiology, and are involved in sexual differentiation of embryos (Yamamoto, 1969), sexual maturation (Van den Hurk, Lambert & Peute, 1982; Patino & Schreck, 1986), and reproductive behavior (Hutchinson, 1993). Long-considered to be principally a female reproductive hormone due to its roles in vitellogenesis and choriogenesis (Chester-Jones, Ingleton & Phillips, 1987), the identification of estrogen receptors (ERs) in brain and reproductive tissues of male vertebrates (Callard & Mak, 1985; Cooke, Young, Hess, & Cunha, 1991) point to estrogens' importance in male reproduction as well.

Estrogen homeostasis is critical to the normal function of male and female gonads (Van den Hurk et al., 1982), and to reproductive behavior (Hutchinson, 1993). Exogenous estrogen and xenoestrogens appear capable of disrupting this homeostasis. Administration of exogenous estradiol, the pharmaceutical estrogen 17 $\alpha$ -ethynylestradiol (17 $\alpha$ -EE2), or various xenoestrogens has been correlated with adverse reproductive effects in fish including: decrements in fertility (Gray, Teather & Metcalfe, 1999a; Van den Belt, Verheyen & Witters, 2001); testicular degeneration and atrophy (Christiansen, Korsgaard & Jespersen, 1998; Miles-Richardson, Pierens, Nichols, Kramer, Snyder, Snyder et al., 1999); inhibition of spermatogenesis (Jobling, Sheahan, Osborne, Matthiessen, & Sumpter, 1996; Kinnberg, Korsgaard & Bjerregaard, 2000; Sohoni, Tyler, Hurd, Caunter, Hetheridge, Williams et al., 2001); inhibition of testicular development (Gimeno, Komen, Venderbosch & Bowmer, 1998; Panter, Thompson & Sumpter, 1998); development of intersex testes (Metcalfe, Metcalfe, Kiparissis, Nii, Foran & Benson, 2000; Yokota, Seki, Maeda, Oshima, Tadakoro, Honjo et al., 2001); and ovarian degeneration or suppressed ovarian development (Lange, Hutchinson, Croudace, Siegmund, Schweinfurth, Hampe et al., 2001; Van den Belt et al., 2001). In general, the physiological alterations underlying these effects have not been identified. However, certain studies have implicated administration of exogenous estradiol or xenoestrogens in the suppression of gonadotropins (Lessman & Habibi, 1987; Harris, Santos, Janbakhsh, Pottinger, Tyler & Sumpter, 2001), adverse effects on reproductive behavior (Gray et al., 1999a; Gronen, Denslow, Manning, Barnew, Barnes & Brouwer, 1999; Bjerselius, Lundstedt-Enkel, Olse, Mayer & Dimberg, 2001; Van den Belt et al., 2001); and alterations in p450arom activity (Melo & Ramsdell, 2001) or expression (Scholz & Gutzeit, 2000).

Medaka (*Oryzias latipes*) are commonly chosen as a model organism for use in reproductive studies due to their high and relatively consistent reproductive capacity. Under specific culture conditions of photoperiod, water temperature, and regular feeding, reproductively mature females produce eggs approximately daily on a year-round basis (Koger, Teh & Hinton, 1999). Medaka eggs, typically produced in clusters of 10-25, develop externally. Their transparent chorion allows simple visual determination of fertilization, detectable within 0.2 h by the presence of yolk and a perivitelline space. In our laboratories, medaka larvae hatch within 9-12 days.

As newly-identified xenoestrogens, it is important to characterize the effects of Surflan<sup>TM</sup> and oryzalin on indices of reproductive fitness such as fertility and fecundity. Measurement of these parameters can give the first indication of the likelihood that effects of exposure may extend beyond the individual and potentially impact a population. Accordingly, the present study was designed to determine whether exposure of reproductively-active, mature male and female medaka to Surflan<sup>TM</sup> and oryzalin would affect fertilization success, fecundity, viability of eggs, time-to-hatch, or number of

deformities in the offspring. Gonadal tissues of males and females were also evaluated in order to identify and characterize any exposure-related effects.

## Materials and Methods

The medaka used in this study were the golden strain of *O. latipes*, originally purchased from Carolina Biological Supply and maintained at the University of California, Davis Aquatic Center for 17 y. The colony's brood stock has been out-crossed twice with wild-type Northern strain golden medaka to prevent inbreeding (Davis, 2001).

### Reproductive effects study design.

Sexually mature, five month-old medaka ( $n = 168$ ) were separated into six replicate groups for each treatment, with two female and two male fish per replicate. Only reproductively-active females were utilized, and were identified by the presence of an attached, fertilized clutch of eggs on the day of collection. Each group of four medaka was placed in an aerated 1-l glass beaker of U.S. EPA reconstituted water (Horning & Weber, 1985), and maintained at  $25 \pm 1^\circ\text{C}$ . Glassware was cleaned before use by soaking for 24 h in a detergent solution, rinsed, soaked for an additional 24 h in a 1% nitric acid solution, and rinsed. A photoperiod of 16h light, 8h dark was maintained throughout the experiment, with light provided at 400-450 lux by Vita-Lite (Coralife Chromatic™) bulbs.

Final exposure concentrations of Surflan™ and oryzalin were selected from the results of a preliminary study in which 168 medaka were exposed for 21d to nominal concentrations of Surflan™ (8.5, 5.8, or 3.8  $\mu\text{l/l}$ ), or oryzalin (2.0, 1.3, or 1.0  $\text{mg/l}$ ). Final concentrations for the high-dose group of each chemical were selected from the preliminary study as the 21-day maximally-tolerated concentration (MTC) where tolerance was considered to be those concentrations associated with <15% lethality during the exposure period (3.8  $\mu\text{l/l}$  and 1.0  $\text{mg/l}$  for Surflan™ and oryzalin, respectively). Final exposure concentrations for each of the mid- and low-dose groups were set at approximately half of the concentration of each higher dose.

Exposure to nominal concentrations of oryzalin (1.0, 0.50, or 0.25  $\text{mg/l}$ ) or Surflan™ (3.8, 2.0, or 1.0  $\mu\text{l/l}$ ) or reconstituted water was initiated after fish had been allowed to acclimate for five days, and it had been established that each replicate group was actively producing eggs. Exposure solutions were made fresh daily, and the entire solution of each beaker was replaced every day for the 21-d exposure period. On a daily basis, fish were fed a purified casein (PC)-based diet (DeKoven, Nunez, Lester, Conklin, Marty, Parker et al., 1992) at 2% of their body weight prior to solution change, then received brine shrimp at approximately 3.5% of body weight after solution replacement.

At the end of the 21-d exposure period, fish from each treatment replicate were separated by dose group and gender. Two untreated males were placed with each pair of treated females, and two untreated (reproductively active)

females placed with each pair of treated males. Newly-introduced fish were of the same broodstock and hatch date as the treated animals. Each new group of four medaka was placed in a fresh 1-l beaker, and was maintained in reconstituted water for the duration of the experiment. The entire 1-l volume of water in each beaker was replaced daily throughout the remainder of the experiment. All fish received the same diet as described above.

Following the placement of untreated medaka with treated medaka, fish were allowed to acclimate for two days. Egg collection began on day three post-exposure, and continued for five days thereafter. Eggs were siphoned twice daily from each beaker for five days, counted, and evaluated visually under a microscope to determine fertilization status. Egg masses were separated with forceps to obtain individual eggs, and unfertilized eggs were counted, recorded and discarded. For the first three days of egg collection, fertilized eggs from each replicate were counted and transferred to a 600-mL aerated beaker and observed daily for mortality and hatch. To minimize fungal growth, embryos that died were counted and removed from beakers daily. Approximately 75% of the water was exchanged daily from each beaker. Beakers were observed on a daily basis to detect the presence of newly-hatched larvae. Larvae were counted and the date of hatch noted. Larvae were transferred to an aerated 40-l aerated tank for grow-out (one tank per treatment group). The water temperature and photoperiod were maintained as previously described. Larvae were fed a PC diet, and were raised until approximately 30 days of age. At that time, a sample of 50 juvenile fish from each tank were sacrificed and examined visually for the presence of any gross abnormalities.

### **Histologic effects study design**

Eighty four, seven month-old sexually mature medaka were separated into three replicate groups for each treatment, with two male and two female fish per replicate. Each group of four medaka were placed in an aerated 1-l beaker of reconstituted water, and maintained as described above. Following a two-day acclimation period, exposures to Surflan™ and oryzalin were initiated. Exposure concentrations, solution preparation, solution exchange procedures, and feeding regimens were the same as those used in the reproductive effects study.

At the end of the 21-d exposure period, medaka were euthanized by immersion in tricaine methane sulfonate (MS222, Sigma). Abdominal cavities were surgically opened, and fish were immediately immersed in 10% neutral buffered formalin. Formalin-preserved fish were processed with standard methods for paraffin embedding. Two initial sections were obtained from each paraffin block, mounted on glass slides, and stained with hematoxylin and eosin (HE). If representative sections of gonads were not obtained during the initial processing, paraffin blocks of fish were step-sectioned (at 50  $\mu$ m intervals) for an additional four to six levels as needed to obtain adequate gonadal views.

Glass HE slides were initially sorted by gender and then randomized prior to reading and scoring. All slides were read and scored semi-quantitatively without knowledge of exposure history. Table 1 identifies lesion types and lesion scoring criteria for male and female medaka.

### Statistical analyses

Fertility and fecundity data and both testicular and ovarian lesion scores were evaluated by applying the nonparametric Kruskal-Wallis test for one-way ANOVA using NCCS<sup>®</sup> or JMP<sup>®</sup> statistical software. When comparisons were made on the basis of dose group,  $\alpha$  values were initially adjusted by a Bonferroni correction to account for multiple comparisons, such that  $p < 0.008$  represents a result that is significantly different from controls. The Bonferroni correction is a multiple-comparison correction used to preserve the desired  $\alpha$  value when several independent statistical tests are performed simultaneously. Adjustments of  $\alpha$  values were not made for testicular and ovarian lesion-scores evaluated by treatment category, where an outcome of  $p < 0.05$  was identified as statistically significant. Given that the Bonferroni approach examines only the p-values of each test without accounting for the number of tests within a series that are significant, we also applied the Bernouli equation (Moran, 2003) to determine the probability of obtaining 'n' number of significant results in a series by chance alone. If both the individual ANOVA result and the Bernouli equation applied to a series of tests yielded a p value  $\leq 0.05$ , we concluded that the result was statistically significant. Time-to-hatch data were tested for statistical significance by application of the Wald-Wolfowitz Runs Test, which tests the hypothesis that any two samples (i.e., distributions) came from populations with identical distribution functions. A result of  $p < 0.05$  in the Wald-Wolfowitz Runs Test represents statistical significance.

### Results

Exposure to Surflan<sup>TM</sup> or oryzalin for 21d adversely affected the reproductive outcomes of medaka, significantly affected the hatching time of eggs, and increased the incidence and/or severity of testicular and ovarian lesions.

The daily mean production of non-fertilized (NF) eggs by treated females paired with untreated males, and by untreated females paired with treated males was consistently greater than controls in all dose groups (Table 2). These differences in the mean number of NF eggs were statistically significant in low- and mid-dose oryzalin females, and in mid-dose Surflan<sup>TM</sup> and mid-dose oryzalin males. Considering treated females and males together (F+M), the daily total production of NF eggs was significantly greater than controls in the high- and mid-dose Surflan<sup>TM</sup> groups, as well as in the mid- and low-dose oryzalin groups.

The mean daily total production of eggs was not uniformly affected by exposure to Surflan<sup>TM</sup> or oryzalin. Although several treatment groups produced lower total numbers of eggs than controls, none of these apparent

effects reached statistically significant levels (Table 3). Viability of fertilized eggs was also not affected by treatment, with a mean hatch rate in all groups typically  $\geq 90\%$  (data not shown). Additionally, there were no treatment-related effects on the incidence of gross deformities in juveniles sampled at approximately 30-d post-hatch (data not shown).

The results of the Wald-Wolfowitz Runs Test (Table 4) demonstrate that the time-to-hatch in all Surflan<sup>TM</sup> and oryzalin treatment groups was significantly different from controls. In eggs produced by untreated females paired with treated males, hatching time was significantly increased relative to controls. The opposite trend was apparent in eggs produced by treated females paired with untreated males, where time-to-hatch occurred significantly earlier than in eggs produced by controls.

A 21-d exposure to Surflan<sup>TM</sup> or oryzalin induced intersex lesions with stage one oocytes (S1-Oo) and/or stage 2/3 oocytes (S2/3-Oo's) in male medaka in all treatment groups (Table 5). Pre-intersex lesions (PIL) were also observed in all treatment groups, and there was a low but measurable occurrence of PIL in controls. One control male exhibited a S1-Oo lesion (see discussion). Mean intersex lesion scores were significantly different than controls for S1-Oo (high- and low-dose Surflan<sup>TM</sup> groups); and for S1-Oo and S2/3-Oo's combined (all Surflan<sup>TM</sup> groups as well as the mid-dose Oryzalin group). There was a marked difference in prevalence of S1-Oo's, S2/3-Oo's, or total intersex lesions (S1-Oo or S2/3-Oo) between treated and control groups, where prevalence refers to the number of animals in a treatment group to manifest a specific intersex lesion(s). Intersex prevalence data by treatment category (control, oryzalin, or Surflan<sup>TM</sup>) are presented in Figure 1, and an example of a male with intersex testis is provided as Figure 2.

Some necrosis of spermatids (NST) and necrosis of spermatogonia (NSG) were observed in control animals (Table 5), although the mean severity scores for these lesions were generally higher in animals exposed to Surflan<sup>TM</sup> or oryzalin. The mean lesion severity score for NST approached statistical significance in mid and low dose oryzalin animals. Mean lesion severity scores for NSG were significantly different from controls in the mid-dose Surflan<sup>TM</sup> and low- and mid-dose oryzalin groups. Evaluation of NST and NSG by treatment category ( $n = 18$ ) demonstrated that oryzalin ( $p < 0.05$ ) but not Surflan<sup>TM</sup> caused statistically significant increases in the mean lesion severity score for these categories of testicular lesion (Table 6).

Of the five categories of ovarian lesions; ovarian macrophage aggregates (OMA), oocyte atresia (OA), ovarian follicular necrosis (OFN), immature oocytes (IO), and ovarian hyperplasia or hypertrophy (OHH), three showed little to no effect of exposure to either Surflan<sup>TM</sup> or oryzalin (OMA, OA, OFN). All oryzalin dose groups exhibited mean lesion scores for IO's that were approximately half those determined for controls. None of these results was statistically significant when evaluated on the basis of dose (data not shown); however, when evaluated by treatment category, the



mean lesion severity scores for IO's in oryzalin-exposed animals was significantly lower than in controls (Table 7). The severity of OHH also seemed to be affected by exposure to Surflan<sup>TM</sup> and oryzalin, with mean lesion severity scores in all dose groups greater than those in controls (data not shown). Evaluation of OHH by treatment category (Table 7) demonstrated that exposure to oryzalin was associated with a statistically significant higher mean lesion score for OHH relative to controls.

## Discussion

Male medaka undergo sexual differentiation of the gonad at approximately 2 weeks post-hatch (Okada, 1964). Induction of intersex lesions in genotypic adult male medaka with differentiated testes following exposure to estradiol or xenoestrogens has been reported by Egami (1955), Shibata & Hamaguchi (1988), and Gray, Niimi & Metcalfe (1999b) among others. The gonadal plasticity exhibited by male medaka has been attributed to the existence of bipotential spermatogonia, whereby type B (secondary) spermatogonia retain the ability to form ovarian tissue under estrogenic influences (Shibata & Hamaguchi, 1988).

In the present study, intersex was induced in 10/18 (55.6%) of medaka exposed to oryzalin and in 15/17 (88%) of medaka exposed to Surflan<sup>TM</sup> for 21 d (Figure 1). These are comparatively high values relative to the 17% incidence reported by Gray et al. (1999b) for adult medaka exposed to 200 or 300 µg/l octylphenol for 18 or 36 d, and are generally comparable to the ~ 70% incidence reported by Egami (1955) in medaka exposed to 80 or 160 µg/l estradiol for 31 to 37 d. The biological significance of intersex lesions to an individual fish is unclear, in part because intersex lesions tend to regress once the estrogenic stimulus is withdrawn (Okada, 1964; Shibata & Hamaguchi, 1988). Furthermore, it is not known whether intersex lesions within a testis affect fertility (Gray et al., 1999a).

The spontaneous development of intersex testes in naïve medaka has not been formally reported in the open literature. However, the experience in our laboratory has been that intersex testes do occur – albeit infrequently – in naïve fish (Koger, Teh & Hinton, 2000; Davis, 2002). One possible explanation for the occurrence of intersex testes in one of the control males is that these lesions can be induced by a potentially estrogenic compound present in the PC diet used by our laboratory. The PC diet formulated and prepared in our laboratory contains soy lecithin at approximately 6% of the total dry weight. Soy food products are estrogenic in humans (e.g., see Kurzer, 2002), and it is likely that they would have similar activity in teleosts. Alternatively, estrogens released by females into aquaria water may be able to induce intersex – either singly, or in combination with the soy-derived lecithin. That biologically-active levels of estrogen may occur in aquaria water is indicated by reports of vitellogenin (Vg) in unexposed male fish (Tyler, van der Eerde, Jobling, Panter & Sumpter, 1996; Panter et al., 1998; Tyler, van Aerle, Hutchinson, Maddix & Trip, 1999). Vitellogenin expression is

regulated by estradiol, and typically, Vg is not produced at measurable levels in males in the absence of estrogenic stimulus. Whether biologically active levels of estrogen are present in aquaria water in our facilities has not been established, nor is it known whether levels of estrogen, if present, are sufficient to induce intersex testes. Although the occurrence of the S1-Oo lesion in a control male cannot be definitively explained, it is clear that both Surflan™ and oryzalin dramatically affect the occurrence and severity of intersex lesions in medaka.

In this study, Surflan™ and oryzalin adversely affected spermatogenesis, increasing the incidence and severity of NST and NSG in treated animals above that observed in controls. In mammals, germ cell degeneration is a common phenomenon in adult males (Huchkins, 1978; Kerr, 1992), and Nonclerq, Reverse, Toubreau, Beckers, Sulon, Laurent et al. (1996) documented a low but measurable incidence of apoptosis in the seminiferous epithelium of untreated male hamsters. Although analogous data specific to teleosts does not exist, it is likely that a similar phenomenon occurs and is the underlying basis for the occurrence of NST and NSG in controls.

The effects of Surflan™ and oryzalin on NST and NSG are consistent with previously-reported effects of estrogens or xenoestrogens on spermatogenesis, in which necrotic spermatids (Miles-Richardson et al., 1999), inhibition of spermatogenesis (Jobling et al., 1996; Kinnberg et al., 2001; Sohoni et al., 2001), mild degeneration of testes (Lange et al., 2001), and testicular atrophy (Jobling et al., 1996; Panter et al., 1998) have been reported. These adverse effects on gonad structure and gametogenesis encompass a range of diagnostic criteria and a spectrum of severity of effect. Underlying these differences are the use of separate species, distinct chemicals, different exposure durations, and different dosages. Notwithstanding the existence of these variables, there is considerable consistency in the nature of effects on the male gonad associated with exposure to estrogenic substances. How a xenoestrogen elicits these effects is not clear, but Surflan™ and oryzalin may act directly at the gonad by either suppressing testosterone synthesis, altering testosterone's rate of conversion to estradiol by affecting p450aromatase, or indirectly via feedback effects at the pituitary that impact the synthesis and secretion of gonadotropins and in turn, affect steroid levels within the gonad.

The development of ovarian lesions after estrogenic challenge is both more poorly defined and less frequently identified than effects in males. We identified treatment-related effects on two categories of ovarian lesion; IO and OHH. When analyzed by treatment type, oryzalin but not Surflan™ was associated with a significant decrease in the mean severity score for IO's. The scoring criteria for IO (Table 1) were established such that the lower the score, the fewer the number of immature oocytes present in an ovary. Oocytes are derived from mitotically-active oogonia, stem cells that develop from primordial germ cells, which migrate into the ovary during embryogenesis. Oogonial proliferation and oocyte formation are followed by development of the oocyte within a follicle. Under the influence of gonadotropins,

follicles synthesize testosterone and estradiol, where estradiol is produced via the p450 aromatase-mediated conversion of testosterone (Nagahama, 1987). The decrease in the number of immature oocytes in ovaries of exposed females may be a consequence of oryzalin acting to inhibit the formation or maturation of oocytes – either by some direct action on oögonia, or by interference with estrogen or gonadotropin homeostasis. Van den Belt et al. (2001) reported a qualitatively similar effect in zebrafish exposed to 17 $\alpha$ -EE2 i.e., highly reduced ovarian somatic indices and an absence of “yolky oocytes”, indicating that 17 $\alpha$ -EE2 interferes with oocyte development in this species.

Many of the same treatment groups that displayed a decrease in the number of IO's, also exhibited OHH. The lesion score data for individual animals were reviewed, and in most cases, both lower IO scores and higher OHH scores were documented in the same ovary. Considering the scores for these two criteria, our data indicates that there was both a relative decrease in the number of IO's, and an increase in the number of developing oocytes and/or an overall enlargement of the ovary, primarily in oryzalin-treated animals. From estrogen receptor knockout mice, it has been established that estradiol is not required for the development of primary and secondary oocytes, but is critical to the ability of an oocyte to progress beyond the secondary stage to an antral state (for review, see Drummond & Findlay, 1999). Subsequent oocyte maturation and ovulation then depend on estradiol and FSH-mediated expression of LH receptors, stimulation of an LH surge, and LH-mediated terminal differentiation of granulosa cells (reviewed in Robker & Richards, 1998). Therefore, interference with estradiol, FSH (GtH-I), or LH (GtH-II) synthesis, or xenoestrogen-induced suppression of GtH-I or GtH-II could impair normal ovulation and manifest as an increase in the number or size of maturing oocytes within an ovary as well as a greater ovarian size. However, if oocyte development or ovulation is impaired by treatment with oryzalin, the effect appears to be relatively subtle, as oryzalin did not measurably affect the fecundity of treated females.

Exposure to Surflan™ or oryzalin increased the production of nonfertilized eggs (and thus decreased the production of fertilized eggs) in all treatment groups of both genders. The production of nonfertilized eggs was a highly variable event, and was typically manifested as most or all of a given clutch of eggs failing to be fertilized on the day of collection. This tended to alternate with successful fertilization for a period of days, followed by another failed fertilization. This pattern of fertilization failure was observed in all of the treated groups, and yielded fertilization data characterized by substantial variability. This variability in the response of individuals within all treatment groups made it exceptionally difficult to demonstrate statistical significance.

Exposure to both Surflan™ and oryzalin was associated with reduced fertilization capacity of treated male medaka paired with untreated females. This phenomenon has been observed in zebrafish, where an exposure to 17 $\alpha$ -EE2

caused a dose-related reduction in the number of unexposed females that spawned when paired with exposed males (Van den Belt et al., 2001). Male medaka exposed to the xenoestrogen octylphenol (OP) for 6 months were also found to be significantly less likely to fertilize eggs than untreated controls. These latter data, reported within the context of OP-induced effects on behavior, also documented a significant correlation between impaired courtship behavior in treated males and a lower egg fertilization success rate (Gray et al., 1999a). Bjerselius et al. (2001) showed that a relatively brief exposure to estradiol (24-28 d) negatively impacted a number of courtship parameters in treated male goldfish paired with an unexposed, artificially-induced spawning female. Therefore, for males in particular, but also potentially for females, decreased fertility may be due to effects of treatment with estrogenic substances on reproductive behavior. Although certain data indicate that exogenous estrogen may impact brain p450arom activity, a causal link between altered p450arom activity and behavior have not been established (Callard, Kruger & Betka, 1995; Melo & Ramsdell, 2001).

There were statistically significant differences in the time-to-hatch of eggs from all groups of males and females treated with Surflan™ and oryzalin (Table 4). In all Surflan™ and oryzalin dose groups, eggs produced by untreated females paired with treated males took significantly longer to hatch than eggs from controls, and eggs produced by treated females paired with untreated males hatched significantly earlier than controls. For exposed medaka of both genders, the effects on time-to-hatch were remarkably consistent, occurring in all dose groups. The biological significance of these effects is not clear, given that survival of progeny was not affected by exposure, nor were there any effects on the incidence of deformities in juveniles grown to approximately 30 d post-hatch.

Although we have documented gonadal lesions in both males and females and altered fertilization success in both genders of medaka exposed to Surflan™ or oryzalin, there is not a complete correspondence between the groups that manifested the most severe lesions and the groups that exhibited the greatest effects on fertility. However, in general, treatment with oryzalin at 0.5 mg/l or 0.25 mg/l, and treatment with Surflan™ at 2.0 or 1.0 µg/l had the greatest adverse effects on male and female fertility as well as on the severity of non-intersex gonadal lesions.

The maximum dose of oryzalin and maximum concentration of Surflan™ used to assess reproductive toxicity were selected on the basis of range-finding studies that identified an apparent MTC for each compound. Mid- and low-dose amounts of Surflan™ and oryzalin were set at approximately 50% decrements of the MTC or of the mid-dose quantity, respectively. Surflan™ is an emulsion that contains 40.4% oryzalin, and at the concentrations used, gave nominal concentrations of oryzalin of 1.5 mg/l, 0.8 mg/l, and 0.4 mg/l, levels that are approximately 50-60% higher than were administered for oryzalin given alone. Despite the administration of greater amounts of oryzalin in the Surflan™ groups, in general, Surflan™ did not elicit more severe effects than the lower doses of oryzalin administered alone. The

one clear exception to this is seen in the prevalence and severity data for induction of intersex lesions, where Surflan™ consistently caused more severe lesions as well as a greater prevalence of these lesions than oryzalin alone. It is also noteworthy that the high-dose oryzalin group (1 mg/l) had no statistically significant or marginally significant effects on non-intersex testicular lesions or on any measure of male or female fertility, and in only one category of ovarian lesion, OHH, did effects approach statistical significance. In fact, the most severe lesions often occurred in the low-dose oryzalin group, or the effects in that group were similar in magnitude to those observed from treatment with the next higher dose. Similarly, the highest concentration of Surflan™ was not associated with significant effects on non-intersex testicular lesions or on any ovarian lesion. These results suggest that oryzalin administered alone, or administered within the mixture Surflan™, has a distinctly non-linear dose response for the reproductive parameters assessed here. This phenomenon has been documented for certain other xenoestrogens, but is not a universal characteristic of these compounds (Andersen, Conolly, Faustman, Kavlock, Portier, Sheehan et al., 1999; Melnick, Lucier, Wolfe, Hall, Stancel & Prins, 2002). The National Toxicology Program's Panel on low-dose effects and nonmonotonic dose-response relationships for endocrine-disrupting chemicals (EDCs) focused on mammalian testing of EDCs, and did not extend their review to fish. Nonetheless, the panel concluded that current testing 'paradigms' for the assessment of reproductive and developmental toxicity may require reassessment to account for this fact (Melnick et al., 2002). It is clear from these data that a lowest-observed-adverse-effect-level for reproductive effects of oryzalin and Surflan™ has not been defined by our work. Further testing to evaluate the consequences of exposure to environmental levels of oryzalin must consider the dose-response phenomenon suggested by our data and structure studies accordingly.

## Conclusion

A relatively brief (21 d) exposure of mature, reproductively-active medaka to Surflan™ and oryzalin impaired fertilization success of both males and females, and affected the hatching time of eggs. Fecundity, viability of eggs, and incidence of deformities of offspring were not affected by exposure. Exposure to Surflan™ or oryzalin increased the prevalence and severity of intersex lesions, and exposure to oryzalin increased the severity of NST and NSG. Whether the testicular lesions caused by exposure led directly to the observed decrements in fertility in males, or whether there are indirect effects of treatment that adversely affect fertility –such as impairment of reproductive behavior—are not known.

Oryzalin affected the number of immature oocytes present in the ovaries of exposed females, potentially via inhibition of the mitotic division of oogonia. Alternatively, oryzalin may inhibit oocyte development and/or impair ovulation, leading to the observed paired phenomena of decreased IO and increased OHH scores. Oryzalin also adversely affected spermatogonia, and thus it appears that oryzalin may be capable of affecting the viability or function of germ cells

of both males and females. Although the mechanism(s) of these effects remains to be identified, it appears plausible that a phenomenon experienced by both genders – such as alteration in estrogen homeostasis– may underlie the toxicity elicited by these compounds.

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Table 1. Histologic lesion definitions and criteria. A criterion score of 0 was assigned if no lesion was present.

Gender	Lesion (criterion score) <sup>a</sup>	Definition
Males	Pre-intersex lesion (PIL) (1: < 2 PIL cells; 2: 2-5 PIL cells; 3: >5 PIL cells).	Hypertrophied spermatogonia; karyomegaly; prominent single nucleoli; increased euchromatin; numerous provitelline nucleoli.
	Intersex: stage 1 oocytes (S1-Oo) (1: < 1 S1-Oo; 2: 1-2 S1-Oo clusters; 3: > 2 S1-Oo clusters).	Clusters of small cells as discrete acini distinct from normal seminiferous tubules. Pale eosinophilic to amphophilic cytoplasm; higher than normal nucleus to cytoplasm ratio; prominent oval nuclei with more euchromatin and an absence of distinct nucleoli.
	Intersex: stage 2/3 oocytes (S2/3-Oo) (1: 1-2 stage 2 oocytes; 2: >2 stage 3 single oocytes or 1 oocyte cluster; 3: >2 clusters of stage 3 oocytes).	Stage 2 oocytes: 5-15 $\mu$ m, usually solitary; large central nuclei with diffuse euchromatin and small amounts of intensely basophilic cytoplasm; Stage 3 oocytes: clusters of 15-45 cells, to 30 $\mu$ m; cells with abundant intensely basophilic cytoplasm, marked karyomegaly (nuclei 4-30 $\mu$ m). Translucent euchromatin and provitelline nucleoli.
	Necrosis, spermatids (NST) (1: < 2 dead spermatids; 2: 2-20 dead spermatids; 3: > 20 dead spermatids. Numbers are per 40X field).	Spermatids with irregular hyperchromatic heads (2-5 times normal size).
	Necrosis, spermatogonia (NSG) (1: 2-10 dead spermatogonia; 2: 11-20 dead spermatogonia; 2: >20 dead spermatogonia. Numbers are per 40X field).	Rounding of individual spermatogonia, hyper-eosinophilic cytoplasm, and nuclear pyknosis.
Females	Ovarian macrophage aggregates (OMA) (1: < 3 OMA; 2: 3-10 OMA; 3: > 10 OMA Numbers are per 20X field).	Large macrophages ( $\geq$ 50 $\mu$ m) with foamy pale eosinophilic debris concentrated in the medullary portion of the ovary.
	Ovarian follicular necrosis (OFN) (1: < 4 necrotic follicles; 2: 4-5 necrotic follicles; 3: > 6 NF). Numbers are NF per 4, 10X fields.	Presence of large necrotic follicles (several hundred $\mu$ m diameter), collapsed, distorted, and empty of oocyte. Follicles lined by necrotic columnar epithelium.
	Oocyte atresia (OA) (1: < 3 atretic oocytes (AO per ovary); 2: 3-5 AO; 3: > 5 AO).	Atretic oocytes or follicles with distinct chorions and abundant yolk protein. Chorion distorted and collapsed, yolk disorganized and fragmented; variable influx of macrophages.
	Ovarian hypertrophy/hyperplasia (OHH) (1: ovary 20-30% of abdominal cavity (AC); 2: ovary 31-40% of AC; 3: ovary >40% of AC).	Overall enlargement of ovary, with an increased number and density of developing oocytes.
	Immature oocytes (IO) (1: IO 5-10% of total oocytes (TO); 2: IO 11-50% TO; 3: IO >50% TO).	Characterized by scoring the percentage of immature oocytes within an ovary by small size, absence of definitive chorion, absence of distinct yolk protein or oil droplets, abundant basophilic cytoplasm, distinct nuclei.

Table 2. Daily mean production of non-fertilized eggs in medaka after exposure to either Surflan™ or oryzalin.

Exposure concentration	Treated medaka Females and Males <sup>a</sup>	Treated Females <sup>b</sup>	Treated Males <sup>c</sup>
Control	1.7	1.7	1.8
Surflan™ 3.8 µl/l	3.7* <sup>ψ</sup>	3.1 n.s.	4.4 n.s.
Surflan™ 2.0 µl/l	3.7 ** <sup>ψ</sup>	2.9 n.s.	4.6* <sup>τ</sup>
Surflan™ 1.0 µl/l	2.7 n.s.	2.3 n.s.	3.2 n.s.
Oryzalin 1 mg/l	5.1 n.s.	5.3 n.s.	4.9 n.s.
Oryzalin 0.5 mg/l	4.8 *** <sup>ψ</sup>	4.5 * <sup>τ</sup>	4.9 * <sup>τ</sup>
Oryzalin 0.25 mg/l	4.7 ** <sup>ψ</sup>	5.2 ** <sup>τ</sup>	4.2 n.s.

<sup>a</sup> Data are from 12 replicates; 6 containing treated males (paired with untreated females) or 6 treated females (paired with untreated males). Each replicate contained 2 males and 2 females.

<sup>b</sup> Data are from 6 replicates containing treated females paired with untreated males. Each replicate contained 2 males and 2 females.

<sup>c</sup> Data are from 6 replicates containing treated males paired with untreated females. Each replicate contained 2 males and 2 females. n.s. = not significant.

\*p < 0.05 determined by ANOVA.

\*\*p < 0.005 determined by ANOVA.

\*\*\*p < 0.001 determined by ANOVA.

<sup>ψ</sup> Probability of obtaining 4 of 6 tests with p < 0.05 by chance = 0.00008 (Moran, 2003).

<sup>τ</sup> Probability of obtaining 2 of 6 tests with p < 0.05 by chance = 0.03 (Moran, 2003).

Table 3. Mean daily total egg production of medaka after exposure to either Surflan™ or oryzalin. The tests of significance are Kruskal-Wallis one-way ANOVA nonparametric tests of differences in the medians between the individual treatment and control groups. All  $\alpha$  values for determining statistical significance were adjusted by a Bonferroni correction to account for multiple comparisons.

Exposure concentration	Treated medaka Females and Males <sup>a</sup>	Treated Females <sup>b</sup>	Treated Males <sup>c</sup>
Control	28.0	30.8	25.1
Surflan™ 2.5 µl/l	23.3 *	23.6 *	23.0 n.s.
Surflan™ 1.3 µl/l	26.1 n.s.	24.8 n.s.	27.4 n.s.
Surflan™ 0.67 µl/l	28.8 n.s.	29.7 n.s.	27.8 n.s.
Oryzalin 1 mg/l	28.4 n.s.	35.6 n.s.	21.1 n.s.
Oryzalin 0.5 mg/l	28.2 n.s.	31.7 n.s.	24.7 n.s.
Oryzalin 0.25 mg/l	22.8 *	26.9 n.s.	18.6 *

<sup>a</sup> Data are from 12 replicates; 6 containing treated males (paired with untreated females) or 6 treated females (paired with untreated males). Each replicate contained 2 males and 2 females. Kruskal-Wallis ANOVA results for Treated medaka, Females and Males: F ratio, 1.81; p = 0.095; DF = 6

<sup>b</sup> Data are from 6 replicates containing treated females paired with untreated males. Each replicate contained 2 males and 2 females. Kruskal-Wallis ANOVA results for Treated Females: F ratio, 2.44; p = 0.0269; DF = 6

<sup>c</sup> Data are from 6 replicates containing treated males paired with untreated females. Each replicate contained 2 males and 2 females. Kruskal-Wallis ANOVA results for Treated Males: F ratio, 1.72;  $p = 0.1164$ ; DF = 6

n.s = not significant.

\*  $p < 0.05$ . Not significant by the criterion for statistical significance ( $p < 0.008$ ) established by use of the Bonferroni correction for multiple comparisons; data are included to indicate that a value approached statistical significance.

Table 4. Results of time-to-hatch data evaluated by the Wald-Wolfowitz Test. All results are statistically significant ( $p < 0.05$ ).

Exposure concentration	Treated Females <sup>a</sup>		Treated Males <sup>b</sup>	
	No. of eggs	No. of runs <sup>c</sup>	No. of eggs	No. of runs <sup>c</sup>
Controls	368		324	
Surflan <sup>TM</sup> 3.8 µl/l	323	2 (-)	317	1 (+)
Surflan <sup>TM</sup> 2.0 µl/l	286	2 (-)	369	3 (+)
Surflan <sup>TM</sup> 1.0 µl/l	438	2 (-)	394	3 (+)
Oryzalin 1 mg/l	578	2 (-)	242	3 (+)
Oryzalin 0.5 mg/l	256	4 (-)	314	1 (+)
Oryzalin 0.25 mg/l	381	4 (-)	210	2 (+)

<sup>a</sup> Data are from 6 replicates containing treated females paired with untreated males. Each replicate contained 2 males and 2 females.

<sup>b</sup> Data are from 6 replicates containing treated males paired with untreated females. Each replicate contained 2 males and 2 females.

<sup>c</sup> The sign of the outcome of the Wald-Wolfowitz Test indicates whether the time-to-hatch was significantly increased (+) or decreased (-) by treatment.

Table 5. Mean testicular lesion severity scores in medaka exposed to Surflan<sup>TM</sup> or oryzalin. Lesions and lesion scoring criteria are defined in Table 1.

Treatment group <sup>a</sup>	Pre-intersex lesion	Intersex stage 1 oocyte	Intersex stage 2/3 oocyte	Intersex stages 1 & 2/3 oocytes	NST <sup>b</sup>	NSG <sup>c</sup>
Control	0.33	0.17	0.00	0.2 (17%)	1.00	0.83
Surflan <sup>TM</sup> 3.8 µl/l	0.67 <sup>n.s.</sup>	1.50* <sup>τ</sup>	0.67*	2.2* <sup>ψ</sup> (83%)	1.17 <sup>n.s.</sup>	1.33 <sup>n.s.</sup>
Surflan <sup>TM</sup> 2.0 µl/l	0.40 <sup>n.s.</sup>	0.60 <sup>n.s.</sup>	0.20 <sup>n.s.</sup>	0.8* <sup>ψ</sup> (80%)	2.00 <sup>n.s.</sup>	1.80* <sup>φ</sup>
Surflan <sup>TM</sup> 1.0 µl/l	0.67 <sup>n.s.</sup>	1.33** <sup>τ</sup>	0.33 <sup>n.s.</sup>	1.7* <sup>ψ</sup> (100%)	1.33 <sup>n.s.</sup>	0.83 <sup>n.s.</sup>
Oryzalin 1 mg/l	0.67 <sup>n.s.</sup>	0.83 <sup>n.s.</sup>	0.17 <sup>n.s.</sup>	1.0 <sup>n.s.</sup> (50%)	1.33 <sup>n.s.</sup>	1.33 <sup>n.s.</sup>
Oryzalin 0.5 mg/l	0.83 <sup>n.s.</sup>	0.67 <sup>n.s.</sup>	0.83 <sup>n.s.</sup>	1.5* <sup>ψ</sup> (67%)	2.17*	2.0* <sup>φ</sup>
Oryzalin 0.25 mg/l	0.50 <sup>n.s.</sup>	0.33 <sup>n.s.</sup>	0.33 <sup>n.s.</sup>	0.7 <sup>n.s.</sup> (50%)	2.17 <sup>n.s.</sup>	2.0** <sup>φ</sup>

<sup>a</sup> Scores are the mean of observations on 6 males per treatment group.

<sup>b</sup> NST refers to necrosis of spermatids.

<sup>c</sup> NSG refers to necrosis of spermatogonia.

n.s. not significant.

\* p < 0.05 determined by one-way ANOVA.

\*\*p < 0.005 determined by one-way ANOVA.

<sup>ψ</sup> Probability of obtaining 4 of 6 tests with p < 0.05 by chance = 0.00008 (Moran, 2003).

<sup>φ</sup> Probability of obtaining 3 of 6 tests with p < 0.05 by chance = 0.04 (Moran, 2003).

<sup>τ</sup> Probability of obtaining 2 of 6 tests with p < 0.05 by chance = 0.03 (Moran, 2003).

Table 6. Mean testicular lesion scores in medaka compared by category of treatment.

Treatment category	NST <sup>a</sup>	NSG <sup>a</sup>	NST & NSG <sup>a</sup>
Control <sup>b</sup>	1.0	0.83	1.83
Oryzalin <sup>c</sup>	1.89 <sup>d</sup>	1.78 <sup>d</sup>	3.67 <sup>d</sup>
Surflan <sup>TM</sup> <sup>c</sup>	1.47	1.29	2.76

<sup>a</sup> NST refers to necrotic spermatids; NSG refers to necrotic spermatogonia.

<sup>b</sup> Scores are the mean of observations on 6 males per treatment group.

<sup>c</sup> Scores are the mean of observations on 18 males per treatment group.

<sup>d</sup> Result is statistically significant (p < 0.05) by one way ANOVA.

Table 7. Mean ovarian lesion scores in medaka compared by category of treatment.

Treatment category	OMA <sup>a</sup>	OA <sup>a</sup>	OFN <sup>a</sup>	IO <sup>a</sup>	OHH <sup>a</sup>
Control <sup>b</sup>	1.83	0.17	1.83	1.2	2.2
Oryzalin <sup>c</sup>	2.10 <sup>n.s.</sup>	0.17 <sup>n.s.</sup>	2.01 <sup>n.s.</sup>	0.67 <sup>d</sup>	2.80 <sup>d</sup>
Surflan <sup>TM c</sup>	2.30 <sup>n.s.</sup>	0.17 <sup>n.s.</sup>	1.67 <sup>n.s.</sup>	1.28 <sup>n.s.</sup>	2.30 <sup>n.s.</sup>

<sup>a</sup> OMA, ovarian macrophage aggregates; OA, oocyte atresia; OFN, ovarian follicular necrosis; IO, immature oocytes; OHH, ovarian hyperplasia or hypertrophy.

<sup>b</sup> Scores are the mean of observations on 6 females per treatment group.

<sup>c</sup> Scores are the mean of observations on 18 females per treatment group.

<sup>d</sup> Result is statistically significant ( $p = 0.007$ ) by one way ANOVA.

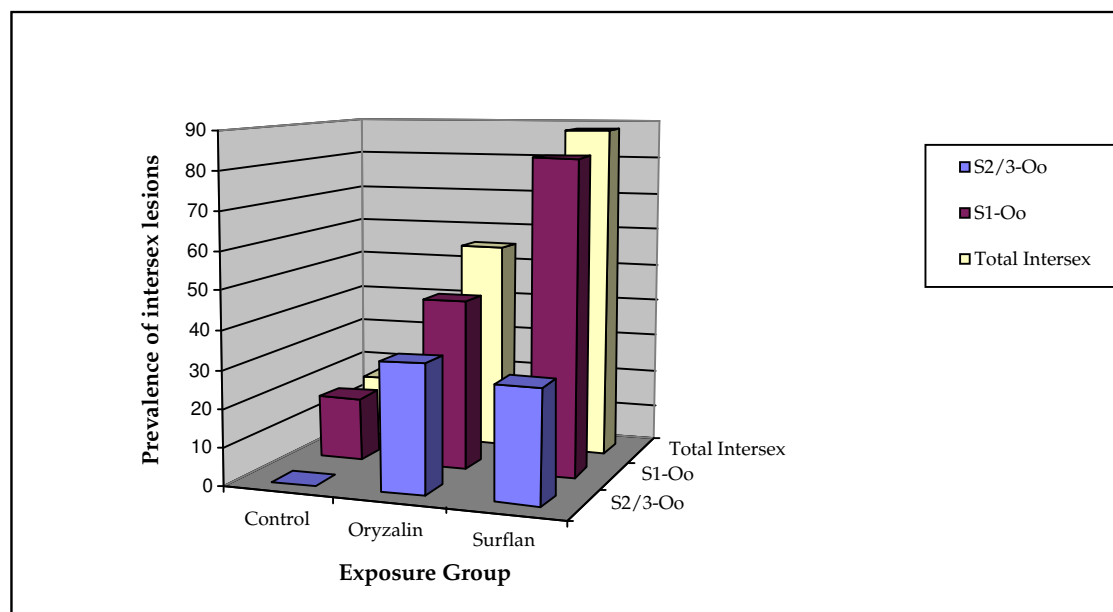


Figure 1. Prevalence of intersex lesions in testes of control medaka and those exposed to oryzalin and Surflan<sup>TM</sup>. S1-Oo = stage 1 oocytes; S2/3-Oo = stage 2 or 3 oocytes; Total Intersex = combined stage 1 and stage 2 or 3 intersex lesion severity scores.

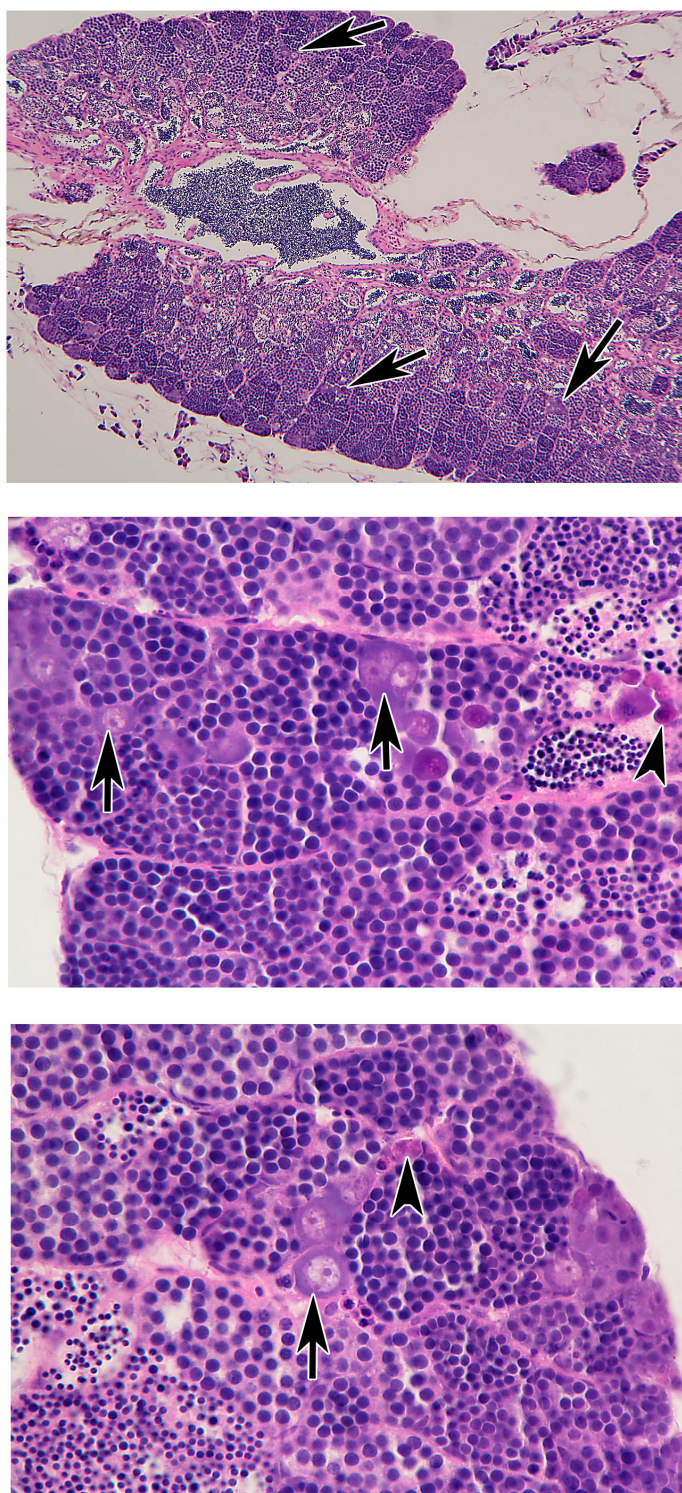


Figure 2. A. Intersex testis in a mature, sexually active male medaka exposed to 0.5 mg/l oryzalin. Arrows point to primary oocytes (100X). Higher magnification view (600X white box) reveals B. a significant number of primary oocytes (>10) seen within a testicular tubule (arrows), and C. several degenerated primary oocytes. All views from hematoxylin and eosin stained tissue.