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Atrazine disrupts gonadal development in a live-bearing lizard

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Abbreviations: ATZ, Atrazine; DES, diethylstilbestrol; EDC, Endocrine disrupting chemical; POF, Polyovular follicle; ST, seminiferous tubule; SVL, snout vent length.

Atrazine (ATZ) is an endocrine disruptor that increases aromatase activity. In viviparous (live-bearing) vertebrates, embryos may be exposed to endocrine disruptors via the placenta. Studies of the effects of ATZ in viviparous amniotes have focused on rodents, which are relatively insensitive to ATZ: studies on other viviparous amniotes are therefore required. We aimed to determine the effects of gestational exposure to a single dose of ATZ at 10 ppb on gonadal development in a viviparous skink, *Niveoscincus metallicus*. Pregnant skinks were exposed to ATZ, the synthetic estrogen diethylstilbestrol (DES) (positive control), vehicle solvent or no treatment. Gonads were examined histologically at birth. Females born to ATZ and DES exposed mothers were more likely to exhibit ovaries with abnormal oocytes than were females whose mothers received vehicle solvent or no treatment. Males born to ATZ and DES exposed mothers were equally more likely to exhibit testes devoid of germ cells with reduced organization of seminiferous tubules (ST) compared to males born to mothers receiving vehicle solvent or no treatment. However, ATZ treatment significantly increased the number of male neonates born with testicular lesions compared to males born to mothers in any other group. We conclude that atrazine disrupts gonadal differentiation in the viviparous lizard, *N. metallicus*. The similar effects of DES and ATZ suggest that the developmental effects of ATZ in *N. metallicus* reflect increased estrogen signaling. Atrazine should be used with caution as exposure of wildlife to this EDC is likely to have adverse effects on reproductive health.

Introduction

Endocrine disrupting chemicals (EDCs) in the environment pose major threats to wildlife, particularly via impairment of reproductive success. The most profound effects of EDC exposure on the reproductive system occur during sexual differentiation; exposure to EDCs at critical stages of embryogenesis can result in impaired gonadal development of both male and female embryos,[1-3] sex reversal,[4,5] impaired fertility[6,7] or reproductive cancers[8,9] although these effects might not be apparent until later life.

To date, analyses of the effects of EDCs in vertebrates have focused on oviparous species, primarily because of the ease with which manipulations of the embryonic environment may be performed. However studies on viviparous vertebrates are urgently required. In viviparous vertebrates, the placenta allows transfer of maternal hormones to the embryo,[10-12] while placental hormone synthesis is critical to embryonic development.[12,13] Therefore the cumulative effects of disruption of maternal and placental endocrine function have the potential to magnify the effects of EDCs on the developing embryos of viviparous species.

The herbicide atrazine (ATZ) is one EDC that impacts on reproductive function in vertebrates. Atrazine disrupts the hypothalamo pituitary gonadal (HPG) axis via multiple mechanisms. This EDC increases expression of the enzyme aromatase[14-17] which converts aromatisable androgens to estrogens.[18] Atrazine also decreases circulating HPG hormones including gonadotrophin releasing hormone (GnRH), luteinizing hormone (LH)[19-21] and the androgen dihydrotestosterone (DHT),[22] and alters DHT availability.[23]

Consistent effects of ATZ have been identified in representatives of all the major vertebrate groups,[22] but the only viviparous vertebrates in which the effects of ATZ have been studied are laboratory rodents. Although laboratory rodents may be useful models for assessment of the effects of some EDCs,[3,9] they are not the most appropriate with which to study the effects of ATZ in viviparous vertebrates. Firstly, no rodent placenta studied to date expresses aromatase and is thus atypical compared with other

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viviparous vertebrates including humans.[12,18,23] The rodent placenta is therefore unlikely to be a site of disruption by ATZ. Secondly, ATZ disrupts aromatase expression in tissues which express aromatase via the ArPII promoter in cells that also express steroidogenic factor 1 (SF 1)[14,15] but in female rat embryos SF 1 is not active in the differentiating ovary until just before birth.[24] Thus disruption to various reproductive indices in laboratory rodents occurs only at concentrations of ATZ (1000 ppb 100 ppm) which are far above those of ecological relevance.[25,26] We therefore require other model species with which to examine the effects of ATZ in viviparous vertebrates.

Reptiles exhibit a diverse range of parity modes, with some 30% of reptilian species being viviparous. Most viviparous reptiles utilize both a yolk and a placenta to support embryonic development and, accordingly, 4 main reptilian placental types have been defined.[27-31] Embryos of Type I species are entirely dependent upon yolk, while Type IV species are almost entirely placentotropic.[32,33] The intermediate Types II and III exhibit varying degrees of placental complexity and dependence upon yolk as a source of embryonic nutrition.[27-31] Viviparous reptiles are therefore of particular interest in EDC studies due to the multiple pathways through which EDCs may affect development: via altered maternal endocrine signals transferred to the embryo via the yolk[34] or the placenta;[10,11,35] by altered placental function[36] or via direct embryonic contamination via the yolk or the placenta. However, to date there have been no studies of the effects of EDCs on reproductive function in viviparous reptiles.

Niveoscincus metallicus is a small (adult mass ~ 3 5 grams, 45 65 mm snout vent length) viviparous skink with a moderately complex Type II placenta and a moderately sized yolk[29,37-39] so both yolk and placenta are significant sources of embryonic nutrition. The reproductive physiology of this species is well studied;[29,38,40] importantly, placental aromatase activity is high (comparable with that in maternal ovaries), at the time of embryonic sexual differentiation,[36] although the specific promoters of aromatase have not yet been characterized. *Niveoscincus metallicus* can be collected in large numbers, is easy to maintain in captivity, and therefore provides an excellent first model for examining the impacts of EDCs in viviparous reptiles. Furthermore, the wide distribution of *N. metallicus* means that some populations may occur in locations where ATZ is used, meaning that the ecological impacts of ATZ could potentially be assessed in the future.[41,42]

This study aimed to determine the effects of a single low dose of ATZ (10 ppb) on embryos of *Niveoscincus metallicus* exposed *in utero* during the initiation of sexual differentiation. We have previously established the effects of increased estrogen signaling during embryonic development in this model species using the potent estrogen mimic diethylstilbestrol (DES)[43]: DES was therefore utilised as a positive control in this study. We examined the effects of ATZ and DES on gonadal development in neonatal *N. metallicus* born to exposed mothers. We also measured neonatal locomotor performance as proxy for a range of physiological traits,[44,45]plus the weight of abdominal fat bodies, snout to vent length (SVL) and body weight, all of which relate to body condition and can be affected by altered hormone regimes.[46-49] As in many other squamate reptiles, embryos of both sexes of *N. metallicus* develop hemipenes although in the females the hemipenes regress at a species specific stage of development.[50-52] We therefore measured the size of the hemipenes of both male and female neonates; reduced phallus size is a known marker of endocrine disruption.[3,53]

Results

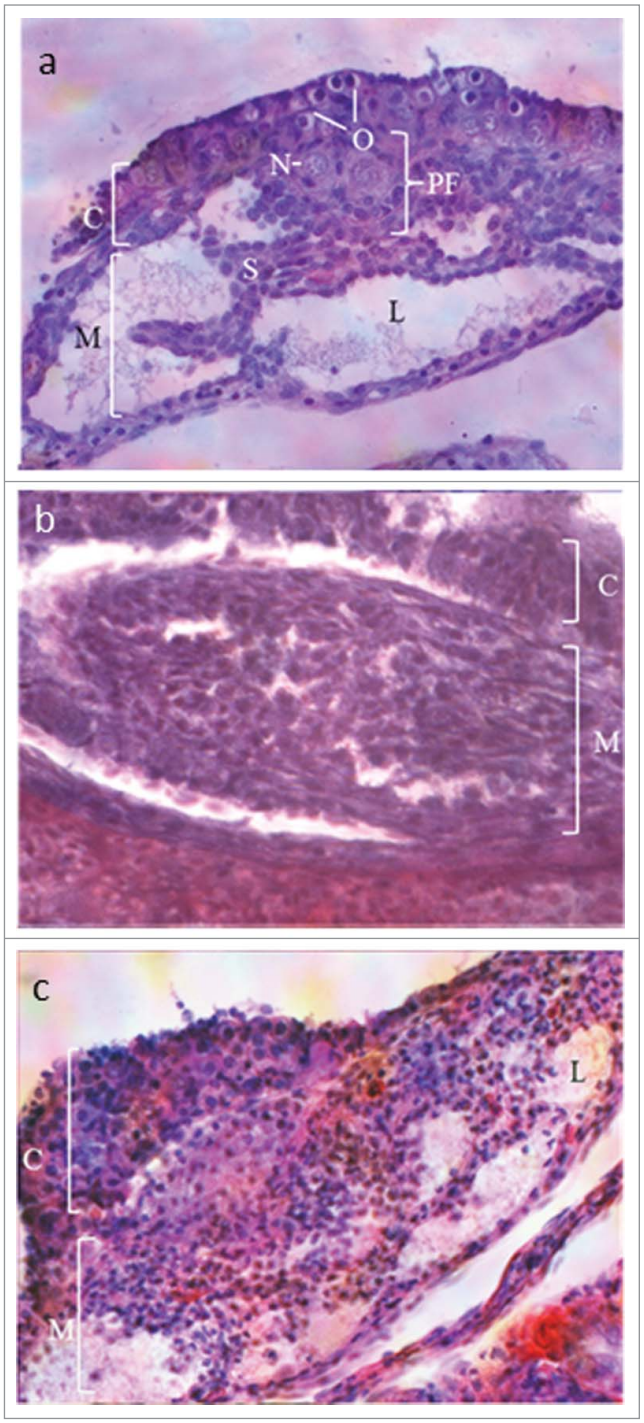
Maternal and neonatal biometrics

There was no significant difference in maternal SVL among all 4 groups at dosing ($F_{3, 76} = 0.22$; $p = 0.8789$; $n = 80$). There was no significant effect of treatment on parturition date among treatment, control injection and control groups ($F_{3, 58} = 0.44$; $p = 0.7254$; $n = 59$). Similarly, there was no significant effect of treatment on the weight of neonates ($F_{3,130} = 2.18$; $p = 0.0938$; $n = 190$), SVL ($F_{3, 161} = 1.78$; $p = 0.1525$; $n = 232$), the weight of neonatal fat bodies ($F_{3, 150} = 1.31$; $p = 0.2743$; $n = 220$), or sprint speed ($F_{3, 107} = 0.07$ $p = 0.9767$; $n = 163$) at birth among treatment, control injected and control groups.

Gonad morphology and hemipenis size

Both ATZ and DES treatment resulted in significant disruption of ovarian structure (as assessed by cumulative score) and of the micro anatomy of oocytes and follicles compared to both control treatments; examples of abnormal ovarian structures are depicted in **Figure 1**. There was a significant effect of treatment on ovarian structure, with females born to ATZ or DES exposed mothers averaging cumulative scores 4 times higher than females born to control mothers of both groups ($F_{3, 36} = 21.27$; $P < 0.0001$; $n = 86$). However, there was no significant difference between the cumulative ovarian scores of the female neonates born to ATZ or DES treated mothers ($F_{1,19} = 0.59$; $p = 0.4531$; $n = 43$), or between females born to control injection or control mothers ($F_{1,17} = 0.15$ $p = 0.7045$; $n = 42$; **Fig. 2**). We observed POFs in the ovaries of some neonatal *N. metallicus* (**Fig. 3**), however, there was no significant effect of ATZ or DES treatment on the presence of POF ($F_{3,37} = 1.08$; $p = 0.3677$; $n = 86$).

In male neonates, there was a significant effect of both ATZ and DES treatment on ST formation ($F_{3, 47} = 15.19$; $P < 0.0001$; $n = 111$) (see examples in **Fig. 4**). Males born to ATZ and DES treated mothers were 50% more likely to score one (moderately differentiated) compared to males born to control or control injection mothers, which were likely to score zero (highly differentiated) on this character. There was no significant difference in level of ST organization between males born to ATZ or DES treated mothers ($F_{1, 21} = 0.17$, $p = 0.6803$; $n = 52$), nor between control injection or control mothers ($F_{1, 26} = 0.15$; $p = 0.7039$; $n = 59$) (**Fig. 5**). There was a significant effect of treatment on the presence/absence of primordial germ cells, with males born to both ATZ and DES treated mothers having a 50% mean probability of being born with testes devoid of primordial germ cells ($F_{3,45} = 7.08$; $p = 0.0005$; $n = 108$), but there was no difference between males born to ATZ or DES exposed mothers ($F_{1,20} = 4.23$; $p = 0.0530$; $n = 50$), nor between males born to control injection or control mothers



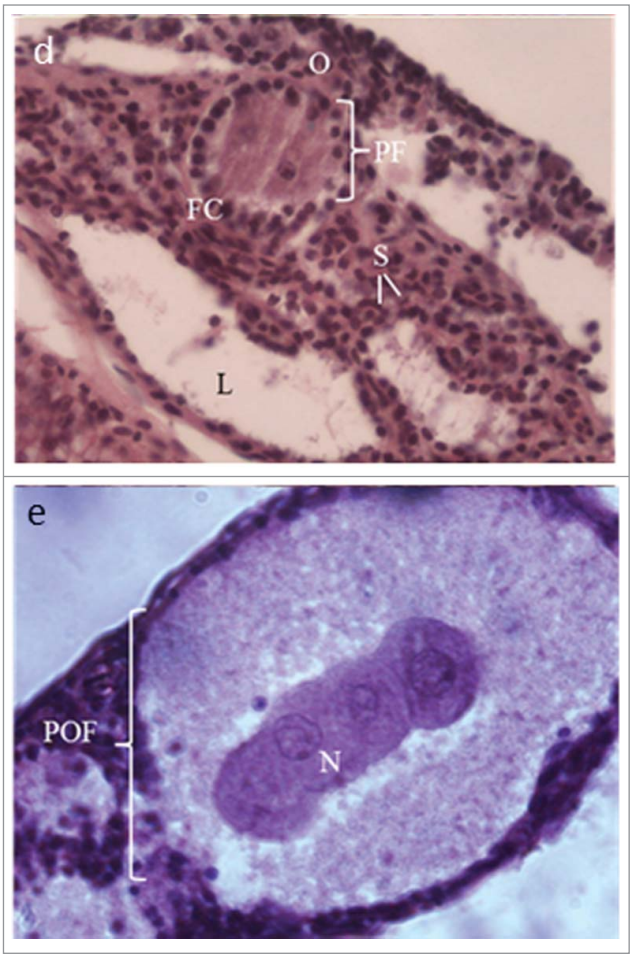


Figure 1. Ovaries from neonatal *N. metallicus* illustrating abnormal phenotypic traits which are utilised in the scoring system. (**A**) illustrates normal phenotype, (**B**), illustrates reduced lacunae and presence of medullary cords, (**C**) illustrates crenated, elongated and heavily granulated stroma; (**D**) illustrates oognia with reduced ooplasm, elongated nuclei and heavily granulated nuclei, and heavily granulated follicular cells. (**E**) illustrates a POF with 4 oocytes. C, cortex; M, medulla; O, oogonia; PO, primary oocyte; PF, primordial follicle; N, nucleus; FC, follicular cell; S, stroma; L lacunae. All ovaries are stained with haematoxylin, eosin and sectioned at 6 μm, and magnified at 400 ×.

Figure 1.

($F_{1,25} = 0.74$, $p = 0.3987$; $n = 58$) (**Fig. 6**). There was a significant effect of treatment on the presence/absence of testicular lesions ($F_{3,43} = 5.51$, $p = 0.0027$; $n = 106$), with more males born to ATZ treated mothers exhibiting testicular lesions compared with males born to DES treated mothers ($F_{1,17} = 7.38$ $p = 0.0147$; $n = 47$). Atrazine and DES treatment resulted in elongation of the nuclei in some unidentified cells of the testes ($F_{3,45} = 10.28$; $P < 0.0001$; $n = 109$), but there was no significant difference in the occurrence of such cells in males born to ATZ and DES treated mothers ($F_{1,19} = 1.66$; $p = 0.2134$; $n = 50$) or in males born to control injected and control mothers ($F_{1,26} = 0.16$; $p = 0.6914$; $n = 59$). There was no significant difference in the presence/absence of testicular lesions in males born to the control injection and control groups; $F_{1,26} = 0.11$; $p = 0.7454$; $n = 59$ (**Fig. 6**). There was no effect of either ATZ or DES treatment on the width of hemipenis size ($F_{3,125} = 1.48$; $p = 0.2220$; $n = 195$).

Discussion

We investigated the effect of atrazine (ATZ), a known endocrine disruptor, on embryonic development in the viviparous

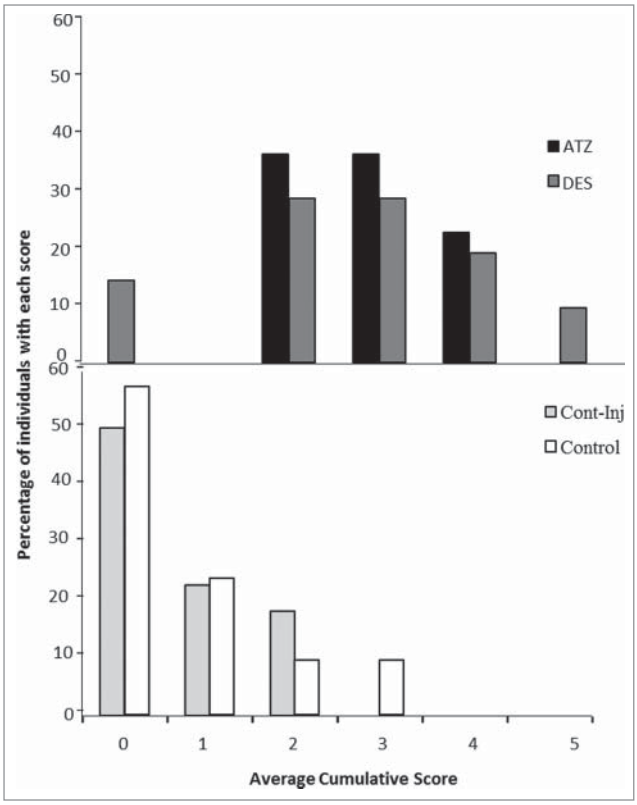


Figure 2. The accumulation of abnormal phenotypic traits relating to the cells and structure of the ovary comprising the 'cumulative score' of one randomly selected ovary of *N. metallicus* from each of 86 females. The mean cumulative score of female *N. metallicus* born to ATZ (n= 22), DES (n = 21), Control Injected (sesame oil n = 22) and Control (n= 21) treated mothers.

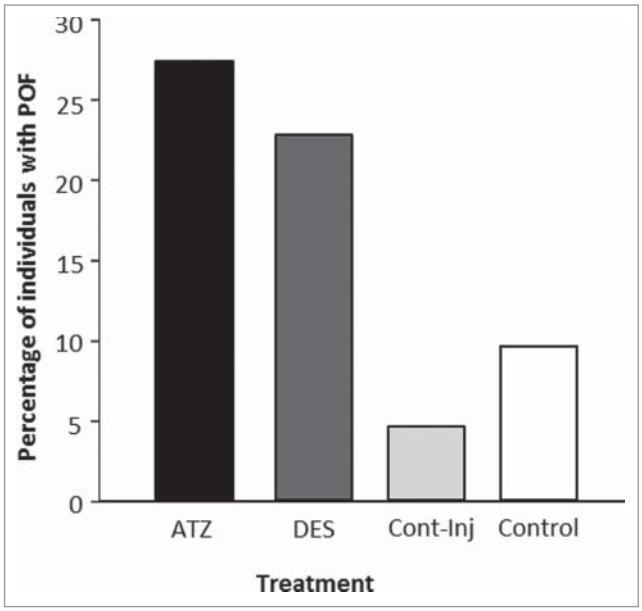
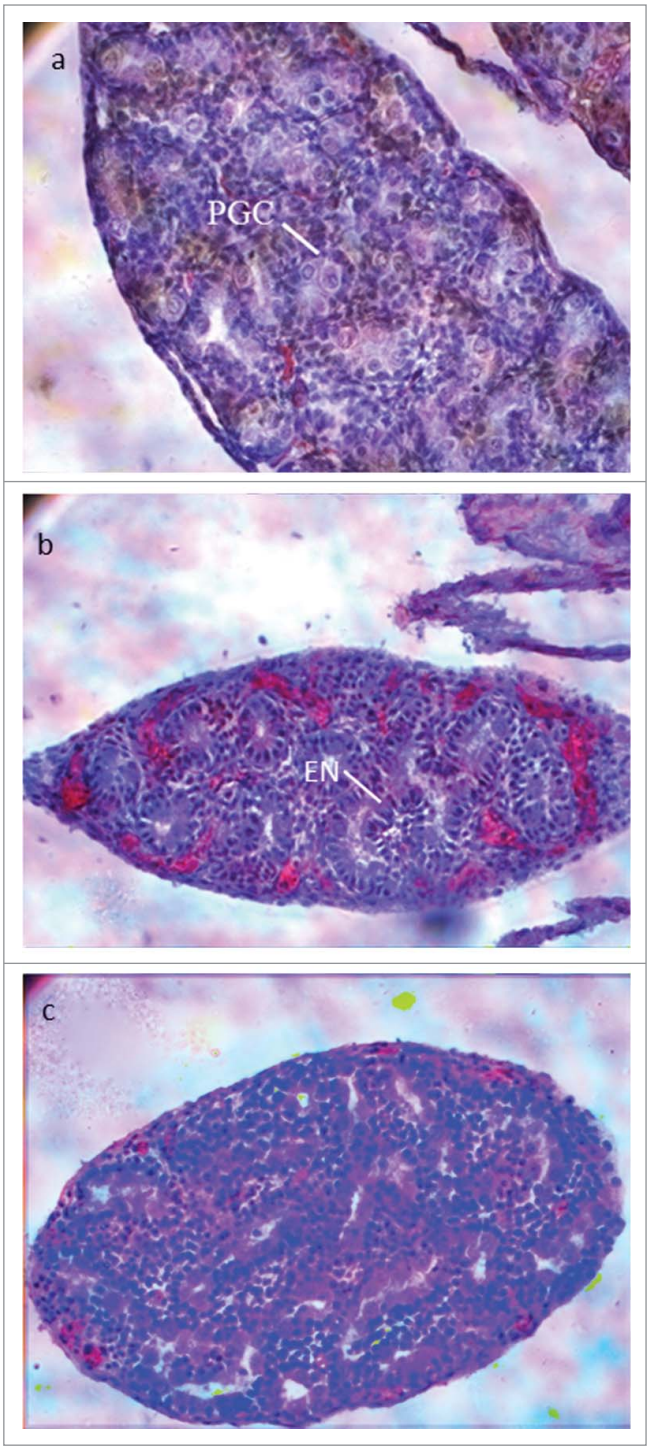


Figure 3. The occurrence of polyovular (POF) and normal follicles containing one oocyte in ovaries of neonatal *N. metallicus* born from ATZ (n = 22), DES (n = 22), Control Injected (sesame oil; n = 22) and control (n = 21) treated mothers. Neither ATZ nor DES treatment had a significant effect on POF formation.

lizard, *Niveoscincus metallicus*. Neonatal *N. metallicus* of both sexes born to mothers exposed to a single dose of ATZ at 10 ppb during sexual differentiation exhibited gonadal abnormalities. Female neonatal *N. metallicus* born to ATZ exposed mothers had a higher incidence of a range of developmental abnormalities of the ovary compared to females born to control mothers. Exposure to ATZ *in utero* increased the occurrence of polyovular follicles (POFs), atretic follicles and cells with aberrant nuclei. Furthermore, in contrast to females born to control mothers, females born to ATZ exposed mothers exhibited ovaries without a distinct medulla and cortex and lacunae. Ovaries of females born to ATZ treated mothers also retained medullary cords, while medullary cords in the ovaries of females born to control injection and control mothers had regressed. We also assessed the effects of ATZ on other key factors which contribute to fitness and offspring survival in lizards: birthdate,[54,55] locomotor performance,[44,45] size of offspring[56] and offspring body condition at birth.[48,49] There was no effect of developmental exposure to ATZ on these additional predictors of fitness. The significant effects that we observed relate to reproductive fitness, specifically gonadal development, and impacts were evident in both female and male gonads.

Studies on the effects of ATZ on ovarian development in other taxa are limited in number, focus on oviparous taxa, and have yielded contradictory results. For example, Crain et al.[57] reported no effect of *in ovo* exposure to ATZ on the developing ovary of *Alligator mississippiensis*, but *in ovo* exposure of female *Gallus gallus* resulted in persistence of the right gonad which would normally regress.[58] Exposure of embryonic *Caiman latirostris* to ATZ *in ovo* altered ovarian follicular dynamics such that follicles from exposed female neonates were at advanced stages compared with controls.[59] In contrast to the observations of Stoker et al.[59] many of the abnormalities that we observed *in N. metallicus* suggest a delay in ovarian development rather than an advance in maturation of follicular cells. For example, indistinct medulla and cortex, presence of medullary cords and absence of lacunae are all features observed during early ovarian differentiation of other reptilian species,[52,60,61] including the congeneric species *N. ocellatus*.[52,60,61] Retarded ovarian development has also been reported in the fish *Coregonus lavaretus* exposed to estradiol during gonad development[62] and in Wistar rats exposed to ethynylestradiol *in utero*.[63] Similarly, exposure to estrogenic EDCs prevents follicle nest breakdown, thus delaying follicular maturation, and results in the formation of POF in laboratory rodents.[64,65] Our observation of POF in the ovaries of *N. metallicus* thus also supports the hypothesis that the effects we observed reflect delayed ovarian development in ATZ exposed embryos.

Our observations of the effects of *in utero* exposure to ATZ on testis development in *N. metallicus* are in direct agreement with several other studies in male vertebrates. Testes with poorly defined ST, devoid of germ cells, containing testicular lesions have been observed in male *Xenopus laevis*[66] and *Caiman*



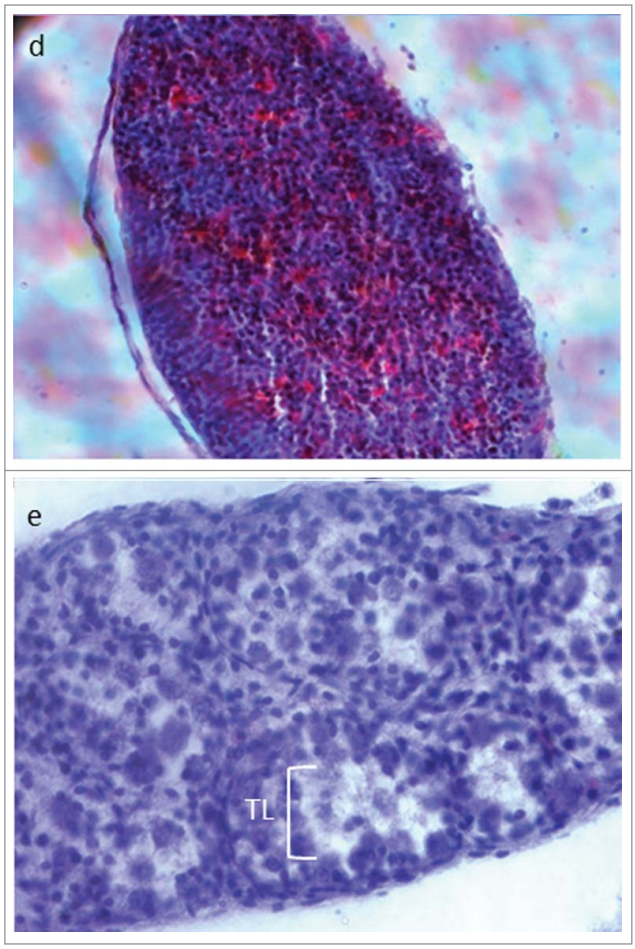


Figure 4. Testes from neonatal *N. metallicus* illustrating (**A**) normal testis with well defined seminiferous tubules (ST score = 0), (**B**) testis with moderately defined seminiferous tubules (ST score = 1), (**C**) testis with poorly defined seminiferous tubules (ST score 2), (**D**) testis with no semi niferous tubules (ST score = 3), (**E**) testis demonstrating testicular lesions. PGC, primordial germ cell; EN, elongated nucleus; TL, testicular lesion. All testes are stained with haematoxylin, eosin and sectioned at 6 μm, and magnified at 400 ×.

Figure 4.

latirostris[67] following developmental exposure to ATZ. Similarly, exposure to ATZ resulted in germ cell loss in reproductively mature rodents exposed during adulthood.[68,69] Furthermore, tes ticular lesions following ATZ exposure have been observed in reproductively mature *Carassius auratus*[70] and laboratory rodents.[68,69] Despite differences in life stages and class of the experimental subjects, ATZ appears to have consistent effects on the testes of vertebrates;[22] our results extend these findings to viviparous reptiles.

The mechanisms by which ATZ disrupts the vertebrate endo crine system have been extensively examined. Increased aroma tase expression in tissues which utilize the ArPII promotor and SF 1 is one major pathway of ATZ effects.[14-17,71] Previously, we have documented the effects of DES, a known estrogen mimic, on gonadal development in *N. metallicus*;[43] DES was therefore used as the positive control agent in the present study. We found no significant difference in any aspect of ovarian development between neonates exposed to DES or ATZ *in utero*. Furthermore, we found no significant difference between males born to DES or ATZ mothers in the organization of ST, the presence of germ cells or the presence of cells with elongated nuclei. Our results therefore support the hypothesis that the majority of the effects

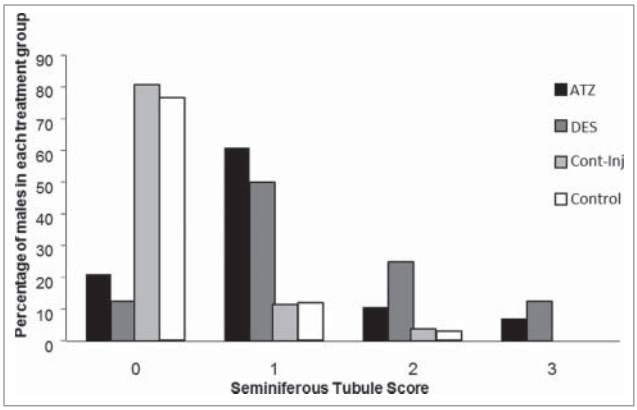


Figure 5. The percentage of male neonatal *N. metallicus* born to ATZ (n = 28), DES (n = 24) Control injected (n = 26) and control (n = 33) treated mothers exhibiting seminiferous tubule scores of 0 (normal) 1, (moderately differentiated) 2, (poorly differentiated) and 3 (undifferenti ated). Males born to ATZ and DES treated mothers were statistically more likely to score 1 (moderately differentiated), compared with males born to control injected or control treated mothers who were more likely to score 0, (well differentiated) on this character.

of ATZ on gonadal development in *N. metallicus* are mediated via increased estrogen signaling.

In contrast, we observed significantly greater incidence of tes ticular lesions in males born to ATZ exposed mothers compared with males born to DES mothers. We therefore suggest that the formation of testicular lesions is not a result of increased aroma tase activity with subsequent increase in estrogen signaling. Rather, we suggest that testicular lesions may reflect decreased availability of dihydrotestosterone (DHT). Atrazine decreases

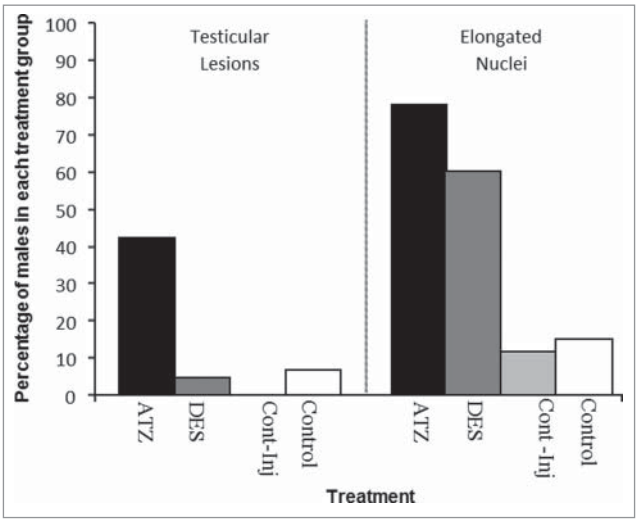


Figure 6. The percentage of male neonatal *N. metallicus* born to ATZ (n = 28), DES (n = 24) Control Injected (sesame oil, n = 26) and control (n = 3) treated mothers with testes exhibiting testicular lesions (left); or testicular cells with elongated nuclei (right). *In utero* ATZ exposure resulted in more males exhibiting testicular lesions compared with males born to DES treated mothers, while exposure to ATZ and DES resulted in more males exhibiting cells with elongated nuclei.

androgen synthesis via multiple pathways (reviewed in Hayes et al.[22]). Atrazine inhibits 5α reductase activity,[72] which reduces dihydrotestosterone (DHT) synthesis,[22] and inhibits DHT bind ing to binding proteins and receptors *in vitro,*[23] thus reducing DHT synthesis and potency.[22] Sex differences in the action and effects of ATZ are therefore likely because testis formation is largely dependent on DHT[73-76] but ovarian differentiation is not,[78,79] so reduced DHT synthesis and potency are therefore unlikely to affect gonadal development in females. Sex differences in the effects of EDCs have been reported in other taxa,[77] pre sumably reflecting the different hormonal milieu in which males and females develop. Future studies are required to determine the molecular mechanisms which underpin ATZ effects in *N. metallicus*.

In adult males, reproductive success can be impaired by reduced phallus size. Hemipenis regression or proliferation is mediated via sex specific production of gonadal steroids in other reptilian species,[50,51] but we found no significant effect of ATZ exposure on the size of hemipenes in neonates of either sex. Simi larly, there was no effect on hemipenis size of the positive control agent DES in this or in our previous study of DES.[55] These results may reflect the timing of the experimental exposure to the endocrine disruptor. We exposed gestating females to a single dose of ATZ or DES during the early phases of sexual differentia tion. However, hemipenes do not develop until a much later stage and begin to be sexually dimorphic in the final stages of development prior to birth in both *N. metallicus* (Parsley personal observations) and the congeneric *N. ocellatus.*[50] Thus, despite the overt effects of ATZ and DES on gonadal development in both sexes, the development of hemipenes was not impaired under the dose regime employed in this initial study.

In conclusion, we have found that ATZ is a potent EDC in the lizard *N. metallicus*. A single dose of ATZ at 10 ppb adminis tered to the mother during gestation demasculinises the gonads of male embryos, probably via 2 main mechanisms, and delays the development of female gonads, most likely via increased aro matase activity. These results demonstrate that in this viviparous species the endocrine disrupting effects of ATZ are transmitted from mother to embryo. *N. metallicus* exposed as embryos to the low level dose of ATZ utilized here exhibited subtle phenotypic changes in gonad histology but no changes were observed in the other phenotypic measures taken. Assessments of the impact of herbicides such as ATZ should therefore examine gonad histol ogy as the most relevant endpoint rather than other phenotypic parameters relating to reproductive fitness. Our results suggest that ATZ should be used with caution, as prolonged exposure of wildlife to this EDC is likely to have devastating effects on reproductive health.

Methods

Animal collection and husbandry

Eighty female *N. metallicus* were collected in late October 2010 from Old Farm Rd (42°53′38.33”S, 147°19′21.29”E) in greater Hobart, Tasmania. In *Niveoscincus* species, sex

determination and subsequent differentiation in embryos occurs at stages 30 32 which is during the middle third of develop ment,[53] and typically occurs in late October. After ovulation, embryos of female lizards in the same population and microhabi tat vary only by 2 3 embryonic stages.[34,36] To check the stage which the embryos had reached, we dissected 10 female lizards from the site of capture: those animals were used in another study.[32] After collection, lizards were transported to the Univer sity of Tasmania Herpetology facilities. The lizards were housed as detailed in Parsley et al.[43]

Atrazine exposure

Lizards were allowed to acclimatise to captivity for 7 d. After acclimatisation the lizards were weighed (± 1 mg) and their snout to vent length (SVL) was measured (± 1mm). They were then randomly allocated into treatment groups of 20. The ATZ group were administered a single dose of ATZ (Sigma Aldrich, Australia) dissolved in sesame oil at a concentration of 0.0016 ppm: an intraperitoneal injection at 6.25 μl/g delivered a 10 ppb dose. Such levels of ATZ have been reported in surface waters and drinking water systems in the Midwestern United States.[78] The positive control DES (Sigma Aldrich, Australia) group also received a single dose of DES at a concentration of 0.0016 ppm in sesame oil, which was administered intraperito neally at 6.25 μl/g to yield a10 ppb dose. A dose of DES at 10 ppb has been linked with phenotypic abnormalities in gonads of both male and female embryos of *N. metallicus.*[43] The Control Injection group received a single dose of sesame oil alone at 6.25 μl/g, while the Control group received no treatment. All injections were performed with a Hamilton gas tight micro syringe. The lizards were then maintained as described above until parturition.

Locomotor performance testing, dissection, hemipenis measurement and gonad preparation

The locomotor performance of the neonates was assessed by sprint testing[45] on the day following birth as described in Parsley et al.[43] After performance testing, the neonates were humanely killed with an intraperitoneal injection of sodium pentobarbital at 500 ng/g diluted 1:100 in saline solution. After death, the weight (± 10 μg), total length (TL), and SVL were measured (0.01 mm). The hemipenes were exposed by removal of the sur rounding skin and muscle tissue and photographed on a Zeiss Stemi SV11 microscope with a Leica DFC 425 camera attach ment at 32 × magnification. Photos were captured with Leica Application Suite V3 7.0. The width of hemipenes was measured thrice with ImageJ software, and the mean measurements for both hemipenes were used in the data analysis. The abdominal fat bodies adjacent to the adrenal kidney gonad complex (AKG) were removed and their wet weight obtained (± 10 μg).[79] The AKG was removed and processed for histology as in Parsley et al.[43] The serially sectioned (at 6 μm) gonads were recoded to prevent biasing data collection. Neonates were identified as female by the presence of ovaries, Müllerian and Wolffian ducts, which have not regressed at birth in the female.[52,61] Male neo nates were identified by the absence of Müllerian ducts and the presence of Wolffian ducts and testes.

Examination of ovarian histology

Estrogenic EDCs can cause polyovular (POF) follicles in developing ovaries, and POF is a marker of endocrine disruption in females.[1,80] We have previously described multiple phenotypic changes, including the presence of polyovular follicles (POFs) in the developing ovary as markers of endocrine disruption in *N. metallicus* and have developed a scoring system for the effects of exposure to 'estrogenic' EDCs *in utero.*[43] The criteria for scoring ovarian phenotype are presented in **Table 1**. All sections of the ovaries were examined 3 times to ensure accurate scoring; the total cumulative scores for one randomly selected ovary of each female neonate were utilized in the statistical analysis.

Examination of testis histology

The effects of ATZ on testis development include disruption to the organization of ST and the presence of testicular lesions.[22] We have identified the demasculinising effects of an estrogenic EDC (DES) on testis development in *N. metallicus* as the absence

Table 1. The 5 criteria used to determine the cumulative score of ovarian abnormalities in neonatal *Niveoscincus metallicus* Parsley et al.[43]

Criterion	The aspect of the ovary	Phenotype 1: normal, score = 0	Phenotype 2: abnormal score = 1
1	Structure	Distinct medulla cortex, lacunae present, no medullary cords	Indistinct medulla cortex, or medullary cords present, or absent lacunae
2	Oogonia	Spherical or ovoid, prominent ooplasm, spherical nucleus	Ooplasm absent, or crenate, or elongated nuclei
3	Primary oocytes	Spherical or ovoid, lightly stained ooplasm, spherical nucleus	Heavily stained ooplasm, or granulated and elongated nuclei
4	Primordial Follicle	Cuboidal follicular cells with visible organelles, one oocyte, oocyte contacting follicular cells, not atretic	Crenate elongated follicular cells, or poly ovular, or atretic receding oocyte
5	Stoma	Ovoid with visible organelles	Heavily granulated (organelles no longer visible), crenate or elongated cells

Each criterion relates to one aspect of the ovary and any of the listed abnormal phenotypes for each criterion results in a score of one for that criterion. Female *Niveoscincus metallicus* with ovaries with the normal phenotype for each criterion scored a zero, while neonates with one or several of the abnormal phenotypic traits for each aspect of the ovary scored one for that criterion. For each female, a cumulative score of all of the criteria was obtained. An ovary with a completely normal phenotype would have a total score of zero, while an ovary with a completely abnormal phenotype would score 5.

of primordial germ cells, and disruption to the organization of ST, and have defined 4 levels of disruption to ST organization: 0 = highly differentiated (normal), 1= moderately differentiated, 2 = poorly differentiated, and 3 = undifferentiated.[43] We there fore scored the organization of ST using this scale, and noted the presence or absence of primordial germ cells and testicular lesions. We randomly selected data from one testis to include in the statistical analysis.

Statistical analyses

Data analysis was performed using SAS 9.2 for Windows. Dif ferences between groups in adult female SVL at dosing and in parturition date were examined individually using general linear models (GLM). Data from all neonates were included in the analysis; maternal identity was therefore included as a random factor and thus data from neonates were analyzed with a general linear mixed model. Neonatal weight and SVL were analyzed separately on untransformed data. Sprint speed, the weight of neonatal fat bodies, and the mean width of hemipenes were ana lyzed using log transformed data. Neonate sex was included as a fixed factor in the analysis of hemipenis width. Normality of dis tribution for all parametric models was identified by examining plots of standardized residuals against predicted values and nor mal probability of the residuals. Significant differences were iden tified using Tukey's Honest Significant Difference.

The cumulative score of ovarian abnormalities was analyzed with a generalized linear mixed model with a Gaussian distribu tion. We included the occurrence of POF as a criterion in the average cumulative score, but as POF are a known marker of disruption by estrogenic EDCs,[1,34] we also performed a separate analysis on the occurrence of POF. The presence or absence of POF, the organization of ST, the presence or absence of primor dial germ cells, the presence or absence of lesions and the occur rence of cells with elongated nuclei in neonatal testes were analyzed separately with maternal identity as a random factor by generalized linear mixed model with a binomial distribution and a logit link function. Where analyses yielded significant results, appropriate preplanned post hoc comparisons were made and α was adjusted accordingly.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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