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An endocrine disrupter increases growth and risky behavior in threespined stickleback (*Gasterosteus aculeatus*)

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Abstract

There is considerable concern that endocrine disrupting chemicals (EDCs) can affect wildlife and humans. While several studies have reported that acute exposure to EDCs can cause changes in reproductive traits, we are in the early stages of discerning whether such changes have significant deleterious fitness consequences. In this study, chronic exposure of threespined stickleback (*Gasterosteus aculeatus*) to an environmentally relevant level of an EDC used in the birth control pill and post-menopausal hormone replacement therapy produced changes in growth and behavior that were related to fitness. Exposure to 100 ng/l ethinyl estradiol accelerated growth rate and increased levels of behavior that makes individuals more susceptible to predation (activity and foraging under predation risk). Moreover, the costs of exposure to ethinyl estradiol took their ultimate toll via mortality later in life, and were particularly high for females and for one population. The ecological approach taken in this work revealed heretofore unexamined effects of EDCs and has direct implications for the way we evaluate the impact of EDCs in the environment.
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Introduction

Alarming effects of estrogenic endocrine disrupting chemicals (EDCs) on reproductive traits have been reported, such as altered gonadal morphology (Guillette et al., 1995; Hayes et al., 2002) and reduced sperm production (Bayley et al., 2002). However, estrogens also mediate other important physiological processes throughout development including the stress response (Pottinger et al., 1996), growth (Nelson, 2000), and immunity (Grossman, 1985). As a result, chronic exposure to low levels of estrogenic EDCs has the potential to influence a wide variety of traits with effects on fitness. Here, I report that chronic exposure to a very low level of an EDC (ethinyl estradiol) increases growth, risky behavior, and mortality in a model freshwater fish, the threespined stickleback (*Gasterosteus aculeatus*).

Ethinyl estradiol is present at low concentrations (5–200 ng/l) in sewage effluent and waterways in the United States and Europe (Aherne and Briggs, 1989; Kolpin et al., 2002; Stumpf et al., 1996; Tabak et al., 1981). At very low levels, this potent chemical can affect animals: exposure to environmentally realistic levels of this EDC induced physiological effects on male trout (Hansen et al., 1998) and reduced aggressive behavior of male threespined stickleback (Bell, 2001). Ethinyl estradiol is a powerful EDC because it is a very stable, lipophilic compound that is not easily degraded. As the active ingredient in the birth control pill and post-menopausal hormone replacement therapy, it is designed to mimic naturally occurring 17ß-estradiol. Unlike other EDCs which produce estrogenic effects as a byproduct of other functions, ethinyl estradiol directly binds to the estrogen receptor with the same affinity as naturally occurring estrogen, 17ß-estradiol (Kaspar and Wintzel, 1985). As a result, the effects and mechanism of action of ethinyl estradiol are directly comparable to 17ß-estradiol. In this experiment, fish were exposed to 100 ng/l ethinyl estradiol throughout their entire lives.

Threespined stickleback are quickly becoming a model system in toxicology (Bell, 2001; Katsiadaki et al., 2002) because they are widely distributed, relatively easy to rear and study in the lab, and because much is known about their biology. In particular, stickleback are renowned for their geographic variation in behavior and morphology (Bell and

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Foster, 1994). Studying this geographic variation across different populations of stickleback provides a unique opportunity to ask whether closely related, but phenotypically different, groups are similarly affected by the same EDC. In this experiment, the offspring of stickleback from two Northern California freshwater environments (Putah Creek and the Navarro River) were studied. Wild-caught fish from Putah Creek are larger and have historically experienced lower predation by fish and birds than fish from the Navarro River (Bell, unpublished data). Because fish from these two populations are phenotypically distinguishable and inhabit different selective environments, stickleback from these two populations might not respond to the same EDC in the same way. Currently, ethinyl estradiol is undetectable in both waterways (detection limit 80 ng/l).

Exposure to ethinyl estradiol might increase growth and levels of behavior that increase susceptibility to predation for the following reasons. First, gonadal steroids can have positive effects on growth in fish (Donaldson and Hunter, 1981). Second, gonadal steroids, particularly estrogens, have been shown to increase activity levels in vertebrates (Palanza et al., 1999). Activity level is a simple but critically important behavior that can influence growth and survivorship (Werner and Anholt, 1993). For instance, increased levels of activity can increase encounter rates with food, resulting in increased growth. However, increased activity level can come at a cost: while elevated movement increases a fish's encounter rate with food, it also increases a fish's encounter rate with predators. Therefore, high activity levels in the presence of predators can produce negative effects on fitness because more active individuals may be more likely to be consumed by predators (Werner and Anholt, 1993). In addition, previous studies on stickleback and other fish have shown that an individual's energetic state influences its decisions regarding feeding versus predation risk (Aeschlimann et al., 2000; Jonsson et al., 1996). In particular, faster-growing animals, or animals with greater energy requirements, may be more willing to risk exposure to predators to gain food.

The goal of this laboratory study was to infer whether fish in the natural environment may suffer from heretofore unexamined deleterious changes in behavior as a result of exposure to EDCs. Therefore, this experiment was designed to simulate the conditions experienced by fish chronically exposed to low levels of an EDC in the field. Animals were exposed to ethinyl estradiol throughout their lifetime and raised in a semi-natural environment that mimicked natural conditions as closely as possible. By studying fish in an environmentally realistic but controlled laboratory setting, individual growth, behavior, and life history could be followed without sacrificing ecological relevance.

Materials and methods

Adult threespined stickleback were collected from Putah Creek and the Navarro River between May and June 2000 and brought to the Institute of Ecology on the University of California, Davis campus. The fish were fed frozen brine shrimp, live tubifex worms and trout chow (Silver Cup, Nelson and Sons, Murray, UT) ad libitum. Each male was mated to a different female from the same population to produce twenty-two full sib families (11 families per population). Males were housed in 37.9-l (26 × 29 × 51 cm) aquaria, each of which contained a gravel substrate and string algae for nesting. After a male completed his nest, a single gravid female from the same population was placed in the aquarium with the male. If the pair spawned by the following morning, the eggs were removed from the male's nest, and half the clutch was assigned to the experimental group and half to the control group. To eliminate environmental effects that might arise from differences in paternal care (Tulley and Huntingford, 1987), the eggs were artificially incubated in 1-l incubators fitted with an airstone for aeration. Water in the experimental groups' incubators contained 100 ng/l ethinyl estradiol (see below), while water in the control groups' incubators contained 0 ng/l ethinyl estradiol. After assignment to either the control or the experimental group, each fish was raised in the appropriate treatment until the end of the experiment. After hatching, each half-clutch of fry was placed in separate aquaria until the fry were large enough to be permanently marked by spine clipping (20 mm, 2–3 months of age). The temperature of the breeding tanks and incubators was held constant at room temperature (20°C) and were exposed to a 'summer' (16L:8D) photoperiod. All of the water used in this experiment came from a well on the UCDavis campus and was passed through a packed column aerator to remove excess nitrogen and add oxygen. The pH of the well water was 7.8.

After marking, the fry were transferred to the Putah Creek Aquaculture Facility, UC Davis, where they were raised in one of twelve 106-l tanks (43 × 43 × 49 cm) ('home tank') for the duration of the experiment. There were three replicate home tanks of each population *x* treatment combination (three experimental, three control home tanks per population). Experimental tanks contained ethinyl estradiol at a concentration of 100 ng/l while control tanks did not contain any ethinyl estradiol. Each home tank contained a sponge filter, a gravel and sand substrate, artificial plants, a cinderblock, and a terra cotta pot. There was a representative from each full sib family within a particular population within each home tank. By rearing the fish in mixed family groups, different social conditions that might arise because of behavioral differences among families were controlled for. The home tanks were kept in a water bath and maintained at 16 ± 2°C and exposed to an ambient (Davis, CA) photoperiod throughout the experiment.

The fish were fed a limited ration, which included a variety of food types. As fry, they were fed brine shrimp nauplii and Golden Pearls 'Juvenile Diet' (Brine Shrimp Direct, Ogden UT) twice a day. At approximately 2 months of age, the fish were gradually introduced to a mixed diet, which included trout chow, live and frozen brine shrimp, and live and frozen

tubifex worms. The amount of food provided was adjusted so that the fish received approximately 10%, 5%, 3%, and 1% of their body mass per day as fry, juveniles, subadults, and adults, respectively. Each tank received the same amount of food.

Each fish's standard length was measured on four occasions. Because the fish spawned on different days, the fish differed in age on any given measurement day. To control for this, 'spawn date' was used as a covariate as necessary in analyses below. The measurements were conducted during periods when the fish could be grouped into broad age classes as fry, juveniles, subadults, and adults. The standard length measurements were natural log-transformed to homogenize variances.

The tanks were inspected every day and any dead fish were removed. The tanks were also routinely monitored for sexually mature fish. Mature male fish were detected by checking for territorial behavior and characteristic nuptial coloration, which includes a red chin and blue eye. Mature female fish were detected by looking for signs of gravidity. Sex identification was subsequently confirmed by dissection. There was no apparent effect of treatment on sex determination (Bell unpublished data).

Ethinyl estradiol solution

The stock solution of ethinyl estradiol was made in 100% ethanol. Two milligrams ethinyl estradiol (Sigma) were dissolved in 200 ml ethanol by stirring the solution for 3 days with a magnetic stirrer, following the methods in Bell (2001). A competitive enzyme immunoassay (RIDA-SCREEN® ethinylostradiol, R-Biopharm AG, Darmstadt, Germany) was used to confirm the concentration of ethinyl estradiol in the stock solution. The analysis was carried out by Trilogy Analytical Laboratory, Inc. (Washington, MO) and found that the concentration of the stock solution was 0.0077 g/l, or 77% of the nominal concentration (0.01 g/l). The appropriate volume of this stock solution was added to the incubators or tanks to achieve the nominal concentration of 100 ng/l. The same amount of untreated ethanol was added to the control tanks. Half of the water in the home tanks was changed every month, adding the appropriate volume of the ethinyl estradiol stock solution to maintain the concentration in the experimental tanks. In a separate experiment, it was confirmed that sponge filters do not remove the chemical from water and that the concentration changes minimally through time.

Behavioral observations

In February 2001, the behavior of individual subadults was observed in the absence and presence of predation risk. All of the fish in a given home tank were observed on a single day. The mean age at observation was 211 days (range: 175–245 days of age). None of the fish had yet become sexually mature.

The fish in a home tank were deprived of food 1 day before an observation day. The following day, all of the fish from that home tank were placed singly in 1 of 12 observation tanks ($26 \times 29 \times 51$ cm, 37.9 l) by an assistant. There was no ethinyl estradiol present in the observation tanks. Each observation tank contained two plastic plants, a terra cotta pot, a food cup fastened to the side of the aquarium, an airstone, and a heater that maintained the water temperature at $16 \pm 1°C$. The fish were allowed to adjust to the observation tanks overnight (approximately 12 h). The observations were conducted 'blind' so that the observer did not know the identity, treatment, or population of the subjects.

The following morning, each fish was observed in the absence of predation risk. Preliminary studies found that when individuals were placed singly in an aquarium, they tend to spend most of the time motionless or freezing. When the fish did move, they tended to quickly dart to another area of the aquarium, where they again remained motionless until moving again. Therefore, to measure activity in the absence of predation risk, the number of times the fish moved (swam to a different position) was continuously recorded for 3 min. The number of times the animal moved was divided by three to indicate the activity rate (the number of movements per minute). Below, this variable is termed 'safe activity' because the measurements were made in the absence of predation risk. The proportion of time spent with spines raised was also recorded during this observation. Spine raising in an effective antipredator defense used by stickleback (Bell and Foster, 1994) because erect spines can prevent a predator from piercing the body following capture.

Approximately 3 h following the observation of safe activity, each fish was observed after a simulated egret attack. This method has been widely used by fish biologists and reliably elicits antipredator behavior (e.g., (Godin and Crossman, 1994; Johnsson and Bjornsson, 1994)). A great egret skull (Casmerodius albus) was fastened over the tank and, after 10 min, 10 tubifex worms were added to the food cup. When the fish approached within one body length of the food cup, the skull was released twice in quick succession via a string attached to the skull, simulating a strike within 3 cm of the food cup. At that time, the behavior of the stickleback was recorded for 5 min. Specifically, the number of times the animal moved as well as the number of times the fish bit at the food were recorded. In subsequent analyses, the number of times the fish moved divided by five indicates the activity rate (the number of movements per minute) in the presence of predation risk ('risky activity') and the number of bites at the food indicates willingness to forage under predation risk ('risky foraging'). The proportion of time spent with spines raised was also recorded during this observation.

At the end of an observation day, the fish were removed from the observation tanks and returned to their home tank. The water was changed in the observation tanks and fish from another home tank were transferred singly to the observation tanks as before.
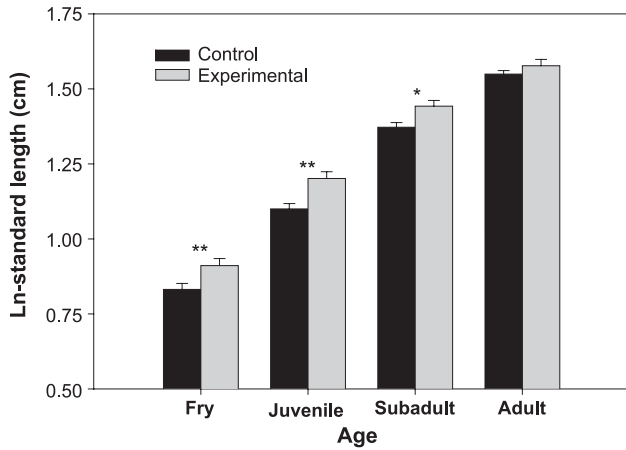
Fig. 1. Mean ($\pm$ 1 SE) natural log-transformed standard length of fry, juveniles, subadults and adults by treatment (control $n = 43$; experimental $n = 20$). **$P < 0.0125$ (the Bonferroni-adjusted significance level), *$P < 0.05$.

Statistical analysis

Replicate home tanks did not significantly differ in behavior, body size, survivorship, or maturity. Therefore, the data from the different replicates were pooled in subsequent analyses.

Standard length at each age was examined with univariate ANOVAs with population, treatment, sex, and all the interactions as fixed factors and with birthdate as a covariate. Separate ANOVAs were employed to test for the effects of the factors on behavior. It is worth noting that the significance level of these ANOVAs may need to be altered to account for the fact that an individual's body size at different ages and behaviors are not necessarily independent. The most conservative correction (Bonferroni) produces a critical significance level of $P = 0.0125$ (0.05/4) for the body size variables, and $P = 0.0167$ (0.05/3) for the behavioral variables. Differences between groups that are significant at both the $P = 0.05$ and corrected significance level are noted in
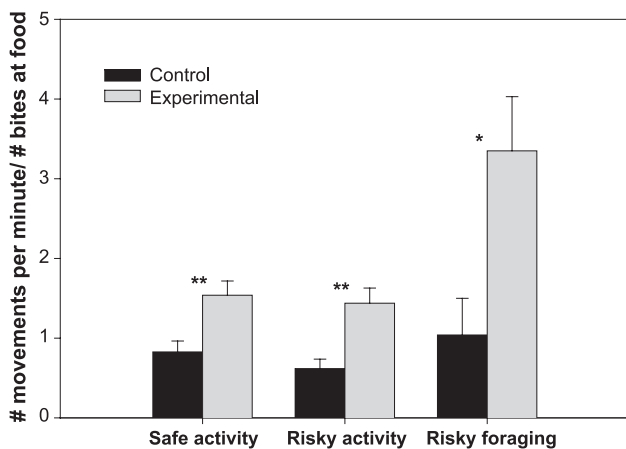
Figs. 1 and 2. All statistical analyses were carried out using SPSS Version 10 (SPSS, Inc. 1989–1999, Chicago, IL).

Results

Exposure to an environmentally realistic concentration of ethinyl estradiol accelerated growth early in life (Fig. 1). The size difference between treatments was most pronounced when the fish were fry ($F_{1,54} = 9.10$, $P = 0.004$) and juveniles ($F_{1,54} = 12.51$, $P = 0.001$), but the effect of treatment on growth attenuated by subadulthood ($F_{1,54} = 3.87$, $P = 0.05$). As a result, control and experimental animals ended up at the same size by the end of the experiment ($F_{1,54} = 1.88$, $P = 0.176$).

Exposure to ethinyl estradiol increased activity levels both in the presence and absence of predation risk (Fig. 2). Individuals exposed to ethinyl estradiol were more active than control fish in the absence of predation risk ('safe activity') ($F_{1,41} = 6.64$, $P = 0.014$). The egret attack produced an antipredator response: fish spent a greater proportion of time with their spines raised following the egret strike than in the absence of predation risk (Paired t test $t_{44} = -2.23$, $P = 0.03$). Despite this increase in predation risk, fish exposed to ethinyl estradiol were more active under predation risk ('risky activity') than control fish ($F_{1,41} = 7.44$, $P = 0.009$). Moreover, ethinyl estradiol increased an individual's willingness to risk exposure to a predator to gain food: fish exposed to the EDC engaged in more 'risky foraging' than fish in the control group ($F_{1,41} = 4.38$, $P = 0.043$).

There was also a strong effect of ethinyl estradiol on survivorship. Overall, fish in the experimental group suffered greater mortality than fish in the control group (backwards logistic regression log-likelihood ratio = -36.331, $P = 0.03$). However, this mortality was disproportional across ages, sexes, and populations. First, individuals that were large as

Fig. 2. Mean ($\pm$ 1 SE) levels of safe activity, risky activity and risky foraging as a function of treatment (control $n = 23$; experimental $n = 26$). **$P < 0.0167$ (the Bonferroni-adjusted significance level), *$P < 0.05$.
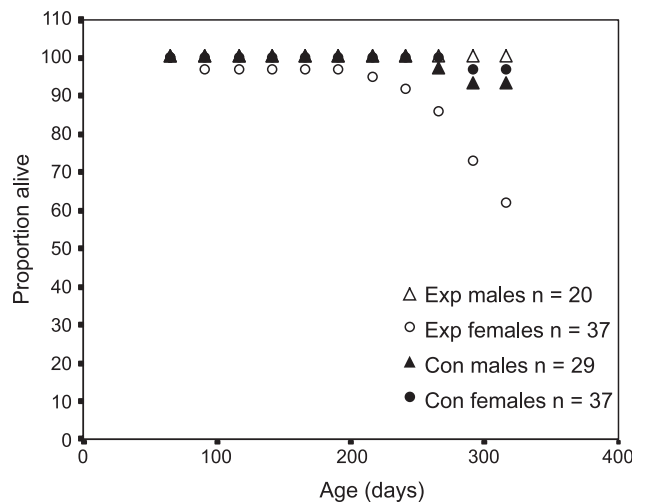

Fig. 3. The proportion of control and experimental males and females surviving over time.

subadults were more likely to die (backwards logistic regression log-likelihood ratio = −12.661, P = 0.004). Second, females were especially susceptible to the negative effects of ethinyl estradiol on survival (backwards logistic regression log-likelihood ratio=−38.921, P=0.002, Fig. 3). Third, fish from the two populations differed in their susceptibility to mortality caused by exposure to ethinyl estradiol: fish from the Navarro River were more likely to die as a result of treatment than fish from Putah Creek (backwards logistic regression log-likelihood ratio=−37.185, P=0.01).

While exposure to ethinyl estradiol had a strong effect on survivorship, it had no discernable effect on the onset of sexual maturity. Overall, 54% (n =123) of the fish became sexually mature by the end of the experiment. There was not a statistically detectable effect of treatment on whether an animal matured by the end of the experiment. Among just those animals that matured by the end of the experiment, there was no effect of treatment on age or size at maturity. However, animals that were large as subadults were more likely to become sexually mature by the end of the experiment (backwards logistic regression log-likelihood ratio = −41.865, P = 0.003).

Discussion

In this study, exposure to a very low concentration of an EDC produced strong effects on several ecologically important traits. Animals exposed to ethinyl estradiol grew faster early in life, engaged in behavior that is likely to make them more susceptible to predation and suffered greater mortality than fish in the control group. The positive effect of ethinyl estradiol on growth is consistent with aquacultural research which has shown that steroids can have anabolic effects on early growth in fish through both improved food conversion efficiency and increased food consumption (Donaldson and Hunter, 1981). Likewise, the positive effect of ethinyl estradiol on activity is consistent with studies of mice which found that exposure to estrogenic xenobiotics increased activity (Palanza et al., 1999). Because increased levels of activity can increase an individual's encounter rate with food, the positive effects of ethinyl estradiol on growth may have been related to its positive effects on activity.

The stimulatory effects of ethinyl estradiol on growth and activity came at a cost: fish exposed to ethinyl estradiol engaged in behavior that would make them more susceptible to predation. That is, experimental fish were more active and more willing to forage under predation risk. This suggests that growth enhancement by ethinyl estradiol increased the level of risk these fish were willing to incur while foraging (Aeschlimann et al., 2000; Jonsson et al., 1996). Collectively, these behavioral effects imply that fish exposed to estrogenic EDCs in the environment may suffer from increased mortality from predators, via increased levels of activity and increased willingness to forage under predation risk (Jonsson et al., 1996; Werner and Anholt,

1993), as well as predator-induced stress (Relyea and Mills, 2001).

Even without a lethal effect of predation, experimental fish and fish that were large as subadults were more likely to die. Together, these results suggest that accelerated growth early in life caused by exposure to the EDC was linked to later mortality. Other studies have also found that individuals which grow rapidly early in life can be more vulnerable to stressors and other sources of mortality later in life (Metcalfe and Monaghan, 2001). For example, early growth rate is negatively related to life span in rats (Jennings et al., 1999) and humans (Eriksson et al., 1999), and escape from predators in rainbow trout (Jonsson et al., 1996). Some of the hypothesized physiological mechanisms that underlie the tradeoff between rapid growth and survival include free radical damage that occurs during rapid growth, and a fundamental tradeoff between cell number and cell size (Mangel and Stamps, 2001; Metcalfe and Monaghan, 2001). Whatever the mechanism, these results add to increasing evidence that fast growth is not always beneficial, and can entail a variety of costs that is not evident until much later in adult life (Mangel and Stamps, 2001; Metcalfe and Monaghan, 2001).

While mortality was heaviest on experimental animals, the burden of exposure to ethinyl estradiol was especially costly for females. Rates of survivorship for experimental females plummeted after approximately 230 days of age (Fig. 3), or when they were starting to become sexually mature. The timing of this mortality may be related to the onset of sexual maturity. In other vertebrate species, the capacity and affinity of the estrogen receptor in females increase just before sexual maturation or the reproductive season (Smith and Thomas, 1991). In addition, fish from the Navarro River were particularly susceptible to the negative effects of ethinyl estradiol on survival. The particular susceptibility of Navarro fish may be related to high levels of predation pressure in that environment. For example, Navarro fish might be particularly sensitive to predator-induced stress (e.g., Relyea and Mills, 2001) because selection by predators in that environment has favored a very reactive, or timid, phenotype (Magurran, 1990).

While exposure to ethinyl estradiol had a strong effect on survivorship, it did not appear to influence the onset of sexual maturity. At first glance, this is surprising because treatment accelerated growth rate and growth rate is often related to the timing of sexual maturity (Roff, 1992) and a study on mice found that exposure to bisphenol A (an endocrine disrupter) accelerated growth and advanced puberty (Howdeshell et al., 1999). However, the effects of treatment on growth were strongest early in life, while maturity was related to body size later in life: animals that were large as subadults were more likely to become sexually mature. In fact, the growth trajectories of animals in this experiment are consistent with 'targeted growth' for size at maturity under a time constraint (Ludwig and Rowe, 1990). Stickleback from these populations are annual fish and

while treatment increased early growth, the growth of experimental animals slowed around subadulthood, perhaps because they were on a growth trajectory which would take them past the size threshold for maturity.

Although the concentration of ethinyl estradiol used in this experiment is at the high end of the range of concentrations found in the natural environment, it can be viewed as representative of the total estrogenic content of sewage discharges, which includes 17ß-estradiol, estriol, estrone, estranol, and ethinyl estradiol (Kolpin et al. 2002). It is also likely that the concentration of ethinyl estradiol in the home tanks decreased slightly between water changes due to adsorption by the materials in the home tanks and biological degradation. However, such slight fluctuations in concentration mimic natural conditions and it is worth considering that the effects were probably due to exposure to a concentration less than 100 ng/l. The precise dose–response relationship between ethinyl estradiol and behavior and growth is an obvious topic for future study.

Despite considerable public and scientific concern about the presence of EDCs in the environment, we are in the early stages of discerning how much concern is warranted. While lab studies have revealed subtle changes in sensitive endpoints such as the production of vitellogenin in male fish (Hansen et al., 1998), and reproductive abnormalities in amphibians and reptiles (Guillette et al., 1995; Hayes et al., 2002), it remains to be seen whether such changes caused by exposure to EDCs have important consequences for wildlife health and reproduction. In this experiment, chronic exposure to a very low level of an EDC produced changes in nonreproductive endpoints that are directly tied to fitness. Ethinyl estradiol promoted activity and willingness to incur risk to gain food, which leads to increased predator exposure and thus a higher risk of being preyed upon. Moreover, accelerated growth due to exposure to ethinyl estradiol was accompanied by increased mortality.

The results of this study have direct implications for the way we evaluate the impacts of EDCs on wildlife. First, there were deferred costs of exposure to a very low level of an EDC that would not be detected in standard acute toxicity tests. Therefore, to detect deferred effects later in life, we may need to expose animals to EDCs over their entire lifetime. Second, the costs of exposure were especially high for females and for one population. Such intraspecific variation urges us to pay careful attention to sex-specific effects of EDCs and to be cautious in extrapolating effects across even closely related groups of animals (Spearow et al., 1999). Finally, there were strong deleterious effects of ethinyl estradiol on nonreproductive endpoints. Exposure accelerated growth rate and caused individuals to engage in behavior that makes them more susceptible to being preyed upon, and those changes were associated with unanticipated costs in terms of survivorship before sexual maturity. As a result, some of the most alarming impacts of EDCs may be on heretofore unexamined endpoints, such as foraging and antipredator behavior, that have direct consequences of individual and population viability.

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