Feminization of male frogs in the wild

Water-borne herbicide threatens amphibian populations in parts of the United States.

Atrazine is the most commonly used herbicide in the United States and probably in the world[1]. Here we investigate the effects of exposure to water-borne atrazine contamination on wild leopard frogs (*Rana pipiens*) in different regions of the United States and find that 10–92% of males show gonadal abnormalities such as retarded development and hermaphroditism. These results are supported by laboratory observations, which together highlight concerns over the biological effects of environmental atrazine on amphibians.

We exposed *R. pipiens* larvae to different concentrations of atrazine (0, 0.1 or 25 parts per billion, p.p.b.) in the laboratory by immersion (30 larvae per treatment; $n = 3$) from just after hatching until tail resorption was complete. Only exposed males developed testicular oocytes (29% and 8%, respectively, at 0.1 and 25 p.p.b.); retarded gonadal development (gonadal dysgenesis) was evident in 36% and 12% of exposed males, respectively, and in one control animal (results not shown).

These findings are consistent with the more marked effects reported for endocrine-disrupters at lower doses (see ref. 2, for example). They also support previous indications that atrazine can cause gonadal abnormalities in males of *Xenopus laevis*[3,4] and *Acris crepitans*[5] in the laboratory. As its effects are not restricted to a single species, it is possible that this herbicide may pose a threat to amphibians in general.

We also examined leopard frogs, sampled from eight different sites in a transect running from Utah to Iowa, for abnormalities comparable to those seen under laboratory conditions. We used records of atrazine sales to identify potentially contaminated sites (Fig. 1). As control sites, we used various non-agricultural regions in Utah, Wisconsin and Nebraska that reported atrazine sales of less than 0.4 kg km^{-2}, as well as a non-agricultural area in Iowa. A golf-course pond in Cache county, Utah (the only county reporting atrazine sales of more than 0.4 kg km^{-2}), and cornfields in Nebraska and Iowa were considered to be likely sites of contamination. Water sampling revealed that only one site (Juab county, Utah) had atrazine levels below our detection limit (0.1 p.p.b.).

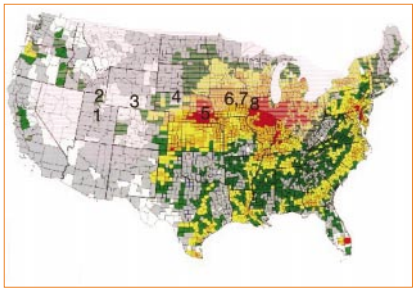

Figure 1 Use of the herbicide atrazine in the United States, on the basis of sales[11]. Pink overlay shows the natural range of leopard frogs (*Rana pipiens*)[12,13]. Regional coloration indicates atrazine usage (in kg km^{-2}): white, zero or no data; grey, <0.4; olive, 0.4–2.4; yellow, 2.5–9.2; orange, 9.3–28.7; red, >28.7. Numbers indicate the eight sites at which frogs were collected (for histological analysis of gonads) and water was sampled for atrazine analysis. We collected 100 animals at each site, selecting small individuals in order to sample newly metamorphosed animals, and killed them immediately in benzocaine; after fixation (Bouin's) for 48 h and preservation in 70% ethanol, animals were measured and their sex was determined. The histology of the gonads of 20 males and a subset of females taken from each site was analysed. Further details are available from the authors.

This site was the only locality where testicular oocytes were not observed in the local population of leopard frogs. All sites associated with atrazine sales exceeding 0.4 kg km^{-2} and with water-borne atrazine contamination above 0.2 p.p.b. were found to contain males with testicular oocytes (Fig. 2a, b). These abnormalities were of similar morphology to those induced by atrazine in the same species in the laboratory. This hermaphroditism was not evident in the absence of atrazine exposure. We conclude that atrazine is responsible for these effects in wild populations, even though other contaminants may be present that could produce similar effects.

Atrazine may affect sex differentiation by inducing aromatase, the enzyme that converts androgens into oestrogens, and can cause inappropriate synthesis and secretion of oestrogens in males at the expense of androgens. This occurs in fish[6], reptiles[7] and mammals[6,8], with inhibition of spermatogenesis probably being a secondary effect associated with the depletion of androgens and synthesis of oestrogens in exposed males, rather than a direct effect of atrazine. Evidence for this mechanism of toxicity in three out of five vertebrate classes, and possibly in amphibians as well, generalizes the possible environmental risk associated with atrazine.

Most water sources in the United States, including rain, contain more atrazine than the effective doses determined in laboratory studies[1]. Although the locality in Wyoming (North Platte River) with the highest frequency of sex reversal (92% of males) is not in the vicinity of farms and is not in a county that reports significant atrazine usage, hermaphrodite frogs are prevalent there because the North Platte River is fed by atrazine-contaminated[9] streams that originate in Colorado.

The frequency of abnormalities at site 2 is much lower than at site 3, although the contamination measured at these sites was comparable. It may be that intermittently exposed populations are more susceptible to atrazine-induced hermaphroditism, whereas continuously exposed populations undergo adaptive resistance.

Applied to crop fields as a pre-emergent, atrazine contamination in water sources peaks with spring rains, which also coincide with breeding activity in many amphibians. Given the adverse effects of atrazine on the gonads of male frogs, this pattern of atrazine application may increase its impact on amphibian populations. In the light of growing evidence that these populations are in decline[10], the contribution of atrazine to this decline warrants further investigation.

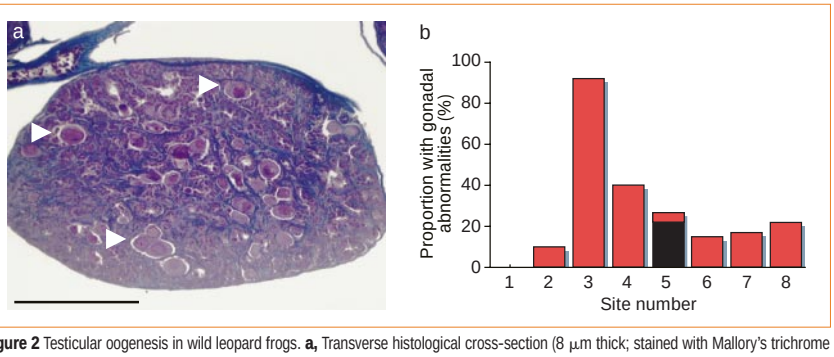

Figure 2 Testicular oogenesis in wild leopard frogs. **a,** Transverse histological cross-section (8 μm thick; stained with Mallory's trichrome stain) of the left gonad of a male frog collected from Carbon County, Wyoming (site 3), showing testicular oocytes (arrowheads). More than 30 oocytes are present in this section. Serial sections reveal that every lobule has at least one oocyte, and some lobules (as shown) have as many as three. Scale bar, 250 μm. **b,** Frequency of occurrence of gonadal dysgenesis (black; poorly developed testes that lack distinct lobules) and testicular oogenesis (hermaphroditism; red) in frogs collected from the eight different sites shown in Fig. 1. The water-borne contamination by atrazine (measured in p.p.b.) was: site 1, 0.14; site 2, 0.20; site 3, 0.20; site 4, 0.30; site 5, 0.80; site 6, 6.70; site 7, data not available; site 8, 0.5.

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Brain development

Memory enhancement in early childhood

Regions of the brain's frontal lobe that are associated with memory retention and retrieval[1,2] begin to mature during the last quarter of the first year in humans. This implies that infants younger than 8 or 9 months should have difficulty in registering an experience and retrieving it after a long delay[3,4]. Here we show that 13-month-old children are unable to recall a sequence of actions performed in front of them when they were 9 months old, whereas 21- and 28-month-olds are able to retrieve representations of the same acts when these were witnessed at 17 and 24 months. Our findings indicate that long-term retention increases during the second year and support the idea that maturation of the frontal lobe at the end of the first year contributes to memory enhancement during this period.

Infants of 6 months old can remember events for up to 24 hours[5], which extends to up to a month when they are 9 months old[6]. It has been proposed that early deficiencies in registering and retaining memories for events in the long term might be related to the protracted maturation of the neocortex[7]. In humans, the brain undergoes important changes towards the end of the first year, including the growth and differentiation of cortical pyramidal neurons and of dendrites in the hippocampus[8–10], which continue into the second year[11,12]. These developmental processes should increase the efficiency of integration and registration of information in the neocortex, and in the prefrontal cortex in particular[1,2].

To test this hypothesis, we evaluated the ability of infants to retrieve representations after a delay of 4 months of motor acts first experienced at 9, 17 or 24 months of age. We used a deferred-imitation procedure in which the experimenter performed a sequence of actions with the aid of props while describing these actions verbally (for example, "Clean-up time!" was used for wiping the table with a paper towel and then throwing the towel into the waste basket; "Make a rattle!" was used for inserting a ring into a slot in a bottle and then shaking the bottle). We estimated the children's recall of these events four months later on the basis of the number of actions they successfully re-enacted and on the number of pairs of actions performed in the proper sequence.

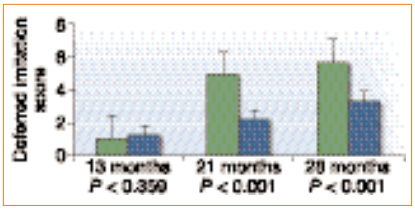

Figure 1 Deferred imitation by infants in three different age groups ($n = 12$) for sets of familiar and new action-sequences. The mean deferred-imitation scores (sum of target actions and ordered pairs reproduced) are shown according to age group for familiar (left bars) and novel (right bars) sequences. Older infants perform better than younger ones on sequences that are new to them, presumably because they are better able to deduce the appropriate target actions without the aid of memory. Note that the performance of the 13-month-old group is weak for both familiar and new action-sequences. The 21- and 28-month-old groups, however, perform significantly better on sequences that they have seen 4 months earlier than they do on novel sequences. Further experimental details are available from the authors.

We exposed infants at 9, 17 or 24 months of age (12 in each group) to three of five possible action-sequences and encouraged them immediately to imitate each sequence. Infants aged 17 and 24 months witnessed four demonstrations of each sequence; 9-month-olds witnessed an additional two demonstrations (for a total of six demonstrations per sequence) to compensate for their immaturity.

After a 4-month delay, we tested the children's ability to re-enact each sequence in response to the same verbal cues when presented with the props for all five action-sequences (the sequences were not demonstrated again after the delay). A comparison of the children's performance on the familiar and novel sequences served as a control for effects unrelated to memory. Five children from the first session were unable to participate in phase two, so their data were not included in the analysis. Six age-matched children, for whom all five action-sequences were novel in session two, were recruited to serve exclusively as controls.

As expected, the 21- and 28-month-olds showed a robust memory for events experienced 4 months earlier, whereas the 13-month-olds did not (Fig. 1). Subjects from the 21- and 28-month-old groups produced more target actions ($F(1,183) = 9.95$, $P < 0.002$) and more ordered pairs ($F(1,183) = 24.55$, $P < 0.001$) on familiar action-sequences than on novel ones. The 13-month-olds failed to produce a greater number of target actions ($t = -1.05$, $P < 0.15$) or correctly ordered pairs ($t = -0.27$, $P < 0.39$) on familiar sequences. In contrast, the 21-month-old ($t = 3.00$, $P < 0.002$) and 28-month-old ($t = 3.17$, $P < 0.001$) groups produced a significantly greater number of target actions on familiar sequences than on novel ones. This trend was even more pronounced for ordered pair scores, where 21-month-olds ($t = 4.60$, $P < 0.001$) and 28-month-olds ($t = 3.55$, $P < 0.001$) had consistently higher scores on sequences they had watched 4 months earlier.

Our findings that long-term memory in infants improves during their second year could be due to compromised registration or poor retrieval in the first year. In either case, our results support the popular belief that at 9 months the hippocampus and regions of the frontal cortex are not yet fully mature. They also indicate that there is a neurobiological component to memory enhancement across the second year, contrary to early assumptions that this is entirely attributable to experience[13].

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