

Clues from Wildlife to Create an Assay for Thyroid System Disruption

Theo Colborn

Wildlife and Contaminants Program, World Wildlife Fund, Washington, DC, USA

In 1996 the U.S. Congress charged the U.S. Environmental Protection Agency to develop a screening program to test chemicals for their possible estrogenic and other endocrine effects. Shortly thereafter, the Chemical Guidelines Program of the Organisation for Economic Co-operation and Development's (OECD) Environmental Directorate organized a Task Force on Endocrine Disruption Testing and Assessment to coordinate development of internationally harmonized screening and testing protocols. Most of the research devoted to this effort has focused on detecting impaired estrogenicity, androgenicity, and/or steroidogenesis, with little progress toward developing assays to detect chemicals that might interfere with thyroid function. Despite the fact that wildlife biologists have been reporting abnormal thyroid gland development and unusual thyroid hormone (TH) and retinoid ratios in fish and birds since the early 1960s, few studies have demonstrated an association between an environmental contaminant and a particular health end point other than reduced reproductive success at the population level. This article is a review of the literature that specifically examines THs and their role in normal behavior and development in wildlife. It presents several studies that associated changes in the thyroid gland, TH concentrations, and behavior with contaminant exposure. The goal of this article is to provide fodder for the creation of simple screens to detect possible thyroid system agonists and antagonists. **Key words:** behavior, brain, development, organochlorine contaminants, retinoids, thyroid, wildlife, xenobiotics. *Environ Health Perspect* 110(suppl 3):363–367 (2002). <http://ehpnet1.niehs.nih.gov/docs/2002/suppl-3/363-367/colborn/abstract.html>

As early as the 1960s, wildlife were exhibiting evidence that prenatal or prehatching exposure to synthetic chemicals could interfere with the endocrine system as well as with other vital systems during development (1). Indisputable peer-reviewed reports have since revealed the effects of a number of synthetic chemicals on the reproduction and development of alligators (2), birds (3), fishes (4), and mammals (5–7). In several instances, the developmental damage has been corroborated through the use of confined wild animals and *in situ*, *in vitro*, and *in vivo* studies. The majority of these studies focused on the disruption of sex hormones, sexual differentiation, and the reproductive system (8). This information eventually led to national legislation and international cooperation to begin removing such chemicals from food and water. The U.S. Congress in 1996 charged the U.S. Environmental Protection Agency to develop a screening program to test chemicals for their possible estrogenic and other endocrine effects (9). Shortly thereafter the Chemical Guidelines Program of the Organisation for Economic Co-operation and Development's (OECD) Environmental Directorate organized a Task Force on Endocrine Disruption Testing and Assessment to coordinate development of internationally harmonized screening and testing protocols.

Thus far, the majority of the research effort has focused on anti- and estrogenic effects, anti- and androgenic effects, and

steroidogenesis. Less research has focused on the disruption of the thyroid system, despite the fact that many species of birds and fish in the U.S. and Canadian Great Lakes and other water bodies around the world suffer unusual thyroid gland development and ratios of circulating thyroid hormones (THs). In 1998, Brouwer and co-workers published an extensive review of their work and others on the many interactive mechanisms of a suite of organochlorine chemicals (OCs) that interfere with the thyroid system (10). Also in 1998, Brucker-Davis published a list of widely used agricultural and industrial chemicals that interfere with the thyroid system (11). THs are acknowledged as critical for the development of the brain, intelligence, and behavior (12). Consequently, it is prudent to develop screens to detect synthetic chemicals that could possibly interfere with the thyroid system as well. To date, thyroid experts from many disciplines are still searching for new and rapid screens and assays to detect thyroid system disruption. This article is a review of studies that specifically examined THs and their role in normal behavior and brain development among wildlife species and in some cases includes links that were made with contaminant exposure. This literature was not as extensive as that on the sex hormones and development. Nonetheless, when considered with the other articles in this supplement, the data should provide fodder for the creation of sorely needed relatively simple screens to detect possible thyroid system agonists and antagonists.

Background

Early in the 1970s a Canadian biologist reported severe reproductive failure and chick deformities in colonies of common terns (*Sterna hirundo*) on Lake Ontario islands (1). These pathologies included chick mortality, growth retardation, and gross deformities of the bills, eyes, and legs, and subcutaneous, pericardial, and peritoneal edema, as well as porphyria and liver necrosis. Further field and laboratory work was undertaken on herring gulls (*Larus argentatus*) that revealed similarities of the pathological conditions with those previously reported for chicken eggs injected with dioxins. In subsequent field research, these and additional functional impacts were reported in other species as well, including double-crested cormorants (*Phalacrocorax auritus*), Caspian terns (*Sterna caspia*), and Forster's terns (*Sterna forsteri*) from other Great Lakes. The effects were not always identical among colonies. All these avian species had elevated concentrations of OCs, including dioxins, furans, polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethane (DDT), dieldrin, mirex, toxaphene, and so forth (13). As technology improved, quantification of the OCs in animal tissue led to more and more associations between the concentrations of OCs in the tissue of the animals and their health problems (14). The persistent, lipophilic, and bioaccumulative nature of the OCs causes them to biomagnify to extremely high concentrations from the water in aquatic systems up through the trophic system to the tissues of top predators (15). Although the OCs may be close to or below detection limit in the water, they are easy to measure in the animals.

Since 1974 the Canadian Wildlife Service has been monitoring OC concentrations in eggs of herring gulls, a top predator in the Great Lakes (16). Because most herring gulls remain in the Great Lakes basin

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Address correspondence to T. Colborn, Wildlife and Contaminants Program, World Wildlife Fund, 1250 24th Street NW, Washington, DC 20037 USA. Phone: (202) 778-9643. Fax: (202) 530-0743. E-mail: colborn@wwfus.org

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throughout the year, they provide an integrated assessment of the concentration of OCs in the ecosystem. This research-based monitoring has also monitored concentrations of plasma thyroxine (T_4), thyroid mass, and incidence and severity of thyroid epithelial hyperplasia in the adult gulls (17). Trend analyses of residue data reveal a peak in OC contamination in the birds in the late 1970s, which tapered off until approximately 1985. Since 1985 there have been only slight reductions in OC concentrations and little improvement in fish and bird health (18).

Wildlife biologists have not been able to find an adult top predator fish in the Great Lakes that does not have a goiter, an enlarged thyroid gland (19). Even so, no long-term monitoring program of contaminants in fish has been established by either Canada or the United States. Despite the reductions in OCs in Lake Erie in the late 1970s, the thyroid glands in Lake Erie fish have become larger and are now visibly rupturing (20). To date no causal link has been made with the OCs or with any other synthetic chemical in the fish and the condition of their thyroids.

Rolland (21) reviews a large body of literature about the association between alterations in the thyroid gland and vitamin A (retinoids) concentrations and OCs in wildlife and fish. The literature does not mention effects on the nervous system or brain in the animals where the abnormal retinoid/thyroid conditions were observed. As Rolland points out, T_4 and retinol (the circulating form of vitamin A) are carried on the same protein complex, transthyretin, in mammals and birds. Lower plasma retinol concentrations and diminished T-cell response were associated in a dose-related manner with increasing PCB concentrations reported in Great Lakes Caspian terns and herring gulls (22). The thyroid glands of herring gulls from Western Lake Erie exhibit the most severe histological changes in relation to reduction in retinols (17). And in many studies, retinoid concentrations do not change in relation to total contaminant loading, although the ratio of retinol to retinyl palmitate (the storage form of vitamin A) does. Egg retinyl palmitate concentrations are the most affected by OC exposure, with the molar ratio of retinol to retinyl palmitate increasing as exposure increases. In other words, the retinoid profile changes but not the total retinoids (23).

As a result of regulatory action in both the United States and Canada to ban the manufacture or reduce the use of the persistent OCs, obvious mortality and morbidity among Great Lakes birds was considerably reduced by the mid 1980s (24). Reproductive success improved as

measured by the number of fledging offspring per maternal animal, but in many instances the offspring were not, and still are not, surviving to adulthood (18,25). Mora et al. (26) reported a negative correlation of blood plasma PCB concentrations in Caspian terns with the percentage of offspring returning to their natal locale. In the case of top predator fish, those fish that do survive to adulthood do not reproduce well or not at all. Without visible evidence of damage, biologists are now finding it far more difficult to determine the specific impairment that is causing the lack of recruitment in certain wildlife populations (24). Consequently, documentation is lacking in the contemporary literature concerning the less visible impacts of contaminants on wildlife such as reduced function and changes in behavior. The following is what I was able to find in the literature on behavior and brain development associated with the thyroid system and contaminants in wildlife.

Amphibians

Over the years a great deal of attention has focused on the role of THs in amphibian metamorphosis (27,28). These reviews make no specific remarks about amphibian behavior or brain development. However, Robertson and Kelley (29) demonstrated that TH controls the development of the larynx in *Xenopus*, which is androgen dependent but does not control the development of the gonads. Hayes (27) discovered also that in anurans TH acts as a master hormone, primary to estradiol (E_2) or testosterone, during development of some anuran systems; that TH is necessary in the liver before E_2 effectively induces vitellogenin production, vitellogenin mobilization to the eggs, and development of the embryo; and that TH induces testosterone receptors in the larynx (gular pouch).

Little was known about neural or brain development in amphibians until 1997, when Denver et al. (30) isolated 34 TH-regulated genes in the diencephalon in *Xenopus* tadpoles. These include transcription factors, a TH-converting deiodinase, metabolic enzymes, a neural-specific cytoskeletal protein, hypophysiotropic neuropeptides, and an isomerase-like disulfide compound. These genes have also been isolated in neonatal mammals and chicks. This should open the door for further comparative developmental studies of the brain in any species and exploring novel screening approaches for thyroid disruption.

It has been suggested that amphibians might provide a short-term assay to detect endocrine disruption. However, because of their complex life cycles and their susceptibility to long-term delayed effects

from exposure to the parent animal and early life stages, frog assays are proving more difficult to develop than envisioned 5 years ago. Gutleb and co-workers (31) demonstrate this difficulty in a series of exposure studies with *Xenopus laevis* and *Rana temporaria*. They found increased incidence of mortality in tadpoles weeks after they ceased dosing the animals. Over an 80-day period, 47.5% of the tadpoles died. The *X. laevis* exposed to 7.7 pM and 0.64 nM PCB 126 exhibited swimming disorders prior to death. Both increased mortality and reduced T_4 concentrations occurred in a dose-response manner in *X. laevis*. Retinoid ratios were not different between the two species, although the concentrations significantly increased at the higher doses in both. Severe eye and tail malformations increased in the froglets in a dose-response manner after approximately 60–68 days. These assays demonstrate the vulnerability of amphibians to PCB exposure but can in no way serve as short-term screens.

Birds

Thyroid hormones in birds have been investigated for their role in migration and courtship. Photorefractoriness, “a reversible state of unresponsiveness to gonado-stimulatory daylengths,” initiates and then terminates breeding in photoperiodic species of birds (32). Preventing migrating species from breeding out of season is especially critical for their survival. THs maintain photorefractoriness in species that reproduce on a seasonal basis (33). Thyroidectomies in seasonal breeders such as starlings (*Sturnus vulgaris*), tree sparrows (*Spizella arborea*) (32), English sparrows (*Passer domesticus*), and red deer (*Cervus elaphus*) (34) cause them to become continual breeders. For seasonal breeders, short-day conditions stimulate the reproductive system to prepare for spring when favorable food and climate conditions are conducive for raising young. Under long-day conditions that commence in the spring, TH production increases concurrent with increases in leutinizing hormone (LH) and follicle-stimulating hormone (FSH) levels and testes size. The testis growth, which is solely testosterone driven, is separate from the refractory response. In the case of starlings, bill color shifts from black to yellow during breeding, when LH and gonadal volume are also high, and is solely androgen controlled. As the breeding season terminates, plasma prolactin increases, FSH and LH decrease, the testes regress, and the birds molt. At the end of the breeding season, THs are elevated once again, a signal that refractoriness has begun. Refractoriness will not end again until signaled by another series of short days.

Species near the equator are continual breeders, whereas species of birds that nest in the Northern Hemisphere have evolved with refractoriness, which restricts their breeding to the most benign season of the year. We still do not know where THs or TH-dependent ligands exert their effect in the photoneuroendocrine system in the birds. Wilson and Reinert (35) suggest that THs interact somewhere between the photoreceptors and neuroendocrine cells in the brain. On the basis of what we do know in mammals, Bentley (33) points out that T_4 mediates nerve growth factor synthesis, which controls microtubule and axonal growth in the central nervous system, and this may in some way affect gonadotropin-releasing hormone secretion (32), which in turn influences LH/FSH secretion (33).

Detecting loss of photorefractoriness in migrating species is almost impossible for field biologists. However, many agricultural and industrial chemicals can affect TH production and ratios. Many pesticides are applied in the spring during breeding season. In most cases, no two active ingredients have the same effect on the thyroid system (36).

From the 1950s through to the 1970s, fish-eating birds in the Great Lakes were experiencing very poor reproductive success (37). Keith (38) suggested that the high embryo mortality and low chick survival in herring gulls nesting in upper Green Bay in the mid 1960s was both the result of *a*) the effects of the chemical residues from the mother on the embryo and *b*) the effects of the adult's contamination on its parental behavior. Peakall et al. (39) switched gull eggs between contaminated and uncontaminated colonies and clearly demonstrated that both factors were involved. "Clean" eggs incubated by "dirty" parents had much lower hatchability than when they were incubated by "clean" parents. Remote monitoring of incubation behavior using telemetry also revealed nesting behavior differences between the control and contaminated colonies (40). Kubiak et al. (41) switched eggs between a Forster's tern colony in Lower Green Bay and an inland site in 1983 and a laboratory, and implicated developmental toxicity and poor parental behavior for the very low reproductive success in the Green Bay colony. The incubation period was extended by 8 days in the Green Bay colony, consistent with poor incubation. These authors associated these effects with dioxin toxicity equivalents (TEQs) contributed by high levels of non-*ortho* PCB congeners.

When captive ring doves (*Streptopelia risoria*) were fed a Lake Ontario-like OC mixture, dose-related abnormalities were found in their plasma hormone levels (including T_4), some of which were

associated with the anomalies in "... breeding synchrony, nest construction, courtship behavior, incubation attentiveness, and parental care that resulted in marked decreases in reproductive success" (42). The diet mixtures contained 0.07 (control), 1.67, and 4.51 ppm dichlorodiphenyldichloroethylene (DDE); 0, 8.02, and 28.0 ppm PCB (Aroclor 1254); 0, 0.297, and 0, 0.90 ppm mirex; and 0, 0.095, and 0.32 ppm photomirex. The breeding cycle was extended from 39 days to 48 days, with clutch completion extending from 9 to 19 days after pairing. Failure to hatch in some groups led to renesting. Plasma T_4 concentrations increased significantly in a dose-related manner and doubled in the highest dosed birds. T_4 concentrations were directly related at mid-incubation with extensions in time allotted to wing flipping ($p < 0.001$), the pattern of which was altered considerably in what is called "in-bowl" activity ($p < 0.05$) that included rearranging nesting material, during the first 39 days after the birds paired. The authors noted reduced incubation and brooding attentiveness, which included spending more time away from the nest, and it appeared that the birds were suffering from T_4 -induced hyperactivity (42).

The female/female pairing associated with a shortage of territorial males in western gulls (*Larus occidentalis*) on the Channel Islands off the California Coast in the late 1970s may have been due, in part, to a contaminant-induced absence of normal territoriality, courtship, and mating behavior in the males (43). Sex-skewed ratios among bird colonies and nocturnal abandonment of incubation by terns (making owl predation easy) were also reported (40,44).

Fox et al. (24) provide an overview of the condition of herring gulls in the Great Lakes in the early 1990s. Thyroid glands in herring gulls are still enlarged but not as pronounced as they were in the late 1970s. Liver concentrations of retinyl palmitate, the principal storage form of vitamin A, have improved in some locations, whereas the depletion of retinyl palmitate has worsened in others. Mild to moderate highly carboxylated porphyria is still a problem, as is the birds' reduced immune competency. Most of the improvements in bird conditions reported by Fox and co-workers occurred before 1985, reflecting the regulation mentioned above of some of the OCs. However, little improvement in bird health has occurred since 1985, which was confirmed with biochemical analyses (measuring dioxin TEQs) and monitoring for obvious deformities in chicks (18). The lesions frequently found in the chicks provide evidence that the problem is still the result of exposure to contaminants during organization prior to birth or hatching.

Concern was directed recently to the risks posed to terrestrial avian species in the Great Lakes basin as the result of exposure to aerial application of pesticides. THs were monitored in nesting tree swallows (*Tachycineta bicolor*) from three apple orchards in southern Ontario, Canada (latitude $43^{\circ}15'N$ /longitude $80^{\circ}20'W$) (45). The concentrations of OCs in the swallows were low. Increasing numbers of sprays increased triiodothyronine (T_3) in chicks if applied during egg development (positive correlation $r^2 = 0.44$; $p = 0.005$). As the number of mixtures of carbamates sprayed increased, T_3 increased in male chicks (positive correlation $r^2 = 0.74$; $p = 0.001$) but not in adult males or females. With the addition of each mixed-spray applied, the formation of Sertoli cells decreased, ultimately reaching significance ($p = 0.02$). In the same study, female tree swallow nestlings showed a correlation between increases in T_3 and increases in body mass but not with increased application of sprays (45).

Fishes

A review by Iwata (46) reveals that migration of salmonids is linked with THs effecting a sequence of behaviors. In the laboratory, increases in T_4 led to less display of aggressive behavior such as territoriality. Elevated concentrations of both T_3 and T_4 reduced the fishes' preference for shade to more open areas (phototaxis). T_3 treatment caused the fish to swim with the current rather than against the flow (rheotaxis).

In the field, a surge of T_4 in sockeye salmon (*Oncorhynchus nerka*) takes place about 2 weeks before downstream migration. The more aggressive fish stay in fresh water longer, whereas the less aggressive species head out to sea earlier and school more (46). The salmon also tend to school more and more as they move toward the ocean seeking open water. Behavioral changes such as these are critical for anadromous species to survive. There is also evidence that T_4 plays a role in olfactory learning and imprinting in migrating smolts (46).

An immersion study comparing the survival of fasting walleye (*Stizostedion vitreum*) larval stocks from four different rivers revealed that the stock with the highest natural T_4 responded with the highest survival and significantly more cannibalism (8-fold more following exposure to T_4 and 10-fold more following exposure to T_3). This stock was taken from "a highly contaminated river" and compared with the other three rivers (47). The authors did not discuss the nature of the contaminants. They found no association between endogenous T_4 concentrations in the fish and cannibalism. This

study raises the question of whether increased cannibalism is an example of selection in a polluted system, where food would be less available for fish.

Coho (*Oncorhynchus kisutch*), chinook (*O. tshawytscha*), and steelhead (*O. mykiss*) salmon are annually stocked in the Great Lakes because they cannot sustain natural populations through reproduction. Recently, hatcheries have been experiencing increasing problems rearing enough fish to meet their stocking needs (48). Losses occur during the sac fry stage after yolk absorption, which is preceded by what is called early mortality syndrome (EMS). EMS is characterized by the alevin losing its equilibrium and appetite, becoming hyperexcitable to touch, and then becoming lethargic. Hatchery research has revealed that mortality varies among family groups from 5 to 97% depending on species and sex. Females appear to be more sensitive. These effects are associated with decreased levels of total and free thiamine in the egg. Additionally, if newly hatched fry are exposed to 2 mg/L T_4 , EMS is significantly reduced (15%) compared with controls (38%), suggesting that the thyroid system is involved as well. Reduced carotenoids do not seem to be related to this syndrome (48).

Control of growth and survival is a critical management issue in hatcheries (49). Management research manipulating T_3 concentrations, T_3/T_4 ratios, and other hormones such as cortisol and estradiol is providing greater insight into the role of THs and development of the brain and nervous system in teleosts. Further exploration in this field might provide ideas for the development of screens and assays to test compounds for their thyroid system effects.

Similar to the results in the tree swallow study mentioned above, freshwater catfish (*Clarias batrachus*) experienced a decrease in T_4 and an increase in T_3 following exposure to a carbamate insecticide, carbaryl (12 mg/L) (50). In the same study, 0.008 mg/L endosulfan caused an increase in circulating T_4 and a decrease in T_3 , and 0.007 mL/L malathion decreased T_3 without changing T_4 (51). These effects were more pronounced during the spawning phase than during prespawning.

Mammals

Wildlife biologists have begun to measure free T_4 , total T_4 , free T_3 , total T_3 , retinols, seven PCB congeners, and several chlorinated pesticides in polar bears (*Ursinus maritimus*) from Svalbard (52). They found a negative correlation between the ratio of total T_4 /free T_4 and the sum of PCBs, whereas they found a positive correlation with p,p' -DDE. Retinols were negatively correlated with the

sum of PCBs and specifically with PCB 138, PCB 153, PCB 156, and hexachlorocyclohexane. Interestingly, females that entered into denning had significantly higher retinol concentrations ($p < 0.05$) than those that did not. Total T_4 (21–14 nmol/L) and free T_4 (28–21 pmol/L) were noticeably higher in the denning females but not significantly. The authors provided no other behavioral information (53).

Hormone levels were linked with behavior and brain development in a wild mammal, the New Zealand red deer. Seasonal breeding behavior was arrested in thyroidectomized (THX) red deer in a year-long study (34). Plasma T_3 concentrations were undetectable in THX stags, whereas control stags had low plasma T_3 concentrations throughout autumn and winter that increased to high concentrations in spring and summer. Throughout the year, THX stags' blood testosterone concentrations remained constant, and they did not shed their antlers. Cessation of testosterone production is required for the antlers to drop.

Conclusions

In situ studies such as those used in fisheries management as well as amphibian and reptile research should be employed to generate new protocols for developing screens and assays to test for thyroid system impairment. *In situ* avian research could prove to be a great deal more difficult. Nonetheless, helpful glimpses might evolve from collaborations among wildlife biologists and poultry researchers.

The major challenges for biologists studying the effects of contaminant exposure in wildlife include *a*) the difficult logistics associated with field research, *b*) the seclusive behavior of wildlife especially during the breeding season, and *c*) the elusive impairment in function and development from exposure to certain xenobiotics. Individual wild animals are not outstanding sentinels for the impacts of endocrine disruption because of the continuous nature of functional impairment, from subclinical and mild to severe, which has no obvious, visible end point of toxicity. Yet, in retrospect, the effects at the population level among wild animals caught the attention of scientists and led to the discovery of endocrine disruption. Biologists should therefore be thinking in terms of insidious, generational effects and observe wildlife populations carefully for changes in successive age classes that might display alterations in feeding habits and social grouping along with changes in geographic distribution, migration behavior, seasonal activity, population dynamics, and age structure. In all probability, the invisible organizational problems resulting from

thyroid system impairment that reduce an animal's potential would be missed at the individual level. The impairment might more readily be reflected and documented at the population level but only if enough of a year class of animals is affected. However, determining the cause of the impact on a population could take several generations. This suggests that long-term demographic studies are important and could be most informative if the "functionality" of each year class was known.

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