



## Gonadal Feminization and Halogenated Environmental Contaminants in Common Terns (*Sterna hirundo*): Evidence That Ovaries in Male Embryos do not Persist to the Prefledgling Stage

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**Abstract.** Common terns (*Sterna hirundo*) and roseate terns (*Sterna dougallii*) breed on Bird Island, Massachusetts, USA, near a Superfund site highly contaminated with polychlorinated biphenyls (PCBs). Observations of skewed sex ratios and female–female pairings among endangered roseate terns (Nisbet and Hatch (1999) *Ibis* **141**, 307) suggested the possibility of contaminant-related endocrine disruption in these birds and prompted investigation of common terns as a surrogate species. In 1993 and 1994, 60–90% of pipping male common tern embryos sampled exhibited ovarian cortical tissue in their testes (ovotestes) (Nisbet et al. (1996) *Bull. Environ. Contam. Toxicol.* **57**, 895; Hart et al. (1998) *Mar. Environ. Res.* **46**, 174). To examine the possible impact of ovotestes on the reproductive capabilities of common terns, we examined gonadal histology in common tern prefledglings (approximately 21 days old) collected from Bird Island in 1995. As a measure of embryonic contaminant exposure, contaminants were measured in a subset of eggs collected from the same nests as the prefledglings. Concentrations of total PCBs in these eggs ranged from 14.4 to 546 µg/g lipid. No evidence of ovotesticular development was observed in any of the 19 male prefledglings examined. Some gonadal irregularities were observed, including small nodules of testicular tissue within the epithelial capsule of the testes, but these were judged not likely to affect testicular function. There was no relationship between any observed irregularities and levels of contaminants present in the matched eggs. The results suggest that the ovotestes that occur in 60–90% of pipping common tern embryos from this site become fully regressed by approximately 21 days posthatch. Our data from this and previous studies are consistent with the idea that ovotestes occur naturally in some individual common terns at hatching, although the frequency of occurrence may be enhanced by exposure to chlorinated organic contaminants such as PCBs. In either case, we suggest that the presence of ovotestes in common tern embryos from PCB-contaminated sites such as Bird Island does not lead to permanent alterations in gonadal histology that would be expected to impair reproductive function.

**Keywords:** birds; PCBs; gonads; feminization; ovotestes; endocrine disruption

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### Introduction

Many populations of piscivorous birds are exposed to high levels of persistent organic chemicals such as organochlorine pesticides and polychlorinated

biphenyls (PCBs) (Bosveld and Van den Berg, 1994; Hoffman et al., 1996). These chemicals have been suggested as contributing to reproductive impairment, including reduced breeding success and embryonic deformities, in some populations (Peakall and Fox, 1987; Tillitt et al., 1992; Becker et al., 1993; Giesy et al., 1994; Barron et al., 1995; Fry, 1995). Experimental exposure of birds to 1,1,1-trichloro-2,2-bis(chlorophenyl)ethane (DDT), 1,1-dichloro-2,2-bis(chlorophenyl) ethylene (DDE) (Peakall, 1967, 1969, 1970; Fry and Toone, 1981), PCBs (Brunstrom, 1988; Hoffman et al., 1998; Bosveld et al., 2000), or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Walker and Catron, 2000) has been shown to cause biochemical, physiological, morphological, and/or behavioral changes that provide a hypothetical link to the effects observed in wild populations. There is good evidence to support a role for chlorinated aromatic chemicals in the reproductive impairment seen in some populations of piscivorous birds (Peakall and Fox, 1987; Gilbertson et al., 1991; Giesy et al., 1994; Hoffman et al., 1996). It has been suggested that some of the effects of these and other chemicals involve "endocrine disruption", i.e. altered hormone levels or function. However, strong support for the hypothesis that chlorinated aromatic chemicals are causing endocrine disruption in wild birds is lacking (Peakall, 1996; Risebrough, 1999; Dawson, 2000; Brunstrom et al., 2002). Here, we report on one aspect of a possible case of endocrine disruption in piscivorous birds exposed to chlorinated aromatic chemicals.

Roseate terns (*Sterna dougallii*) and common terns (*Sterna hirundo*) breed on Bird Island in Buzzards Bay, Massachusetts, on the northeastern coast of the United States. Some of these terns feed near New Bedford Harbor, a Superfund site highly contaminated with PCBs and heavy metals (Weaver, 1984; Pruell et al., 1990; Lake et al., 1995). High levels of PCBs have been measured in eggs and tissues of these birds (Custer et al., 1983; Nisbet and Reynolds, 1984; Nisbet et al., 1996). Concern about terns nesting on Bird Island arose when endangered roseate terns were found to exhibit a sex ratio skewed toward females, female-female pairing, and supernormal clutches (Nisbet and Hatch, 1999). These same phenomena were also observed in the 1970s in a DDT- and DDE-exposed population of Western gulls (*Larus occidentalis*) breeding off the coast of California (Hunt and Hunt, 1977; Hunt et al., 1980). Subsequently, Fry and coworkers found that male gull

hatchlings from eggs injected with estradiol, methoxychlor, *p,p'*-DDE, or *o,p'*-DDT developed feminized testes (ovotestes) and oviducts (Fry and Toone, 1981; Fry et al., 1987). Similar effects of *o,p'*-DDT have been seen in other avian species (Berg et al., 1998). It was hypothesized (Fry and Toone, 1981; Fry et al., 1987; Fry, 1995) that DDT and DDE exposure led to feminization of male gulls, affecting the reproductive function and/or behavior of males and resulting in the observed female-biased sex ratio at breeding colonies. This experimental link between feminization and exposure to DDT and DDE suggested that exposure to these or other persistent organochlorine contaminants could be causing reproductive dysfunction in the Bird Island terns, as hypothesized for Western gulls.

In 1993 and 1994, contaminant levels and gonadal morphology were studied in common terns from Bird Island. (Common terns were used as a surrogate species for endangered roseate terns.) Histological examination revealed that testes from 60% to 90% of pipping male embryos contained ovarian-like cortical tissue (ovotestes), with severe ovotestes (testes with an ovarian cortical ridge containing primordial germ cells) occurring in 45–60% of male birds (Nisbet et al., 1996; Hart, 1998).

The potential impact of ovotestes on the reproductive success of Bird Island terns (as hypothesized for the Western gull population) might depend on whether the ovotestes observed in the tern embryos persist to adulthood. To test the hypothesis that the embryonic ovotestes documented in Bird Island common terns persist through the post-hatch period, we examined the testes of pre fledgling common terns. Male *S. hirundo* pre fledglings (approximately 21 days old) and adults were collected from Bird Island in 1995 and their gonads were examined histologically. In addition, female pre fledglings were also examined for possible abnormalities in ovarian development. Potential exposure to organochlorine contaminants was assessed by analysis of eggs collected from the same nests as the pre fledglings.

## Methods

### Collection

All samples were collected from Bird Island in Buzzards Bay, MA (41°40'N, 70°43'W) in the 1995 breeding season. Ten pre fledglings (the oldest chick

in each of ten broods) were collected at approximately 21 days of age on July 1 (four males) and July 6 (four males and two females). The third egg from each of the same 10 clutches had been collected previously and the contents stored frozen in acid-washed, hexane-rinsed glass jars. Because we wanted to collect only males, the prefledglings were sexed prior to collection using a PCR-based method with blood in feather tips (Sabo et al., 1994). The sex marker is located on the female W chromosome, and a male is indicated by a negative PCR result. In two cases where a female was collected, the negative PCR result was apparently indicative of an unsuccessful PCR reaction rather than the lack of a W chromosome.

An additional 17 prefledglings (11 males and 6 females), killed by an owl, were collected on July 4 and necropsied within 24 h of death. These birds were approximately 14–21 days old. Nine adult male terns that died of natural causes during the 1995 breeding season were also collected.

Pipping common tern embryos also were collected from Bird Island in 1994 and 1996, and common tern eggs were collected in 1996 (Hart, 1998).

#### *Necropsy*

The 10 prefledglings collected on July 1 and July 6 were weighed and decapitated. Blood was collected, centrifuged, and the plasma stored frozen at  $-80^{\circ}\text{C}$ . After removal of the gall bladder, the liver was removed and weighed. A small slice of liver was placed in 70% neutral buffered formalin (NBF), a portion was frozen at  $-80^{\circ}\text{C}$  in acid-washed hexane-rinsed jars for chemical analysis, and the remainder was frozen in liquid nitrogen. The trachea, esophagus, stomach, intestinal tract, and attached organs/glands were removed and placed into NBF; the back with gonads, kidneys, and cloaca, lungs, and head were also placed in NBF. Within several hours of collection, slices were cut from tissues in NBF and placed into histology cassettes for further fixation in NBF. Fixed tissues were embedded in paraffin blocks within 24 h.

The pipping embryos collected in 1994 and 1996 (Hart, 1998) were processed as above, with the addition that the yolk sac was removed and placed in acid-washed hexane-rinsed jars for chemical analysis.

The prefledglings killed by an owl on July 4 and the adult terns found dead were necropsied within 24 h of death. Gonads were fixed in NBF and embedded in

paraffin blocks. The time between fixation and embedding was within 24 h for owl-killed prefledglings, and between one and three months for adults.

#### *Gonadal histology*

Paraffin-embedded gonads were sectioned (3–5  $\mu\text{m}$  thickness) and stained with hematoxylin and eosin. A minimum of 20 sections through the left gonad were examined for the presence of ovarian tissue in testes of males, and for the presence of double follicles in ovaries of females. Gonad sections were also examined for the presence of other abnormalities.

#### *Egg and yolk sac extraction, contaminant analyses*

Extraction of persistent organic contaminants from egg homogenates and whole yolk sacs was carried out according to the method of Kennedy et al. (1996b), with minor modifications (Norstrom et al., 1986, 1990; Kennedy et al., 1996a). Final extracts contained at least all polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), PCBs, structurally related nonpolar halogenated aromatic hydrocarbons (e.g. polybrominated biphenyls, chloronaphthalenes, and diphenyl ethers), and chlorinated hydrocarbon pesticides (e.g. DDE, Dieldrin, and Mirex). Briefly, egg homogenate (5 g) or whole yolk sac was dried with sodium sulfate, extracted with dichloromethane (DCM)/hexane (1 : 1), and cleaned up by gel permeation chromatography (GPC) to remove lipids. The lipid fraction from GPC was collected and used to determine the percent lipid in the sample. The contaminant-containing fraction collected from the GPC was split; 10% of the sample was used for chemical analysis and 90% for bioassays. The 90% portion of the split was transferred quantitatively into 200  $\mu\text{l}$  of DMSO using a rotary evaporator and water bath heated to  $25^{\circ}\text{C}$ . The DMSO solutions were vortexed, and serial dilutions in DMSO were prepared. The 10% split for chemical analysis was further cleaned on a florisil column according to the standard procedures of pesticide and PCB trace contamination determination at the National Wildlife Research Center (NWRC) (Won, 1992). The sample was eluted from the florisil column in three fractions: Fraction 1 was eluted with hexane, Fraction 2 with 15% DCM in hexane, and Fraction 3 with DCM/hexane (1 : 1). These three fractions were used to measure a suite of

PCB congeners and organic pesticides by gas chromatography electron capture detection (GC-ECD) at the NWRC, Hull, Quebec. Samples were injected into a Hewlett Packard (HP) 5890 GC #1 using an HP 7673A auto injector. Throughout the method, all glassware was rinsed with acetone and hexane three times prior to use.

Control samples were used throughout the extractions of yolk sacs and eggs to monitor recovery. Two solvent blanks and one blank chicken egg showed no detectable contaminants; a second blank egg contained a total PCB level of 0.05 ppm. Results from four spiked chicken eggs and one spiked solvent control showed recoveries ranging from 89% to 104%. These results indicated the extraction method had good recoveries and did not result in contamination of the samples (for details, see Hart, 1998).

Concentrations of PCBs, chlorinated hydrocarbon pesticides, and TCDD-EQs are reported per gram lipid. Concentrations of total PCBs were calculated as the sum of the following congeners (Ballschmiter and Zell, 1980): 31, 28, 29, 52, 49, 44, 42, 64, 74, 70, 66, 60, 101, 99, 97, 87, 110, 151, 149, 118, 146, 153, 105, 141, 137, 138, 158, 129, 182, 183, 128, 185, 174, 171, 200, 172, 180, 170, 201, 203, 195, 194, 206 (Hart, 1998).

#### *TCDD-EQ CEH bioassay*

Primary hepatocyte cultures were prepared from 19-day-old chicken embryos in 48-well plates as described previously (Kennedy et al., 1993). At 24 h, duplicate plates were dosed with each egg extract dilution series (1, 0.3, 0.1, ... 10.00003). Triplicate 48-well plates were dosed with TCDD (range of doses 0.0001–3 nM). After dosing, cells were incubated for another 24 h, at which time the medium was removed, the cells were rinsed, and plates were frozen on dry ice before transferring to a –80 °C freezer. After thawing, ethoxyresorufin O-deethylase (EROD) and total protein assays were carried out in the cell culture plates as described previously (Kennedy et al., 1995), and the reaction products (resorufin and fluorescamine-protein adducts) were measured with a fluorescence plate reader (Cytofluor 2300, Millipore Ltd.). EROD concentration-response curves were fitted empirically to a modified Gaussian curve as described previously (Kennedy et al., 1996b), and the maximal EROD activity and the  $EC_{50}$  were determined for each

concentration–response curve. Bioassay derived dioxin-equivalents (TCDD-EQs) then were calculated as described previously (Kennedy et al., 1996b) according to the following equation:

$$\begin{aligned} \text{TCDD-EQ}_{\text{bio}} (\text{ngg lipid}) &= [\text{TCDD } EC_{50} (\text{ng/ml of medium}) \\ &\quad / \text{extract } EC_{50} (\text{dilution factor})] \\ &\quad \times [\text{volume of medium (ml)/volume of diluted} \\ &\quad \quad \text{extract added to medium } (\mu\text{l})] \\ &\quad \times [\text{volume of extract stock } (\mu\text{l})/\text{mass of lipid} \\ &\quad \quad \text{in sample (g)}], \end{aligned}$$

where “dilution factor” represents the volume of stock egg extract (l) per  $\mu\text{l}$  of dosing solution at the dilution resulting in 50% of maximal EROD induction.

#### *Statistics*

Correlations were tested using Pearson product moment correlation coefficients and Bartlett’s chi-square statistic probabilities. Probabilities of  $p \leq 0.05$  were considered statistically significant.

#### **Results**

Ten pre fledgling common terns (eight males and two females) and same-nest eggs were collected from Bird Island in 1995. An additional 17 pre fledglings (11 males and 6 females) and nine adult male terns dying of natural causes were also collected.

#### *Necropsy findings*

No gross abnormalities were observed in any of the 19 male or 8 female pre fledglings. No males showed development of oviducts, and no testicular abnormalities were evident by macroscopic examination.

#### *Gonad histology*

Gonads from adult and pre fledgling terns were examined histologically; results are summarized in Table 1 and illustrated in Figs. 1–3. The ovaries of all female pre fledglings appeared normal (Table 1). A typical section through the ovary of a common tern pre fledgling depicting the ovarian follicles within the developed ovarian cortex is shown in Fig. 1a. Double follicles may be observed in



Table 1. Gonadal histology from common tern prefledglings and adults collected from Bird Island in 1995

Sample	Normal gonads	Feminized gonads	Gonads with other irregularities
Prefledglings			
Female ( $n = 8$ )	8	0	0
Male ( $n = 19$ )	10	0	9 <sup>a</sup>
Adults-male ( $n = 9$ )	8	0	1 <sup>b</sup>

<sup>a</sup>Irregularities in the nine male prefledglings included:

Eight testes with intracapsular (within the tunica albuginea) nodules of testicular tissue; most very small, and one consisting of a single primordial germ cell (PGC);

One testis with disorganized seminiferous tubules containing low numbers of PGCs, and PGCs located outside of the seminiferous tubules but within the medullary area of the testis;

One testis with a group of similar cells in place of a normal seminiferous tubule, a small extracapsular group of cells, as well as small intracapsular nodules (included in the Eight testes with nodules as well).

<sup>b</sup>One adult testis contained a functioning seminiferous tubule completely surrounded by tunica albuginea.

hormone-injected females (D.M. Fry, personal communication); however, none was observed among common tern prefledglings examined here.

Among male prefledglings, most testes were normal and all lacked ovarian cortical tissue (Table 1). A typical normal testis is shown in Fig. 1b, with the tunica albuginea encapsulating the testis and organized seminiferous tubules within which the primordial germ cells are located. The seminiferous tubules are well organized with a distinctive lining of Sertoli cells, the elongated irregular cells with basal nuclei and cytoplasm extending into the tubule lumen. Numerous primordial germ cells (distinguished by their large nuclei) are found within the seminiferous tubules.

Although most testes appeared normal and none contained ovarian cortical tissue, several slight irregularities were observed among the male testes. At least five males showed small nodules of tissue within the tunica albuginea; these appeared to be isolated seminiferous tubules or seminiferous tubules extending into the tunica albuginea. These intracapsular nodules appeared testicular in nature because of their organization with Sertoli cells lining the base of the nodule. Some nodules contained primordial germ cells while others did not. Most of these nodules were small and appeared quiescent, extending through only

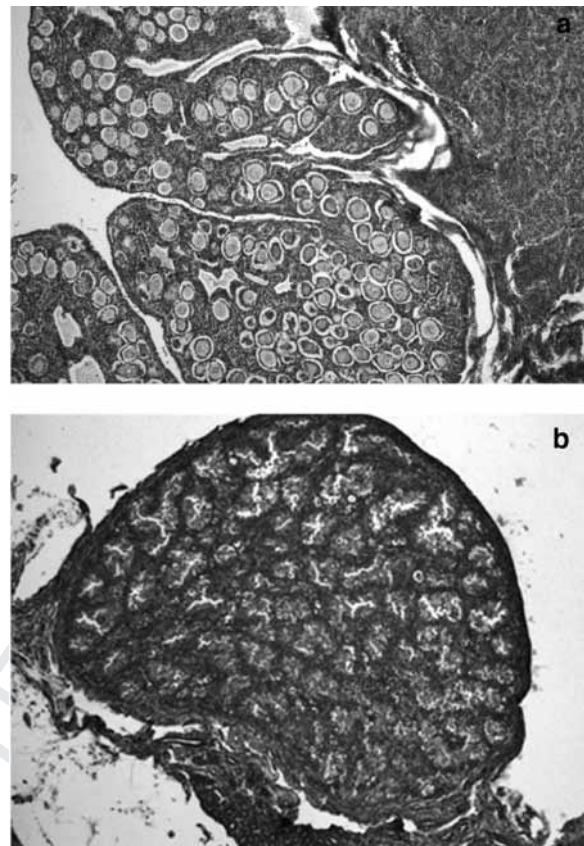
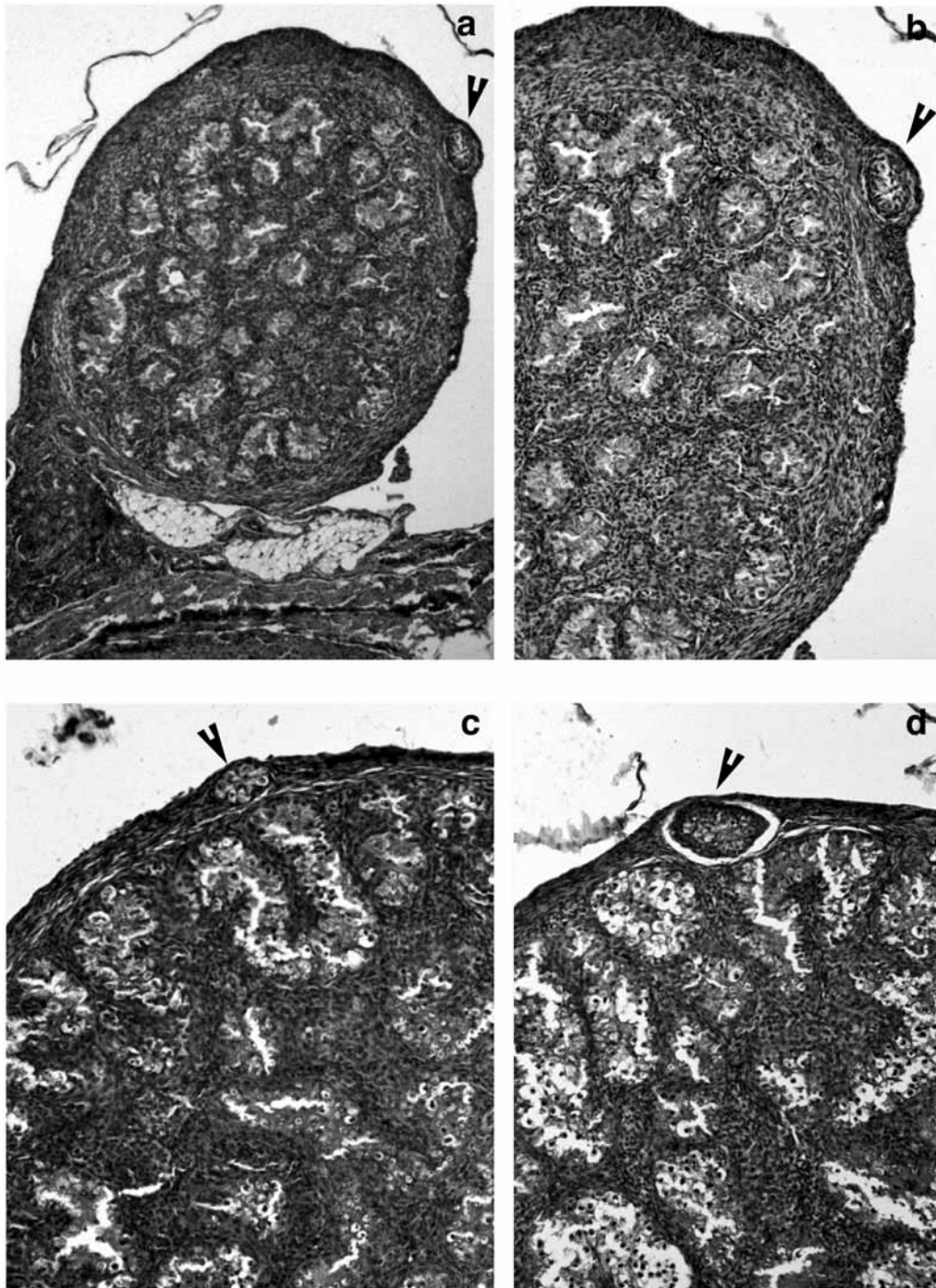
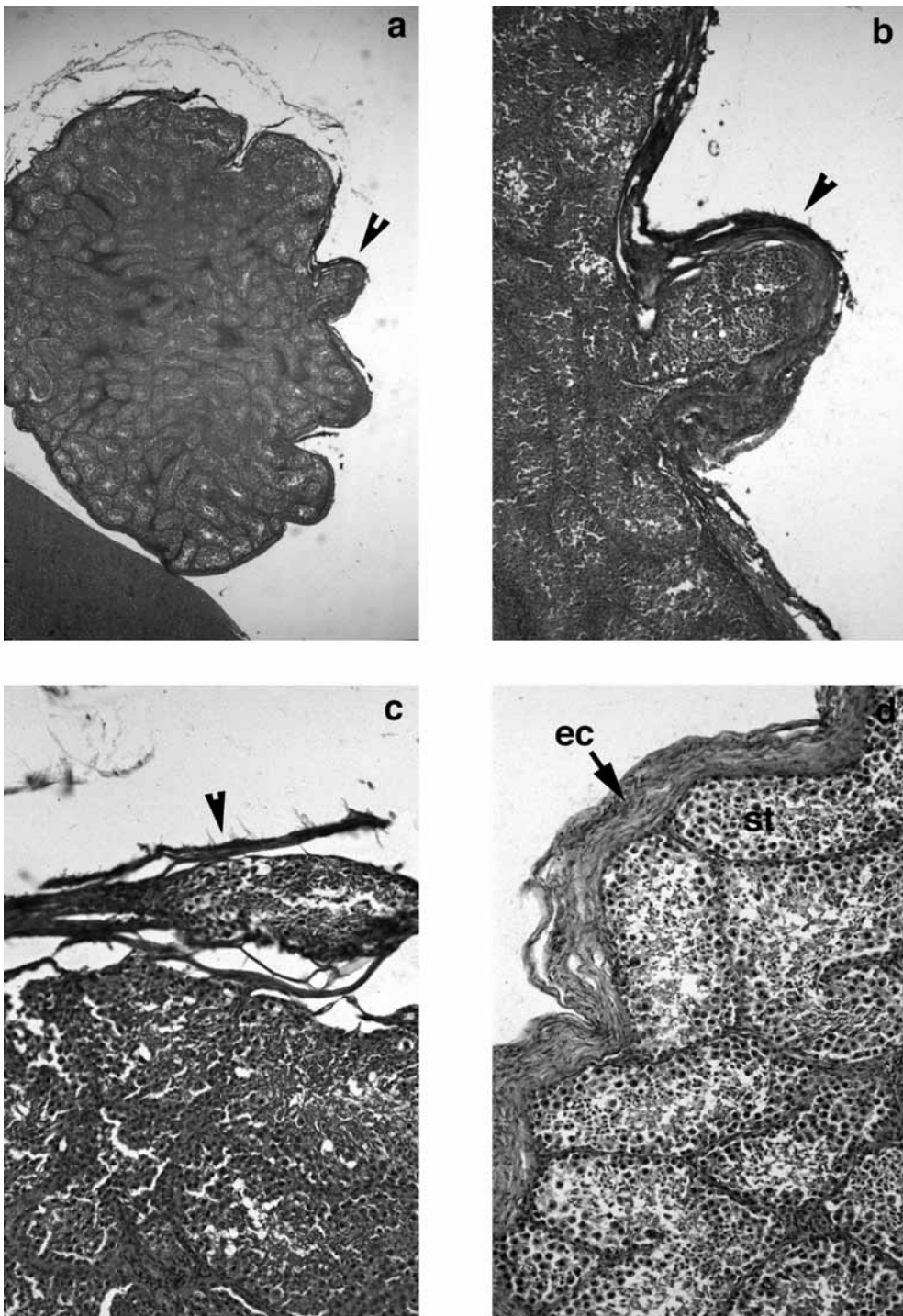


Figure 1. Histology of normal gonads from common tern prefledglings sampled at Bird Island in 1995. Hematoxylin and Eosin stain. a. Normal female ovary. Oocytes can be seen within ovarian follicles. (100 $\times$ ). b. Normal male testis. Epithelial capsule (tunica albuginea) surrounds the testis. Seminiferous tubules are located throughout the medullary area, and primordial germ cells are located within the seminiferous tubules. (100 $\times$ ).

3–15 sections. Figure 2(a–d) shows examples of testes with intracapsular nodules. In addition to the birds with nodules, one male exhibited an abnormal, undeveloped seminiferous tubule that appeared to be a grouping of similar cells, likely Sertoli cells. This tubule contained no primordial germ cells and showed no organization or development of a lumen. The testis that contained this abnormal tubule also showed an area of extracapsular cells, which may have been a cortical remnant, but which contained no primordial germ cells. One male testis had disorganized seminiferous tubules that contained very few primordial germ cells; the same testis also had primordial germ cells located outside the seminiferous tubules.



*Figure 2.* Examples of intracapsular testicular nodules (arrowheads) in male pre fledgling common terns from Bird Island. a. Testis with intracapsular nodule of a large size. (100 $\times$ ). b. Same testis with large intracapsular nodule, organization appears like that of a seminiferous tubule. (200 $\times$ ). c. Testis with intermediate size intracapsular nodule. (200 $\times$ ). d. Testis with large intracapsular nodule. (200 $\times$ ).



*Figure 3.* Histological sections from adult common terns sampled at Bird Island in 1995. a. Normal adult male testis, showing lobules. (25 $\times$ ). b. Same adult testis, enlargement of one lobule (arrowhead). Lobule appears to contain seminiferous tubules like that of the main testicular area. (100 $\times$ ). c. Same adult testis, enlargement of isolated seminiferous tubule surrounded by epithelial capsule (arrowhead). (200 $\times$ ). d. Enlargement of testis showing seminiferous tubules (st) containing developing spermatogonia, spermatocytes, and spermatids. The basal lining of Sertoli cells and lumen of the seminiferous tubules are distinct in the adult testis. The epithelial capsule (ec) surrounding the testis is also shown. (200 $\times$ ).

Although this testis appeared slightly disorganized, there was no evidence of an ovarian cortical area.

In summary, among the 19 male pre fledglings examined, one showed disorganized tubules with extra-tubular primordial germ cells, and one showed a slightly thickened extracapsular area and an abnormal seminiferous tubule consisting of Sertoli-like cells. Five showed intracapsular nodules of testicular tissue but otherwise appeared normal, and the remainder appeared completely normal. Thus, unlike the embryos examined earlier (Nisbet et al., 1996; Hart, 1998), male pre fledglings showed no development of ovarian cortex containing primordial germ cells (i.e. "ovotestes").

As expected, the adult male testes were much more developed than testes of the pre fledglings, showing a dramatically increased area of seminiferous tubules, which contain developing spermatogonia, spermatocytes and spermatids (Fig. 3). The edges of adult testes were often slightly irregular or lobed rather than completely rounded, possibly due to enlargement during the breeding season or handling during dissection, fixation, or embedding (Fig. 3a). However, the tunica albuginea (epithelial capsule) completely

surrounded the testes, and the lobes showed no evidence of ovarian tissue (Fig. 3b). Rather, the lobules consisted of seminiferous tubules containing developing spermatids. In one testis, a seminiferous tubule along the testis edge appeared to be surrounded by tunica albuginea, but this tubule also appeared to be functioning normally and contained spermatids (Fig. 3c). At higher magnification, the developing spermatogonia, spermatocytes and spermatids, as well as the basal lining of Sertoli cells and lumen of the seminiferous tubules, were distinct in the adult male testes (Fig. 3d).

#### *Organochlorines in tern eggs*

Lipid-normalized concentrations of PCBs, organochlorine pesticides, and bioassay-derived TCDD-EQs were measured in individual eggs from 10 nests. Table 2 summarizes the data for total PCBs,  $\sum p,p'$ -DDTs, mirex, trans-nonachlor, and bioassay-derived TCDD-EQs in relation to the gonadal histology of the 10 pre fledglings from same nests. The average total PCB levels were  $75.1 \pm 52.4$  (SE)  $\mu\text{g/g}$  lipid. One egg contained very high levels of total PCBs,  $546 \mu\text{g/g}$

Table 2. Summary egg contaminant data and gonadal histology of paired pre fledglings collected from Bird Island, 1995

Egg number	Total PCBs $\mu\text{g/g}$ lipid	Bioassay- derived TCDD- EQs ng/g lipid	Total $p,p'$ - DDTs $\mu\text{g/g}$ lipid	Mirex $\mu\text{g/g}$ lipid	Trans-non-achlor $\mu\text{g/g}$ lipid	Sex of paired pre fledgling	Gonadal histology
674	547	114	0.73	n.d.	3.24	M	N <sup>a</sup>
855	37.5	16.9	1.16	0.05	0.26	F	N
530	30.3	9.15	1.62	0.03	0.25	M	I (3)
1024	28.7	10.3	0.70	0.06	0.18	M	I (3)
257	24.4	6.36	1.25	0.07	0.28	M	R, I (2), E
952	19.6	6.82	1.08	0.03	0.08	M	I (2)
527	18.2	7.30	0.56	0.06	0.09	M	I (1)
531	16.7	7.26	0.51	0.03	0.07	M	N
765	15.0	3.73	0.35	0.11	0.05	M	I (4)
938	14.4	3.80	0.37	0.12	0.04	F	N
Mean $\pm$ SE	$75.1 \pm 52.4$	$18.6 \pm 10.7$	$0.83 \pm 0.13$	$0.06 \pm 0.012$	$0.45 \pm 0.31$		

<sup>a</sup>Letters represent classification of gonadal histology as follows:

N = Normal;

I = intracapsular nodules of testicular tissue. Number indicates size of nodule:

(1) = 1 PGC;

(2) = nodules contain few cells, and do not persist through more than three sections;

(3) = nodules contain more cells than (2) and persist through 3–7 sections;

(4) = nodules contain many cells, organization is like that of a seminiferous tubule (only much smaller) and persists through many sections.

R = Grouping of similar cells in place of a normal seminiferous tubule.

E = Extracapsular cells.

n.d. = Not detected.



lipid, while the range of the remaining samples was 14.4–37.5  $\mu\text{g/g}$  lipid. Similarly, bioassay-derived TCDD-EQs ranged from 3.7 to 16.9 ng/g lipid except for one sample at 114 ng/g lipid (the same sample which had the highest PCB levels); the average value for TCDD-EQs was  $18.6 \pm 10.7$  (SE) ng/g lipid. Total PCBs and TCDD-EQs were significantly correlated, both including the highest value ( $r^2 = 0.99, p = 0.009$ ) and excluding the highest value ( $r^2 = 0.82, p = 0.02$ ) (Hart, 1998).

Concentrations of organochlorine pesticides were low, with most values ranging from non-detectable to less than 1  $\mu\text{g/g}$  lipid. Total  $p,p'$ -DDTs (sum of  $p,p'$ -DDD,  $p,p'$ -DDE, and  $p,p'$ -DDT) were detected in all samples analyzed and showed the highest mean concentrations ( $0.83 \pm 0.13$   $\mu\text{g/g}$  lipid), with over 95% of the total from  $p,p'$ -DDE (Table 2). Levels of mirex ( $0.06 \pm 0.01$   $\mu\text{g/g}$  lipid) and *trans*-nonachlor ( $0.45 \pm 0.31$   $\mu\text{g/g}$  lipid) were also low but detectable in most samples (Table 2). Other pesticides and their concentrations ( $\mu\text{g/g}$  lipid, mean  $\pm$  SE) were: octachlorostyrene ( $0.08 \pm 0.08$ ), photo-mirex ( $0.06 \pm 0.02$ ),  $\alpha$ -hexachlorocyclohexane (n.d.),  $\beta$ -hexachlorocyclohexane (n.d.),  $\gamma$ -hexachlorocyclohexane (n.d.), hexachlorobenzene ( $0.04 \pm 0.01$ ), oxy-chlordane ( $0.04 \pm 0.004$ ), *trans*-chlordane ( $0.01 \pm 0.01$ ), *cis*-chlordane ( $0.01 \pm 0.01$ ), *cis*-nonachlor ( $0.02 \pm 0.01$ ), heptachlor epoxide ( $0.02 \pm 0.003$ ), dieldrin ( $0.07 \pm 0.01$ ), tris(4-chlorophenyl)methanol ( $0.12 \pm 0.07$ ),  $p,p'$ -DDE ( $0.79 \pm 0.13$ ),  $p,p'$ -DDD ( $0.03 \pm 0.01$ ),  $p,p'$ -DDT ( $0.02 \pm 0.01$ ).

There was no relationship between egg contaminant burdens and prefledgling gonadal histology. The two females exhibited normal gonadal histology, and the eight males showed no development of ovotesticular tissue. Among males, the presence of intracapsular nodules and the abnormal seminiferous tubule was not related to contaminant levels in the corresponding eggs. The egg containing very high concentrations of total PCBs and TCDD-EQs was from the same nest as a male prefledgling with normal gonadal histology.

## Discussion

A high incidence of ovotestes has been observed in pipping male common tern embryos from Bird Island, MA (Nisbet et al., 1996; Hart, 1998). Because similar histological abnormalities occur and can persist in birds experimentally treated with estrogenic compounds,

including organochlorine pesticides (see below), we sought to determine if ovotestes were present in male prefledglings from this site examined at 21 days post-hatch. Same-nest eggs were sampled to provide a measure of PCBs and other organochlorine contaminants, which have been found previously at high concentrations in birds from this site (Nisbet and Reynolds, 1984; Nisbet et al., 1996; Hart, 1998). This study is part of a larger effort to understand the impact of halogenated organic contaminants on common and roseate terns from highly contaminated sites (Nisbet and Reynolds, 1984; Nisbet et al., 1996; Lorenzen et al., 1997; Hart, 1998; Hart et al., 1998; Nisbet and Hatch, 1999; Karchner et al., 2000; French et al., 2001).

## Contaminant data

Previous studies have shown a strong correlation between organochlorine concentrations in paired eggs and embryos from the same nests of double crested cormorants (*Phalacrocorax auritus*) (Custer et al., 1997) and black-crowned night-herons (*Nycticorax nycticorax*) (Custer et al., 1990). Similarly, concentrations of PCBs in individual eggs within three-egg clutches of common terns were highly correlated (French et al., 2001). Therefore, in this study, the “C” (third) eggs collected from the same nests as the prefledglings were considered a good indicator of contaminant exposure experienced by the prefledglings *in ovo*. Because the concentrations of contaminants deposited in C eggs tend to be somewhat greater than that in A or B eggs (Nisbet, 1982; French et al., 2001), the egg data in our study likely provide a slight overestimate of the actual exposure of the prefledglings.

Levels of total PCBs and organochlorine pesticides in common tern eggs collected from Bird Island in 1995 were similar to those in tern embryo yolk sacs collected in 1994 (Table 3). Organochlorine pesticides were fairly low and similar among all samples. In both 1995 eggs and 1994 yolk sacs, most samples had moderate levels of total PCBs, while several showed extremely high total PCB levels. It is likely that some individual terns at Bird Island feed in areas highly contaminated with PCBs [such as near New Bedford Harbor (Weaver, 1984)] and thus accumulate high levels of PCBs that are deposited into eggs, while other Bird Island terns feed in less contaminated areas. This pattern (low organochlorine pesticide levels

Table 3. Summary contaminant data for tissues collected at Bird Island, 1994–1996

Year of collection	Tissue (n)	Total PCBs µg/g lipid	TCDD-EQs ng/g lipid	Total <i>p,p'</i> -DDTs µg/g lipid	Mirex µg/g lipid	trans-non-achlor µg/g lipid
1994 (early)	Yolk sacs (8)	133 ± 77 <sup>a</sup> (31.6–663)	38.4 ± 19.7 <sup>b</sup> (8.3–174.1)	3.42 ± 0.61 (0.91–6.04)	0.28 ± 0.12 (0.05–0.94)	0.88 ± 0.54 (0.15–4.63)
1994 (late)	Yolk sacs (6)	89.8 ± 33.9 (17.2–245)	41.6 ± 20.1 <sup>b</sup> (7.1–138.6)	3.98 ± 0.73 (2.22–6.79)	0.18 ± 0.06 (0.06–0.37)	0.55 ± 0.23 (0.07–1.61)
1995	Eggs (10)	75.1 ± 52.4 (14.4–683)	18.6 ± 10.7 <sup>b</sup> (3.8–114.1)	0.83 ± 0.13 (0.35–1.62)	0.06 ± 0.01 (n.d.–0.12)	0.45 ± 0.31 (0.04–4.02)
1996	Yolk sacs (8)	209 ± 81.3 (16.5–659)	46.3 ± 17.4 <sup>c</sup> (2.4–138.1)	3.27 ± 0.49 (1.23–5.19)	0.13 ± 0.03 (n.d.–0.30)	1.2 ± 0.47 (0.18–3.98)
1996	Egg pool <sup>d</sup> (2)	26.0 (15.5–36.6)	5.5 <sup>c</sup> (2.9–8.0)	1.25 (1.03–1.47)	0.06 (0.06–0.07)	0.14 (0.07–0.21)

<sup>a</sup> Mean ± SE, range in parentheses.

<sup>b</sup> TCDD-EQs were derived using the chick embryo hepatocyte (CEH bioassay).

<sup>c</sup> TCDD-EQs were calculated from PCB and TCDD congener data, using the TEFs derived from the EC-TCDD<sub>10%</sub> values of Kennedy et al., 1996a).

<sup>d</sup> Five eggs per pool.

n.d. = not detected.

combined with moderate to high PCB concentrations) persisted for yolk sacs and eggs collected in 1996 (Table 3) (Hart, 1998). Thus, the pattern of contaminant exposure in common terns nesting at Bird Island has remained relatively constant at least over the period from 1994 to 1996, including years in which a high incidence of ovotestes in pipping male terns has been documented (Hart, 1998).

Mean levels of total PCBs in common tern eggs in this study were 6.5 µg/g wet weight in 1995 and 1.3 µg/g wet weight in 1996. The mean PCB level of Bird Island eggs in 1995 was higher than those in most samples of common tern eggs collected from contaminated sites in North America (Nisbet, 2002), and Europe (Becker et al., 1998) in the period 1988–1998, being exceeded only by samples from Hamilton Harbor, Canada (Weseloh et al., 1995), Green Bay, USA (Ankley et al., 1993), and several sites in The Netherlands (inferred from the data of Bosveld et al., 1995). Thus, some common terns at Bird Island in 1995 were highly contaminated relative to those in other areas at the same period. However, much higher levels of PCBs had been reported in common terns collected prior to 1988 in industrialized areas of North America (Nisbet, 2002), including Bird Island and nearby Ram Island (Nisbet and Reynolds, 1984), and only one of 10 eggs collected at Bird Island in 1995 was within the range of total PCBs or TCDD-EQs associated with adverse effects on reproduction

(Nisbet, 2002). Levels of total DDT (including DDE) and other organochlorines in common tern eggs at Bird Island in 1995–1996 were much lower than those reported in other areas (Nisbet, 2002) and far below any reported as associated with adverse effects (Nisbet, 2002).

#### *Gonadal histopathology: relationship to developmental stage and contaminant exposure*

The male pre fledglings sampled in this study showed no evidence of an ovarian cortical area like that found in female pre fledglings of the same age or observed in pipping male embryos in 1993 (Nisbet et al., 1996, 1994; Hart, 1998). In addition, no evidence of oviducts was found in these male pre fledglings (see below). Thus, the results of this study do not support the hypothesis that the ovotestes described earlier in pipping tern embryos from this site persist through the pre fledgling period. A more definitive test of this hypothesis would require sampling of embryos and pre fledglings from the same nests, which was not done in this study.

Although ovotestes were not observed in any of the birds sampled, approximately half of the male gonads exhibited some minor irregularities. However, none of these abnormalities appeared ovarian in nature. It is possible that some of the histological abnormalities observed in the male pre fledglings might represent remnants of the ovarian cortical areas found in the

embryos (Nisbet et al., 1996; Hart, 1998). For example, the observed quiescent intracapsular nodules in several males and the single intracapsular primordial germ cell could indicate regressing cortical areas of development. The intracapsular nodules in the common terns looked similar to areas identified as regressing cortical areas on the testes of male North American hawks (Stanley, 1937). In addition, cortical areas formed in estrogen-dosed herring gulls (*Larus argentatus*) were described as “transforming into testicular nodules that rose like blisters on the surface of an otherwise normal testis, and microscopically were almost completely enclosed in capsules formed by the fibrous tunica albuginea, but with narrow tubular connections with the main part of the testes” (Boss and Witschi, 1947). Although the intracapsular areas on the common tern testes could not be seen macroscopically, their microscopic appearance is similar to that of testicular nodules that were thought to be regressing cortical areas in the herring gulls. The sample with an extracapsular area of thickened cells also could represent a regressing cortical area, although it is common to see cortical areas of varying thickness among normal males at hatching; therefore regression of this area may not be a good indication of feminization when primordial germ cells are lacking (Fry et al., 1987). Additional time points between the hatching and prefledgling stage might clarify whether these features are related to regression of ovarian cortical areas. Nevertheless, it is clear from the prefledgling observations that the ovarian tissue seen in hatchling male tern embryos does not persist unchanged or continue to develop in a typical ovarian manner in the prefledglings.

None of the abnormalities observed in the male prefledglings would be likely to affect the reproductive function of the testis. (One possible exception is the prefledgling with the disorganized seminiferous tubules and primordial germ cells outside the seminiferous tubules; this bird does not have corresponding egg contaminant data.) The intracapsular nodules of seminiferous tubules/testicular tissue found in some prefledglings appeared to be either quiescent or a typical seminiferous tubule, and likely of little consequence to testicular function. One of the adults examined contained a seminiferous tubule surrounded by tunica albuginea that appeared very similar to the intracapsular nodules found in the prefledglings. This could suggest that the nodules in the prefledglings might be misplaced seminiferous tubules, which in

some cases can continue to develop into a functioning seminiferous tubule. Consistent with the prefledgling results, the adults examined showed no indication of abnormal development, although this might have been expected since they were collected at a breeding colony.

Among the abnormalities observed histologically in the testes of prefledglings, there was no apparent relationship to contaminants. The presence of intracapsular nodules and the abnormal seminiferous tubule found among eight male prefledglings was unrelated to contaminant levels (Table 2). Ovotestes were not found among the eight males with contaminant data from matched eggs, nor in the other 11 male prefledglings for which contaminant data were not available. It may be informative that the prefledgling with the greatest potential exposure to PCBs (as indicated by the high concentration of PCBs in its matched egg) had normal testicular histology. This is in contrast to the data from embryos obtained in 1994, in which the birds with highest PCB exposure (as indicated by PCB concentrations in yolk sacs) all exhibited some degree of ovotestes (Hart, 1998).

#### *Absence of ovotestes in prefledglings: implications for causality*

The absence of ovotestes in prefledgling male terns in 1995 contrasts with the high incidence of ovotestes (60–90%) in pipping embryos collected in 1993 (Nisbet et al., 1996) and 1994 (Hart, 1998). We suggest three possible explanations.

*Ovotestes did not occur in common terns at Bird Island in 1995.* Since only common tern embryos were collected in 1993 and 1994, and only prefledglings in 1995, it is possible that ovotestes were not present in 1995 embryos. However, the embryos showed similar, high incidences of ovotesticular development in 1993 and 1994 (Nisbet et al., 1996; Hart, 1998), as well as in 1996 (authors' unpublished data). In addition, the concentrations of PCBs in yolk sacs in 1995 were similar to those in yolks in 1994 and 1996, and PCB concentrations in yolks in 1996 were at least as high as those in 1994 (Table 3). Thus, contaminant exposure and gonadal histology in embryos appear to have been relatively constant during the time period from 1993 to 1996. Furthermore, the female-biased sex ratio among

roseate terns at Bird Island, which originally prompted this study (see Introduction), has remained at similar levels through at least 2001 (authors' unpublished data). It thus appears unlikely that ovotestes were absent in 1995 Bird Island tern embryos.

*The presence of ovotestes in embryos is caused by exposure to high levels of organochlorine or other contaminants, involving either (a) disrupted hormonal regulation of gonad development or (b) a general delay in development, but the effects are transient.*

(a) The presence of ovotestes at hatch might result from contaminant exposure by alteration of the hormonal control of gonadal differentiation. This could occur at many levels, including the level of hormone receptors. Persistence of ovotestes has been observed in estrogen dosing studies where reproductive success was affected. Adult chickens, dosed with estrogen during embryonic development, showed abnormal copulatory behavior and infertile copulations at two years of age, and also possessed ovotestes when examined (Domm, 1939, 1940; Domm and Davis, 1941). In another study, chickens feminized by treatment with exogenous hormone showed reversion of secondary sex characteristics back to the male form, but ovotestes were still present when examined at nine months (Snedecor, 1949). Other studies with chickens exposed to estrogenic chemicals suggest that male ovotestes may begin reversion to testes shortly after hatching, with complete reversion to testes occurring anywhere from a few weeks to nine months posthatch (Snedecor, 1949; Pincus and Erickson, 1962; Taber, 1964; Haffen et al., 1975; Scheib, 1983). In other avian species, estrogen-induced ovotestes tend to persist longer than in chickens. These include quail (*Coturnix japonica*) (Haffen et al., 1975), ring dove (*Streptopelia risora*) (Riddle and Dunham, 1942), herring gull (Boss and Witschi, 1947), and turkey (*Meleagris gallopavo*) (Taber, 1964), although ovotestes in quail and doves eventually tend to revert back to testes. Evidence from a variety of hormone-dosed bird species suggests that induced ovotestes may persist until the prefledgling stage, although reversion back to testes may occur earlier as indicated by some individuals in most species examined. If contaminants are acting as hormones, ovotestes might regress after hatching because of (i) reduced exposure or (ii) decreased sensitivity to hormonal effects after hatching.

(b) Another possible role of contaminants might be to cause a non-specific delay in embryonic development. It is known that during avian testicular differentiation some degree of ovarian cortical development often occurs; the ovarian tissue normally regresses before hatching. This has been observed in many developing bird species, including Arctic terns (*Sterna paradisaea*) (Hoffman, 1892; as cited in Swift, 1916). The presence of PCBs and TCDD-EQs has been associated with delayed hatching and development in some bird populations (Kubiak et al., 1989; Murk et al., 1996). Therefore, these contaminants might lead to delayed gonadal development as well, resulting in delayed regression of ovotesticular tissue and prolongation of the normal developmental stage in which testes of male birds may possess ovarian cortical-like areas. Thus, the presence of ovotestes could indicate a non-specific developmental delay, which may resolve by fledging.

*The presence of ovotestes at hatch could be a normal part of gonad development in terns.* The lack of persistence of ovotesticular tissue in prefledgling common terns suggests the possibility that the presence of ovotestes in common terns at hatching could be a normal occurrence, either unrelated to contaminants or enhanced by high-level contaminant exposure.

The presence of ovarian cortical tissue in testes at hatching varies among avian species. During embryonic differentiation of the male gonad into a testis, there is a time period where a potential ovarian cortex exists. This tissue usually persists for a short time and may show areas of ovarian cortical development and may contain primordial germ cells. The presence of this cortical area during development of the testes was first observed in the chicken in 1886 (Laulanie, 1886), and was also observed in Arctic terns by Hoffman (1892), as cited in Swift (1916). In chickens this cortical area normally has regressed completely by the time of hatching (Romanoff, 1960; Berg et al., 2001). Thus, many species including chickens, gulls, and pigeons, do not normally exhibit cortical tissue on the testes when examined at hatch (Lahr and Riddle, 1945; Boss and Witschi, 1947; Romanoff, 1960; Fry et al., 1987; Berg et al., 2001).

However, other birds, including quail, doves, ducks, and hawks, are known to have late-persisting areas of ovarian-like cortical tissue that still may be present at hatching (Stanley, 1937; Riddle and



Dunham, 1942; Lahr and Riddle, 1945; Lewis, 1946; Boss and Witschi, 1947; Romanoff, 1960; Haffen et al., 1975; Berg et al., 2001). Ovotestes found in birds at hatch often do not persist for more than a few days to weeks, although in some instances they may persist for many months. Male white pekin duck embryos show a cortical area on the testes, which often persists until hatching and then becomes less prominent by day two posthatch; remnants of the cortical tissue may persist until approximately 30 days posthatch (Lewis, 1946). North American hawks have very persistent traces of cortical tissue on their testes, which may still be observed four months after hatching (Stanley, 1937). Quail testes also may show areas of cortical tissue that persist and proliferate until hatching, and may remain visible in early posthatching. Groups of meiotic primordial germ cells are present in these persistent cortical areas of the quail testes, and are beginning maturation comparable to oocytes in the cortical layer of the ovary at the same age (Haffen et al., 1975). In 76% of hatching male ring doves, testes exhibited traces of ovarian cortex; this ovarian tissue normally did not persist past four days posthatch, and only rarely persisted beyond two weeks posthatch (Riddle and Dunham, 1942). Thus, the terns in this and the previous studies (Nisbet et al., 1996; Hart, 1998) appear to resemble ring doves in the incidence and persistence of ovotestes.

The possibility that the presence of ovotestes at hatching in common tern embryos is normal is also supported by the lack of oviduct development observed in any male common tern prefledglings. In embryos from eggs dosed with estrogenic contaminants or hormones, some oviduct development generally is observed, particularly when ovotestes of intermediate severity are present (Kozelka and Gallagher, 1934; Willier et al., 1937; Gaarenstroom, 1939; Snedecor, 1949; Pincus and Erickson, 1962; Berg et al., 1998; Berg et al., 1999; Berg et al., 2001). Although ovotestes may regress in hormone treated birds, oviduct development does not tend to regress, and would be expected to be present at the prefledgling stage. Thus, if the presence of ovotestes was related to hormonal dysfunction due to contaminant exposure, we might have expected to see some oviduct development in the prefledglings. However, if ovotestes are normal at hatch or are related to delayed development resulting from contaminant exposure, oviduct development would not be expected. The lack of oviducts observed in prefledglings supports the possibility that ovotestes

presence at hatching is normal in common terns, or that contaminants are delaying development in common tern embryos.

Based on the present findings and previous data comparing the presence of ovotestes to contaminant levels in yolk sacs (Hart, 1998), we currently favor the idea that ovotestes occur naturally in some individual common terns at hatching (explanation 3) but that the frequency of occurrence may be enhanced by exposure to chlorinated organic contaminants such as PCBs (explanation 2).

#### *Summary and conclusions*

The goal of this study was to determine if the presence of ovarian cortical tissue observed in the testes of male common terns at hatching persisted into the prefledgling stage. With this information we hoped to predict if ovotesticular development was likely to affect future reproduction in common terns. We also used this information to draw inferences about whether the occurrence of ovotestes is related to contaminant exposure or whether, alternatively, ovotestes are normally present in common terns at hatching.

The apparent regression of ovarian cortical areas on common tern testes by the prefledgling stage indicated that ovotestes were not likely to directly affect reproduction in common terns. With the exception of one male, none of the non-ovarian testicular irregularities observed were judged likely to affect reproduction.

There was no relationship between testicular abnormalities and the concentrations of PCBs and organochlorine pesticides. However, a possible role of contaminants in the development of ovotestes cannot yet be dismissed. A relationship to other contaminants could exist, or modifying factors such as biotransformation could have masked a relationship between ovotestes and the organochlorine compounds we measured. The possibility that contaminants are acting to alter hormonal control of gonad development seems unlikely because of the rapid regression of ovotestes and lack of oviduct development. It is perhaps more plausible that the contaminants could be acting to delay development and, thus, prolong the developmental stage when ovarian tissue is normally present on the testes. In this case, oviduct development would not be expected, and ovotestes would be expected to regress after hatching, as was observed.

Also consistent with the observations on prefledglings (this study) and embryos (Hart, 1998 and recent unpublished observations) is the possibility that the occurrence of ovotestes in this species is normal. The incidence of ovotesticular development appears to change from very high (60–90%) in hatching common tern embryos to non-existent in prefledglings, in approximately three weeks. This is similar to other avian species such as ring doves, in which ovotesticular tissue is normally present at hatching. Although our data are consistent with the possibility that ovotestes development in common terns is normal, it is also possible that contaminants could increase the incidence of ovotestes at hatch or delay the regression of ovotesticular tissue during the early posthatch period. Such a scenario is suggested by comparisons of the incidence and severity of ovotestes at sites with different levels of PCB contamination (Hart, 1998). In either case, the results presented here suggest that the presence of ovotestes in common tern embryos from PCB-contaminated sites such as Bird Island does not lead to permanent alterations in gonadal histology that would be expected to impair reproductive function.

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## Queries

- 1 Please update reference Brunstrom *et al.* (2002)

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