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Source: Journal of Wildlife Diseases, 30(2) : 222-233

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-30.2.222

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*Journal of Wildlife Diseases*, 30(2), 1994, pp. 222–233

# IMPAIRMENT OF GROWTH AND IMMUNE FUNCTION OF AVOCET CHICKS FROM SITES WITH ELEVATED SELENIUM, ARSENIC, AND BORON

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ABSTRACT: Avocets (*Recurvirostra americana*) hatched from eggs collected from the south Central Valley of California (USA) were studied to determine the impact of elevated concentrations of selenium, arsenic, and boron on the immune system and growth to maturity. Corcoran ponds were the reference site with low selenium (1.2 ppb) and arsenic (29 ppb) (boron not measured). Westfarmers Pond had elevated concentrations of selenium (319 ppb), arsenic (127 ppb), and boron (109 ppm). Pryse ponds also had elevated selenium, arsenic, and boron concentrations (13.9 ppb, 1,100 ppb, and 29.4 ppm, respectively). Size at hatch was significantly reduced ($P \leq 0.05$) in birds from Westfarmers and Pryse ponds. The growth rate was faster, but mean adult size was reduced in birds from Pryse ponds. Avocet chicks from Pryse and Westfarmers ponds exposed solely through *in ovo* transfer of these elements had significantly increased heterophil : lymphocyte ratios. The phagocytic activity of macrophages also was significantly reduced in these birds, and Pryse Pond birds had an increased proliferative ability of lymphocytes in the presence of concanavalin A, a T-cell mitogen. Avocet chicks (≤5 wk old) were captured from the various ponds and the same morphometric and immune function measurements made. The birds that were most severely impacted by exposure to these compounds were those that were collected from Pryse ponds.

*Key words:* Avocet, *Recurvirostra americana*, selenium, arsenic, boron, immunotoxicity, morphometrics.

## INTRODUCTION

The Tulare Basin in the southern Central Valley of California (USA) has elevated concentrations of selenium, arsenic, and boron in surface waters due to agricultural practices that collect drain water in low-lying areas. These contaminated, naturally occurring and man-made wetlands attract large numbers of waterfowl and shorebirds for nesting and brood-rearing. Through bioaccumulation and biotransformation processes in plant and invertebrate food sources, birds are exposed to chemical concentrations high enough to cause severe reproductive dysfunction (Ohlendorf et al., 1986a, b; Ohlendorf, 1989). Hatchability of eggs has been depressed as compared to reference sites, primarily due to teratogenic effects leading to early embryonic mortality (Hoffman et al., 1988). Furthermore, chicks that do hatch continue to be exposed to the compounds as they resorb their yolk sac. Chicks that hatch successfully may fail to reach maturity, further decreasing the productivity of the populations of nesting birds in the region.

Questions have been raised about whether immune suppression might be a contributing factor to the continuing occurrence of avian cholera epornitics (Friend, 1989; Fairbrother, 1993). Resident and migratory birds in the California Central Valley appear highly susceptible to this disease and have experienced repeated epornitics since 1944 (Wobeser, 1981). Fairbrother and Fowles (1990) found that mallards (*Anas platyrhynchos*) exposed to 2.2 parts per million (ppm) aqueous selenium experienced dysfunction in the cellular immune response; their delayed hypersensitivity response was suppressed. Whiteley and Yuill (1989) found



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an increase in duck hepatitis virus-induced mortality in mallard chicks hatched from eggs of ducks exposed to streams contaminated with 30 parts per billion (ppb) of selenium as compared to non-selenium exposed controls. Others also have observed immunomodulatory effects of excessive selenium ingestion (Koller, 1980; Kirmeidjian-Schumacher and Stotzky, 1987). Arsenic increases susceptibility of mice to viral infection (Sharma, 1981). There is no information on the potential immunotoxicity of boron.

Our objective was to determine the immune function and growth to maturity of avocet (*Recurvirostra americana*) chicks naturally exposed *in ovo* to elevated concentrations of selenium, boron, or arsenic. This study was part of a long-term effort to examine the possibility that chemical-induced immune system dysfunction is a contributing cause to the high mortality of avian wildlife from infectious diseases.

## MATERIALS AND METHODS

For this study, several agricultural drainwater evaporation ponds in the Central Valley of California were selected from those previously determined to have low (10 to 25 ppb in the water column) and high (100 to 700 ppb) selenium contamination. A reference site was also chosen (<2 ppb). Westfarmers Pond in Kern County (36°10′N, 119°00′W) was the high selenium site, Pryse Pond in Tulare County (35°30′N, 119°00′W) was the medium site, and the reference site was several wastewater settling ponds on the southeast border of the town of Corcoran in Kings County (36°10′N, 119°35′W).

Two avocet eggs from each nest found at the ponds were collected within 5 days of laying in May and June, 1991, packed in an insulated chest warmed with a chemical warm-pack, and shipped by automobile in about 12 hr to laboratory facilities in Corvallis, Oregon (USA). Eggs were candled upon arrival to determine fertility and examined for cracks. Eggs were incubated until hatching in Georgia Quail Farm (Georgia Quail Farm, Inc., Savannah, Georgia, USA) style redwood incubators at 38 C, 80% relative humidity, and a turning frequency of once every 2 hr. Eggs from contaminated areas were placed in a separate incubator from eggs from the reference site. Eggs were candled daily for viability. Cracked eggs and eggs determined to have nonviable embryos were opened and the contents frozen at −70 C until residue analyses were done. Nests from which two viable eggs were collected had one of the eggs opened at 15 days incubation and the contents saved for residue analysis.

Percent hatchability was documented. Newly hatched chicks were moved from the hatcher to brooders (Georgia Quail Farm, Inc.) within 24 hr of hatch. All birds were individually numbered using patagium tags (National Band and Tag Co., Newport, Kentucky, USA). Survival to 2 wk was recorded as were all observed malformations. Eggs that failed to hatch were opened and the chicks examined for malformations. Brooder temperatures were maintained at 38 C for 1 wk and then stepped down 2 C every 5 days. At 2 wk of age, birds were placed in a wooden pen with a sand covered floor until 4 wk old, at which time they were allowed free-run of a 3.5 m × 5.25 m animal room. Food and reverse osmosis, deionized drinking water were provided *ad libitum*. Avocet diets were formulated from high protein dog food (Hi-Pro Glow®, Ralston-Purina, St. Louis, Missouri, USA) supplemented with tuna, grated carrots, cooked egg yolk, and a vitamin B-complex formulation (Interstate Drug Exchange, Plainview, New York, USA).

Chicks were weighed (grams) and measurements of culmen, wing, and tarsus (mm) (Meyers et al., 1992) were taken every 3 days until birds were 6 wk old and every week thereafter until 16 wk old. Chicks that died during the study and all birds euthanized at 16 wk of age were necropsied; muscle and liver tissues were stored at −70 C for residue analysis.

When each chick reached 3 wk of age immune function testing began. Blood samples (≤1% body weight) were drawn by venipuncture of the right jugular vein once each week. Approximately 0.2 ml of blood was put into an evacuated glass blood collection tube containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant (Vacutainer®, Becton-Dickson, Inc., Rutherford, New Jersey, USA) from which thin film blood smears were made and hematocrits determined. Concentration of plasma proteins was determined from the plasma portion of the hematocrit tube following centrifugation. The remainder of each blood sample was put into a polypropylene tube containing 1.0 ml of tissue culture medium RPMI-1640 plus 10% heat-inactivated fetal calf serum and antibiotics (Gibco, Long Island, New York). Because blood sample collection was limited to a maximum of 1% of body weight, there was insufficient blood to perform all the assays from a single sample. Therefore, only the following immune function assays were conducted on every sample: total and differential white blood cell count; hemat-

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ocrit; hemoglobin concentration; plasma protein concentration; and rabbit red blood cell hemagglutination titer. Macrophage phagocytosis indices were determined on samples collected from birds aged 3 and 4 wk (chicks) and 10 and 12 wk (juvenile birds). Lymphocyte blastogenesis assays were performed on samples from birds aged 9 and 12 wk. Conducting tests in this order provided the maximum amount of information concerning the health status of the birds (white blood cell counts, hematocrit, hemoglobin, and plasma protein), humoral immunity (hemagglutination titer), nonspecific immunity (macrophage function), and cell mediated immune functions (blastogenesis). The order was based on prior knowledge of the blood volume required and type of information generated by each assay. Birds were killed by carbon dioxide inhalation at 16 wk of age and liver, kidney, and muscle tissue removed for residue analysis. Tissues were frozen in plastic bags at −70 C until analyzed.

In addition to laboratory-reared birds, avocet chicks ≤5 wk old were captured from the various ponds and morphometric measurements taken of body weight, culmen length, wing length, and tarsus length. Blood samples were kept on wet ice at approximately 4 C and shipped on the same day as they were collected by overnight mail service to Corvallis, Oregon.

#### Immune function assays

Hematocrits were determined by centrifugation of microhematocrit tubes at 1,000 × *g* for 5 min (Schalm and Jain, 1986).

The concentration of hemoglobin in the serum was determined spectrophotometrically by converting the hemoglobin to oxyhemoglobin with ammonium hydroxide and measuring the resultant absorbance at 545 nm, using a commercial kit (Cambridge Instruments, Inc., Buffalo, New York). Whole blood was hemolyzed by placing one drop on the hemolysis stick, mixed for 45 sec, and final concentrations (g/dl) determined in the hemoglobin meter provided.

The concentration of plasma proteins was determined by placing a drop of plasma from the centrifuged microhematocrit tube on the viewing platform of a refractometer and reading the concentration on the eyepiece scale (Schalm and Jain, 1986). The refractometer was calibrated using distilled, deionized water.

White cells were differentiated on thin-film blood smears stained with Diff-Quick (Sigma Chemical Co., St. Louis, Missouri) and classified as heterophil, lymphocyte, eosinophil, basophil, or monocyte according to criteria specified by Dein (1984). One hundred white blood cells were counted per slide to determine the percentage of each cell type. Total white blood cell counts were determined using the eosinophil estimation method (Dein, 1984). Whole blood was mixed with phloxine diluent in the Unopette kit (Becton-Dickinson, Rutherford, New Jersey) and the resulting solution used to charge a Neubauer hemacytometer (American Optical Instrument Co., Buffalo, New York). The number of heterophils and eosinophils (orange-pink, refractile granules) in two chambers were counted, 10% of the count was added, and the total multiplied by $18 \times 10^4$ to get the total number of heterophils and eosinophils per ml. This was divided by the percent heterophils plus eosinophils determined from the thin film smears to generate a total white blood cell count.

Resting serum antibody titers to rabbit red blood cells (RRBC) were determined on birds that had no previous exposure to this antigen (Miller et al., 1989). Chickens have natural antibodies to RRBC (Kassar et al., 1990), and avocets also showed a strong innate response to RRBC in a preliminary study. Serum was separated from whole blood by centrifugation at 3,000 × *g* and total and 2-mecaptoethanol resistant (IgM and IgY, respectively) antibodies to RRBC were measured by microhemagglutination following the methods of Fairbrother and Fowles (1990). All samples were evaluated in duplicate.

The ability of macrophages to engulf polystyrene beads was examined using adhered blood monocytes following the procedures of Fowles et al. (1993). Briefly, leukocytes were separated from whole blood by centrifugation and adhered to tissue-culture-treated polystyrene plates for 24 hr. Adherent cells were incubated an additional 24 hr at 41 C in 5% $CO_2$. Polystyrene microspheres (2 μm) conjugated to fluorescein isothiocyanate (FITC) (Polysciences, Inc., Warrington, Virginia, USA) were added to the media in a ratio of beads : adherent cells of 20:1, with 100 cells counted visually using a fluorescence microscope (Nikon, Inc., Garden City, New Jersey). The number of cells containing 1, 2, 3, 4, or ≥5 beads per cell were recorded.

The ability of lymphocytes to blast in the presence of concanavalin A (Con-A; Sigma Chemical Co.), a T-cell mitogen, was measured in whole blood samples. Concanavalin A was added to 96-well microtiter plates at 5, 10, and 15 μg concentrations. Whole blood was diluted 1:20 in RPMI 1640 (without calf serum, but with antibiotics) and 100 μl was dispensed into each of two wells per Con-A dilution. Nonstimulated and background control wells were included on each plate. Plates were incubated for 60 hr at 41 C in 5% $CO_2$ after which time 25 μl of $^3$H-thymidine (20 microcuries (μCi) per ml) (ICN Biomedical, Irvine, California) was added to each well and the plates incubated for an additional

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12 hr. Cells were harvested onto filter discs using a Brandel cell harvester (Brandel, Gaithersburg, Maryland, USA) following five washes with distilled water. The discs were air dried for 3 hr, placed into scintillation vials with Ecolume scintillation fluid (ICN Biomedical), and counted on a TriCarb 2000 beta scintillation counter (Packard Instrument Co., Downers Grove, Illinois, USA). The blastogenesis stimulation index was derived by dividing the mean disintegrations per minute (dpm) for triplicate mitogen-stimulated wells by the mean dpm for unstimulated wells.

#### Residue analysis

Water samples from Westfarmers and Pryse ponds were collected in glass jars with plastic lids and returned to Corvallis for analysis of inorganic constituents. The U.S. Fish and Wildlife Service provided data for concentration of inorganics in Corcoran ponds and in another similar, nearby reference site (Wilbur ponds). Selenium concentrations in pond water, laboratory diet, and tissue (liver, kidney, egg contents, and whole embryos) were determined using an automated fluorimetric procedure (Brown and Watkinson, 1977) by the laboratory of Dr. P. Whanger, Department of Agricultural Chemistry, Oregon State University, Corvallis, Oregon. Samples were taken in duplicate from frozen tissues, dried overnight in an 80 C oven, and acid digested (Schilt, 1979). Egg contents and embryos were weighed and homogenized in an Omni Mixer (VerTis Co., Gardiner, New York) and duplicate aliquots of homogenate were dried and digested as above. Arsenic and boron concentrations in water and tissue samples were determined by inductively coupled plasma (ICP 3580) atomic emission spectrometry (Fisons, Valencia, California) following the methods of McQuaker et al. (1979). Inductively coupled plasma spectrometry analysis also provided values for water and tissue concentrations of calcium, magnesium, potassium, sodium, sulphur, phosphorus, silicon, iron, magnesium, zinc, aluminum, copper, cadmium, chromium, nickel, lead, molybdenum, tin, titanium, and antimony. Every tenth sample was run in duplicate and a reference standard (bovine layer) and blank were run. Duplicates and standards were required to be within 5% of each other and the expected value, respectively. Quail (*Colinus virginianus*) egg homogenates spiked with a known amount of selenium also were analyzed. Lower limits of detection were 1 ppb for selenium, 15 ppb for arsenic, and 5 ppb for boron.

#### Data analysis

Comparisons were made among collection sites for the hatchability, growth rate, and immunological parameters for birds reared in the laboratory and between laboratory and field collected samples. With the exception of macrophage phagocytosis and differential lymphocyte counts, data from immune function assays were analyzed for differences among collection sites, age classes (chicks were <7 wk old and juvenile birds were ≥7 wk old), and laboratory or field conditions by analysis of variance (ANOVA); *a posteriori* separation of means was determined using Bonferroni's *t*-test (SAS Institute, Inc., 1985). Rabbit red blood cell titers were transformed to $\log_{10}$ to standardize variance and geometric mean titers calculated for comparisons. Macrophage phagocytosis and white blood cell differential counts were analyzed for differences due to collection sites, age classes, and laboratory or field conditions using maximum likelihood estimation (McCullagh and Nelder, 1983) and the software program GLIM (Numerical Algorithms Group, 1987) to fit a log-linear model to the data. Because methods for *a posteriori* separation of means are not defined for maximum likelihood estimation, examination of a table of estimated numbers (based on the statistical model) of each cell type was done to assess how the significant effects were generated. Hatchability data (% hatched) were compared by Chi-squared analysis of several binomial populations (Ostle and Mensing, 1979). For all tests, comparisons with $P \leq 0.05$ were considered significantly different.

Growth curves for weight, tarsus length, culmen length, and wing length were fit using NONLIN in SYSTAT (Wilkinson, 1989). Four potential nonlinear models were considered. The logistic and Gompertz models (three-parameter equations) and the Weibull and Richard's equations (four-parameter equations) were fit to the data and the model with the smallest mean square error was used. In all cases, the four-parameter models did not fit the data well. Both three-parameter equations were similar in fit, although in the majority of cases the logistic model had the smallest mean square error. In addition, the logistic equation is known to have less intrinsic and parameter-effect curvature compared to the Gompertz equation (Ratkowsky, 1983). Only the results for the logistic equation are presented in the paper. The equation (Ratkowsky, 1983) used was:

$$\text{character} = A/[1 + \exp(B - (G \times \text{time}))]$$

where character = weight, tarsus length, culmen length, or wing length and time = day after hatching. The parameter A is the asymptote, G is the growth rate, and B is related to the value of the measurement at time = 0. The parameters (A, B, G) were used separately and together to compare the three groups of avocets with the

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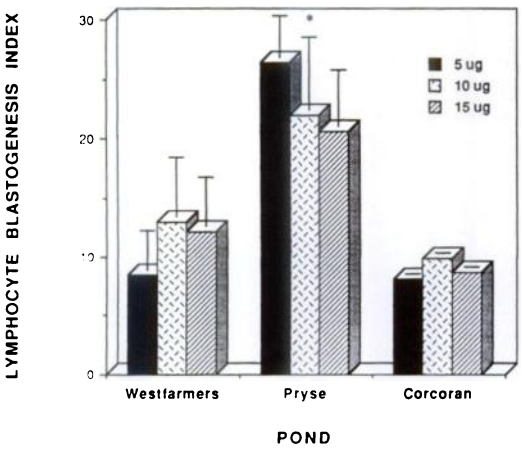



FIGURE 1. Lymphocyte blastogenesis index (mean ± SE) from avocets hatched from eggs collected at two contaminated sites (Westfarmers Pond, $n = 5$; Pryse Pond, $n = 2$) and one reference site (Corcoran Ponds, $n = 1$). The index is shown for three concentrations of mitogen (Concanavalin A) after 60 hr incubation at 41 C followed by a 12 hr pulse with 0.5 $\mu$Ci $^3$H-thymidine. A star designates those that are significantly ($P \leq 0.05$) different from the reference site data.

multi-response permutation procedure (Mielke, 1991). This procedure is a nonparametric randomization procedure based on Euclidean distances. The computer program used to run the test is given in Berry and Mielke (1983).

## RESULTS

Even though chosen for its low selenium concentration (1.2 ppb), the Corcoran ponds also were the lowest in concentration for arsenic (29 ppb); boron concentration was not determined. Corcoran was considered the reference site. Pryse Pond had moderate selenium (120 ppb) and boron (29 ppm) concentrations and a very high arsenic (1,100 ppb) concentration. Westfarmers Pond had extremely high selenium (450 ppb), high boron (109 ppm), and moderate arsenic (127 ppb) concentrations. All of the other elements measured were present at low concentrations (Table 1). Laboratory diets contained 1.1 ppm (dry weight) selenium, 1.9 ppb (dry weight) arsenic, and 12.8 ppm (dry weight) boron.

Macrophage phagocytosis of polystyrene beads was significantly different among ponds and between age classes (Table 2). There were no significant differences between laboratory- and field-reared birds. Chicks from the reference site (Corcoran Pond) had a smaller percentage of cells that phagocytosed at least five beads as compared to juveniles from the same pond, with a concomitant increase in the percentage of cells that phagocytosed one to four beads. Chicks and juvenile birds from Westfarmers and Pryse ponds had lower percentages of cells that phagocytosed at least five beads as compared to chicks and juveniles from the reference site; this shift from ingestion of at least five

TABLE 1. Concentrations (ppm) of inorganic compounds in agricultural drainwater from south Central Valley, California. Corcoran and Wilbur Ponds were located near each other and considered reference sites, although no eggs or chicks were collected from Wilbur Ponds. Westfarmers and Pryse Ponds were considered contaminated sites.

| Compound | Pond | | | |
|---|---|---|---|---|
| | Corcoran | Wilbur | Westfarmers | Pryse |
| Calcium | —[a] | 24.2 | 421.0 | 122.6 |
| Magnesium | — | 25.6 | ND[b] | ND |
| Potassium | — | 8.9 | 31.8 | 69.7 |
| Sodium | — | 272.8 | ND | ND |
| Sulfur | — | 98.8 | ND | ND |
| Phosphorus | — | 0.1 | 5.2 | 3.3 |
| Silicon | — | 1.3 | 1.1 | 1.3 |
| Iron | — | ND | 0.1 | 0.1 |
| Manganese | — | ND | 0.01 | 0.01 |
| Zinc | 0.02 | 0.01 | 0.02 | 0.02 |
| Aluminum | — | ND | ND | ND |
| Boron | — | 1.0 | 109.2[c] | 29.4[c] |
| Copper | ND | 0.03 | 0.02 | ND |
| Cadmium | — | ND | ND | ND |
| Chromium | ND | ND | 0.09 | 0.12 |
| Nickel | ND | ND | ND | ND |
| Arsenic | 0.03 | 0.05 | 0.13[c] | 1.10[c] |
| Antimony | ND | ND | ND | ND |
| Lead | ND | ND | ND | ND |
| Molybdenum | 0.03 | 0.04 | 1.8 | 3.1 |
| Titanium | — | ND | ND | ND |
| Tin | — | ND | 0.11 | 0.18 |
| Strontium | — | 0.05 | 21.2 | 2.3 |
| Selenium | 0.012 | ND | 0.45[c] | 0.12[c] |

[a] Not measured.
[b] Nondetectable (below detection limits).
[c] Denotes concentrations above levels known to have adverse physiological effects in livestock and poultry.

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TABLE 2. Phagocytic index of peripheral macrophages from wild and laboratory-reared avocets hatched from eggs collected at two contaminated sites (Westfarmers and Pryse Ponds) and one reference site (Corcoran Ponds). The index is the percent of 100 cells counted that ingested 0, 1 to 4, or ≥5 particles. Values given are mean ± SE.[a]

| | Number of samples | Phagocytic index | | |
|---|---|---|---|---|
| | | 0 | 1 to 4 | ≥5 |
| Corcoran | | | | |
| Chicks | 3 | 23.3 ± 6.0 | 67.0 ± 5.3 | 18.7 ± 10.3 |
| Juveniles | 2 | 44.0 ± 8.0 | 28.0 ± 13.5 | 23.0 ± 17.0 |
| Westfarmers | | | | |
| Chicks | 3 | 31.3 ± 12.3 | 67.3 ± 13.0 | 2.5 ± 0.5 |
| Juveniles | 10 | 33.4 ± 9.1 | 57.9 ± 7.8 | 20.7 ± 6.0 |
| Pryse | | | | |
| Chicks | 2 | 9.5 ± 2.5 | 89.5 ± 2.5 | 1.0 ± 0.0 |
| Juveniles | 2 | 46.5 ± 3.5 | 47.5 ± 6.5 | 6.0 ± 3.0 |

[a] Significant effects: Pond × index $P = 0.039$; Pond × age $P = 0.003$.

beads towards ingestion of only one to four beads was the greatest in Pryse birds.

Blastogenesis of lymphocytes in the presence of Con-A was increased in birds from Pryse ponds as compared to Westfarmers or Corcoran Ponds (Fig. 1). There was no significant difference among collection sites in resting anti-rabbit red blood cell antibody titers, serum protein, hemoglobin concentration, or hematocrits for either laboratory-reared birds or samples collected from free-ranging birds (Table 3). Hematocrits were elevated in free-ranging birds as compared to laboratory-reared birds from the same ponds, while hemoglobin values were lower in free-ranging birds (Table 3). Plasma protein values did not differ significantly between laboratory-reared birds and free-ranging birds.

Total white blood cell (WBC) counts did not differ significantly between field-collected birds and laboratory-raised birds (Table 4). Juvenile birds (pooled samples from lab and field birds) had significantly ($P = 0.02$) higher mean (±SE) counts than chicks (juvenile = $3.1 \times 10^3 \pm 0.56$, $n = 15$; chicks = $2.0 \times 10^3 \pm 0.24$, $n = 41$; $t = 2.01$). Total WBC counts were not statistically different among ponds. No samples were collected from free-ranging birds at the reference site, due to unsuccessful capturing attempts. Differential WBC counts differed significantly ($P < 0.05$)

TABLE 3. Blood chemistry parameters and resting antibody titers to rabbit red blood cells in wild and laboratory-reared avocet chicks collected from sites contaminated with agricultural drainwater (mean ± SE).

| Pond | Number of samples | Hematocrit (%) | Serum protein (g/dl) | Hemoglobin (g/dl) | Antibody[a] titer |
|---|---|---|---|---|---|
| Westfarmers | | | | | |
| Lab | 21 | 38 ± 5.8 | 4.1 ± 0.3 | 10.7 ± 1.2 | 2.8 ± 0.08 |
| Field | 3 | 43 ± 3.8 | 4.1 ± 0.3 | 9.5 ± 0.1 | — |
| Pryse | | | | | |
| Lab | 12 | 30 ± 0.6 | 3.6 ± 0.1 | 11.5 ± 0.3 | 2.6 ± 0.22 |
| Field | 12 | 44 ± 3.4 | 3.8 ± 0.8 | 10.0 ± 0.5 | — |
| Corcoran | | | | | |
| Lab | 5 | 34 ± 0.5 | 3.9 ± 0.2 | 11.3 ± 0.5 | 2.6 ± 0.08 |

[a] $n = 16$ for Westfarmers, $n = 6$ for Pryse, and $n = 4$ for Corcoran Ponds.

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TABLE 4. Total white blood cell counts and leukocyte percentages in wild and laboratory-reared avocet chicks (<7 weeks old) collected from sites contaminated with agricultural drainwater (mean ± SE).

| Pond | Number of samples | Total WBC ($\times 10^3$/ml) | Number of samples | Lymphocytes | Heterophils | Eosinophils | Monocytes | Heterophil: lymphocyte ratio |
|---|---|---|---|---|---|---|---|---|
| | | | | (%) | | | | |
| Westfarmers | | | | | | | | |
| Lab | 9 | 2.8 ± 0.4 | 3 | 19 ± 3.3 | 5 ± 0.6 | 72 ± 2.7 | 3 ± 0.9 | 0.27 ± 0.54 |
| Field | 3 | 1.3 ± 0.1 | 3 | 29 ± 7.8 | 25 ± 5.2 | 44 ± 3.5 | 2 ± 0.6 | 1.14 ± 0.57 |
| Pryse | | | | | | | | |
| Lab | 5 | 5.3 ± 1.6 | 4 | 17 ± 4.1 | 22 ± 1.5 | 65 ± 3.0 | 1 ± 0.5 | 3.54 ± 1.39 |
| Field | 12 | 2.7 ± 0.8 | 12 | 28 ± 1.3 | 16 ± 2.2 | 56 ± 2.8 | 1 ± 0.4 | 0.57 ± 0.08 |
| Corcoran | | | | | | | | |
| Lab | 12 | 3.0 ± 1.6 | 4 | 23 ± 1.1 | 10 ± 3.5 | 62 ± 5.8 | 2 ± 0.9 | 0.26 ± 0.13 |

among ponds and between lab- and field-reared birds. Corcoran birds raised in the laboratory had lower heterophil and greater lymphocyte percentages than did laboratory-reared birds from Pryse Pond and higher percentages than similar birds from Westfarmers Pond. Monocyte and eosinophil percentages were similar across all ponds. For Westfarmers Pond, birds raised in the laboratory had lower heterophil and lymphocyte percentages than field-raised birds. Pryse Pond lab-reared birds also had lower lymphocyte percentages than field-raised birds, but heterophil percentages were higher.

For the growth parameters, Westfarmers Pond birds were similar to the birds from the reference site at Corcoran; only tarsus length at hatch was shorter and culmen growth rate was slower in Westfarmers Pond birds (Tables 5 and 6). The major differences were seen in Pryse Pond birds. Pryse Pond birds began with lighter hatch weights compared to Westfarmers and Corcoran Pond birds and shorter tarsus lengths at hatch compared to the Corcoran Pond birds (Table 5). Moreover, Pryse Pond birds grew at a faster rate, as measured in the morphological characters, compared to the birds from the other two ponds. Ultimately, the asymptotic tarsus lengths and wing lengths were shorter for Pryse Pond birds; Pryse Pond birds had lighter, and more variable, asymptotic weights compared to Westfarmers Pond birds but not to Corcoran birds.

Hatchability (% hatched) of eggs collected from Pryse and Westfarmers Ponds did not differ significantly from hatchability of eggs collected from Corcoran ponds ($\chi^2 = 6.314$, df = 3, $P = 0.09$). Eleven eggs were collected from the Corcoran Ponds, two of which were used for residue analysis. Of the remaining nine eggs, six hatched. All 12 incubated eggs collected from the Pryse Ponds hatched. From the Westfarmers ponds, 27 eggs were collected of which 13 were used for residue analysis; 10 of the remaining 14 hatched. Only three malformed embryos were seen, all from eggs collected at Westfarmers ponds. One had elongated, hemorrhagic toes and two had their head and neck deviated to one side.

There were no significant differences in selenium liver ($F = 0.14$, df = 9, $P = 0.87$) or muscle ($F = 1.27$, df = 9, $P = 0.35$) residue concentrations among the three ponds (Table 7). Similarly, egg selenium concentration did not differ by pond ($F = 0.17$, df = 17, $P = 0.85$) due to the wide range of values (1.5 to 38.0 ppm dry weight) from all ponds. Liver arsenic was lower ($P = 0.08$) in birds that hatched from eggs collected at Westfarmers Ponds as compared to those from either the Pryse Ponds or the reference site at Corcoran. Arsenic concentrations in eggs, muscle, and

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TABLE 5. Mean and standard error (SE) for the alpha (asymptote), beta (hatch size), and gamma (growth rate) logistic parameters fit to the growth data for avocets from three collection sites (WF = Westfarmers, COR = Corcoran, PRY = Pryse).

| Variable | Number of birds | Pond | | Logistic parameters | | |
|---|---|---|---|---|---|---|
| | | | | Alpha | Beta | Gamma |
| Weight (g) | 6 | WF | Mean | 246.47 | 2.83 | 0.105 |
| | | | SE | 12.82 | 0.37 | 0.011 |
| | 4 | COR | Mean | 237.42 | 3.23 | 0.144 |
| | | | SE | 16.00 | 0.17 | 0.014 |
| | 5 | PRY | Mean | 159.94 | 1.87 | 0.135 |
| | | | SE | 42.40 | 0.24 | 0.029 |
| Tarsus length (mm) | 6 | WF | Mean | 99.12 | 0.57 | 0.054 |
| | | | SE | 1.96 | 0.06 | 0.003 |
| | 4 | COR | Mean | 105.30 | 0.78 | 0.058 |
| | | | SE | 3.19 | 0.07 | 0.001 |
| | 4 | PRY | Mean | 82.76 | 0.42 | 0.096 |
| | | | SE | 6.28 | 0.10 | 0.019 |
| Culmen length (mm) | 6 | WF | Mean | 82.87 | 1.12 | 0.056 |
| | | | SE | 2.73 | 0.04 | 0.001 |
| | 4 | COR | Mean | 80.70 | 1.24 | 0.064 |
| | | | SE | 2.80 | 0.01 | 0.002 |
| | 4 | PRY | Mean | 69.68 | 1.12 | 0.089 |
| | | | SE | 5.63 | 0.07 | 0.015 |
| Wing length (mm) | 6 | WF | Mean | 82.87 | 1.56 | 0.082 |
| | | | SE | 1.08 | 0.09 | 0.007 |
| | 4 | COR | Mean | 86.40 | 1.80 | 0.090 |
| | | | SE | 2.40 | 0.08 | 0.003 |
| | 5 | PRY | Mean | 64.28 | 1.34 | 0.105 |
| | | | SE | 8.38 | 0.26 | 0.007 |

kidney were below detection limits at all ponds. Liver boron concentrations were significantly lower ($F = 17$, df = 9, $P = 0.002$) in birds that hatched from eggs collected at Westfarmers Pond; Pryse Pond was similar to Corcoran. Boron concentrations in eggs ($F = 0.65$, df = 6, $P = 0.46$) and embryos ($F = 2.04$, df = 7, $P = 0.20$) did not differ among sites, were below detection limits in all muscle tissues, and were measurable in kidney tissue only from birds at Pryse Pond.

## DISCUSSION

Concentrations of selenium, arsenic, and boron in agricultural drainwater sampled from Pryse and Westfarmers ponds were well above the water quality criteria of 5 ppb for selenium, 50 ppb for arsenic, and 5 ppm for boron (Environmental Protec-

TABLE 6. Pairwise comparisons of logistic parameters from avocet growth curves; underlined groups are not significantly ($P > 0.10$) different from one another.

| Variable | Logistic parameter | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Alpha | | | Beta | | | Gamma | | |
| Weight | PRY | COR | WF | PRY | WF | COR | WF | PRY | COR |
| Tarsus length | PRY | WF | COR | PRY | WF | COR | WF | COR | PRY |
| Culmen length | PRY | COR | WF | PRY | WF | COR | WF | COR | PRY |
| Wing length | PRY | WF | COR | PRY | WF | COR | WF | COR | PRY |

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TABLE 7. Selenium, arsenic, and boron residues (dry weight, ppm) in avocet eggs and tissues (mean ± SE) from three ponds. Lower limits of detection are 1 ppb for selenium, 15 ppb for arsenic, and 5 ppb for boron.

| Pond | Egg | Liver | Muscle | Kidney |
|---|---|---|---|---|
| Westfarmers Pond | | | | |
| Selenium | 15.965 ± 8.50 | 6.033 ± 0.44 | 1.714 ± 0.02 | —[a] |
| Arsenic | ND[b] | 1.977 ± 0.42 | ND | ND |
| Boron | 10.500 ± 2.20 | 4.492 ± 0.60 | ND | ND |
| Sample size | 13 | 6 | 6 | 6 |
| Pryse Pond | | | | |
| Selenium | 18.156 ± 11.78 | 6.534 ± 0.70 | 1.756 ± —[c] | —[a] |
| Arsenic | ND | 3.055 ± 0.22 | ND | ND |
| Boron | 8.327 ± 0.86 | 10.280 ± 0.59 | ND | 1.86 ± 1.86 |
| Sample size | 3 | 2 | 2 | 2 |
| Corcoran Pond | | | | |
| Selenium | 20.512 ± 23.90 | 6.200 ± 0.83 | 2.101 ± 0.35 | —[a] |
| Arsenic | ND | 3.895 ± 0.48 | ND | ND |
| Boron | —[d] | 9.595 ± 1.28 | ND | ND |
| Sample size | 2 | 2 | 2 | 2 |

[a] Not sampled.
[b] Not detected (below detection limit).
[c] Sample size of one.
[d] Four eggs collected from another reference site had mean (±SE) boron concentrations of 8.48 ± 0.312 (SE) ppm.

tion Agency, 1980, 1984a; National Research Council, 1980). All other elements measured (Table 1) were at concentrations well below those known to have adverse physiological effects (National Research Council, 1980). The reference site, Corcoran Ponds, had very low selenium and arsenic concentrations. Unfortunately, boron was not measured in the reference pond water, making it difficult to implicate boron as a causative factor of observed effects. However, the fact that boron concentrations in Pryse and Westfarmers ponds exceeded water quality criteria and threshold levels for known physiological effects in livestock (National Research Council, 1980), is evidence that it may be a contributing factor. We found that avocet chicks exposed solely through *in ovo* transfer of these elements from adults to their progeny had significantly different white blood cell differential profiles caused by an increased heterophil:lymphocyte ratio that was due to an increased percentage of heterophils and a decreased percentage of lymphocytes. Additionally, the phagocytic ability of macrophages was significantly reduced in both Pryse and Westfarmers Pond birds, while the ability of lymphocytes to proliferate in the presence of a T-cell mitogen was increased in birds from Pryse Ponds. Furthermore, size at hatch and adult size, as measured by body weight and wing, tarsus, and culmen length, were significantly reduced in birds from both sites with high concentrations of these inorganic compounds.

The birds that were most severely affected were those that hatched from eggs collected from Pryse Pond which contained moderately high concentrations of selenium and boron and a very high concentration of arsenic. Based on studies of the avian teratogenic effects of agricultural drainwater in this area of California, Ohlendorf et al. (1986a, b) implicated selenium as the causative agent. Hoffman et al. (1992) found that selenium can retard growth rates of mallards in the laboratory. However, because birds from Pryse Ponds, which had arsenic concentrations 200 times higher than the water quality criteria value and only moderately elevated selenium concentrations, had the greatest number

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of adverse effects it is likely that arsenic played a more significant role in causing the observed effects in this avocet study than did selenium. Alternatively, there could have been an interactive, potentiating effect of the arsenic and selenium and, possibly, boron. Although selenium is a well-known immunomodulator in birds (Whiteley and Yuill, 1989; Fairbrother and Fowles, 1990) as well as in mice (Koller et al., 1980), Fowler (1977) found that arsenic protects against selenium toxicity through increased excretion of selenium from the liver. Both selenium and arsenic exhibit hormetic effects on the immune system, whereby exposure to low concentrations are immunostimulatory and higher levels are immunotoxic. Exposure to high concentrations of arsenic inhibit the synthesis and action of interferon, whereas lower concentrations increase the antiviral activity of interferon (Sharma, 1981). Chronic arsenic poisoning affects the bone marrow resulting in anemia, leukopenia, granulocytopenia, and eosinophilia (Environmental Protection Agency, 1984b; Arnold, 1988). Chronic viral infections such as herpetic lesions and chronic pulmonary infections also have been associated with chronic arsenic exposure (Environmental Protection Agency, 1984b). Arsenic is known to have teratogenic properties (Environmental Protection Agency, 1984b), affect growth rates, and interfere with enzyme function through disruption of disulfide bonds in proteins as well as reducing mitochondrial respiration through disruption of oxidative phosphorylation. Although there have been no reported studies on immunotoxicity of boron, this compound can block prostaglandin synthesis (Larsen, 1988), an important intracellular communication pathway in the immune system. Given this information and the results from this study, we hypothesize that the observed physiological effects in birds that successfully hatched were caused by a combination of the compounds, rather than by selenium alone.

Unfortunately, definitive cause-and-effect relationships could not be established from this study. This frequently is the case in field studies that provide largely observational data. However, hypotheses generated from this study could be tested in controlled laboratory studies, where adult avocets could be exposed to known concentrations of selenium, arsenic, and boron in drinking water and similar parameters measured (Fairbrother and Fowles, 1990). In the current study, the results are further confounded by a lack of differences in contaminant concentration in eggs collected from the three study sites and in the body tissues of the 12 to 16 wk-old juvenile birds. Ohlendorf (1989) and Lonzarich et al. (1992) documented high selenium concentrations in waterfowl and shorebird eggs from these and nearby areas, although these usually were associated with lethal abnormalities. The eggs we collected were biased towards nests that we thought would hatch viable young (>1 egg, presence of adult, away from shore). Therefore, the concentrations in the eggs could be expected to be significantly lower than those measured in previous studies. Moreover, tissue residues of the three compounds were measured in 16 to 20 wk-old birds that had been held in the laboratory on clean diets and consequently had depurated most of the pollutants from their tissues.

Our results provide evidence that exposure to excessive concentrations of inorganic compounds affects the $F_1$ generation of reproductively active birds. Unfortunately, we were unable to monitor the avocet chicks in the field to ascertain the relationship between depressed immunomodulation or growth rates and the fledging success of the birds. However, the study was successful at demonstrating that physiological effects can be measured in wild birds, both under laboratory conditions and from free-ranging animals. The assays described herein now can be applied to other field situations of suspected environmental contamination and an information base developed to document sublethal effects of intoxication. Coupled

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with ecological data on breeding success, recruitment rates, and disease epizootics, no observable adverse effects levels that are protective of wild individuals can more reliably be established for use in hazardous waste site clean-up programs or establishing criteria values for water or sludge-borne contaminants.

## ACKNOWLEDGMENTS

We thank Jeff Fowles and Kimberly Trust for their assistance with the macrophage phagocytosis and lymphocyte blastogenesis assays, Suean Ott for ICP analyses, Dr. Lisa Ganio for statistical support, and Ray Bentley and Susan Schiller for technical assistance and animal care. Dr. Joe Skorupa and Carolyn Marn provided access to the study site and located avocet nests. The manuscript benefited from technical reviews by Dr. J. Zelikoff and Ms. J. Alberico. The information in this document has been funded wholly by the U.S. Environmental Protection Agency. It has been subjected to the Agency's peer and administrative review, and it has been approved for publication as an EPA document. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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*Received for publication 3 May 1993.*

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