

**STUDY PLAN FOR
AVIAN INJURY STUDY
YEAR 4 (2009)**

**HUDSON RIVER NATURAL RESOURCE
DAMAGE ASSESSMENT**

HUDSON RIVER NATURAL RESOURCE TRUSTEES

STATE OF NEW YORK

U.S. DEPARTMENT OF COMMERCE

U.S. DEPARTMENT OF THE INTERIOR

FINAL

JUNE 11, 2009

Available from:

U.S. Department of Commerce
National Oceanic and Atmospheric Administration
Hudson River NRDA, Lead Administrative Trustee
Damage Assessment Center, N/ORR31
1305 East-West Highway, Rm 10219
Silver Spring, MD 20910-3281



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EXECUTIVE SUMMARY

Past and continuing discharges of polychlorinated biphenyls (PCBs) have contaminated the natural resources of the Hudson River. The Hudson River Natural Resource Trustees – New York State, the U.S. Department of Commerce, and the U.S. Department of the Interior – are conducting a natural resource damage assessment (NRDA) to assess and restore those natural resources injured by PCBs.

As part of the NRDA, the Trustees have conducted several investigations focused on birds, including studies on Hudson River tree swallows in 1994-1995, bird egg preliminary investigations in 2002-2003, and avian injury investigations by the U.S. Geological Survey in 2004-2005.

The Trustees have also conducted several years of avian egg injection studies. Year 1 (2006) avian egg injection work focused on injection of test PCBs and development of injection and incubation protocols for eggs from tree swallow, American kestrel and chicken. The Trustees subsequently determined it was appropriate to conduct additional avian egg injection work. Year 2 (2007) work entailed an evaluation of the effects of a PCB mixture relevant to tree swallows from the Upper Hudson River in a controlled egg injection study, an evaluation of the effects of *in situ* PCB exposure in Upper Hudson River hatchling tree swallows, and a pilot study of injection of a PCB mixture into eggs of Eastern bluebirds. Year 3 (2008) work entailed continuation of the egg injection studies conducted on tree swallows, American kestrels and Eastern bluebirds in 2006 and 2007, along with a pilot study of injection of a PCB mixture into eggs of Eastern screech owl, and a comparison of endpoints in tree swallow and Eastern bluebird eggs collected at the Patuxent Wildlife Research Center (PWRC) and Upper Hudson River sites. Analysis of data from these studies is ongoing.

This Study Plan is for Year 4 (2009) of an avian study entailing an assessment of the PCBs in environmentally exposed Eastern bluebird eggs with a focus on heart anatomy and histology. Eastern bluebird eggs collected from the Upper Hudson River will be hatched in the laboratory and compared to hatchlings from the uncontaminated PWRC site. The following endpoints will be assessed in this study: embryo mortality, deformities, heart and respiratory rate, body and organ (heart, lung, liver and bursa) weights, heart histology, CYP450 enzyme induction (liver), and genetic sex.

In the interest of efficiency and to not unnecessarily increase the cost of the NRDA, the Trustees conducted no formal peer review specific to this Study Plan as no new and/or otherwise relevant information regarding the endpoints proposed for study was identified since the conduct of earlier peer reviews on Trustee egg injection study proposals.

Pursuant to the Hudson River NRDA Plan, the results of the work conducted pursuant to this Study Plan will be peer reviewed upon completion of the study, and the results then released to the public.

1.0 BACKGROUND

Past and continuing discharges of polychlorinated biphenyls (PCBs) have contaminated the natural resources of the Hudson River. The Hudson River Natural Resource Trustees – New York State, the U.S. Department of Commerce, and the U.S. Department of the Interior – are conducting a natural resource damage assessment (NRDA) to assess and restore those natural resources injured by PCBs (Hudson River Natural Resource Trustees 2002).

The Hudson River and surrounding area support more than 150 species of birds, including waterfowl, wading birds, shorebirds, songbirds, and rare species such as the bald eagle, peregrine falcon, and osprey (Andrle and Carroll, 1988). Birds are an integral part of the ecosystem and provide a number of important ecosystem services such as seed distribution, plant pollination, and insect control. Birds are also an important source of prey to other species. Birds may be exposed to PCBs through direct ingestion of contaminated water, sediment, and soil. A more important likely exposure pathway is their consumption of food items that contain PCBs derived from the Hudson River and its floodplain. PCB-contaminated food items linked to the river may include fish, amphibians, benthic invertebrates, adult insects that develop from aquatic larvae, plants growing in or near the river, and mammals that forage in the floodplain.

As part of the NRDA, the Trustees have conducted several investigations focused on birds, including studies on Hudson River tree swallows in 1994-1995 (McCarty and Secord 1999a and 1999b, Secord et al. 1999, Stapleton et al. 2001), bird egg preliminary investigations in 2002-2003 (Hudson River Natural Resource Trustees 2004a, 2005a, 2005b), and avian injury investigations by the U.S. Geological Survey in 2004-2005 (Hudson River Natural Resource Trustees 2004b, 2005c).

The Trustees have also conducted several years of avian egg injection studies. Year 1 (2006) avian egg injection work focused on injection of test PCBs and development of injection and incubation protocols for eggs from tree swallow (*Tachycineta bicolor*), American kestrel (*Falco sparverius*) and chicken (*Gallus domesticus*) (Hudson River Natural Resource Trustees 2006 and 2007a). Year 2 (2007) work entailed an evaluation of the effects of a PCB mixture relevant to tree swallows from the Upper Hudson River in a controlled egg injection study, an evaluation of the effects of *in situ* PCB exposure in Upper Hudson River hatching tree swallows, and a pilot study of injection of a PCB mixture into eggs of Eastern bluebirds (*Sialia sialis*) (Hudson River Natural Resource Trustees 2007b). Year 3 (2008) work entailed continuation of the egg injection studies conducted on tree swallows, American kestrels and Eastern bluebirds in 2006 and 2007, along with a pilot study of injection of a PCB mixture into eggs of Eastern screech owl (*Otus asio*), and a comparison of endpoints in tree swallow and Eastern bluebird eggs collected at the Patuxent Wildlife Research Center (PWRC) and Upper Hudson River sites (Hudson River Natural Resource Trustees 2008).

Analysis of data from these studies is ongoing.

2.0 INTRODUCTION

This Study Plan is for Year 4 (2009) of an avian study entailing an assessment of the PCBs in environmentally exposed Eastern bluebird eggs with a focus on heart anatomy and histology. Eastern bluebird eggs collected from the Upper Hudson River will be hatched in the laboratory and compared to hatchlings from the uncontaminated PWRC site.

In the interest of efficiency and to not unnecessarily increase the cost of the NRDA, the Trustees conducted no formal peer review specific to this Study Plan as no new and/or otherwise relevant information regarding the endpoints proposed for study was identified since the conduct of earlier peer reviews on Trustee egg injection study proposals.

Pursuant to the Hudson River NRDA Plan, the results of the work conducted pursuant to this Study Plan will be peer reviewed upon completion of the study, and the results then released to the public.

3.0 PURPOSE AND OBJECTIVE

This study of Eastern bluebirds will be used to evaluate whether the viability of avian resources is affected as a result of exposure to PCBs from the Hudson River. The work will inform the Trustees regarding injury to avian resources and guide their future efforts to identify pathway and specific injuries to birds from PCBs, determine causation, and scale restoration, as defined in the DOI NRDA Regulations. The work will be used to identify and evaluate the type(s) of injury(ies), if any, that PCBs are causing to Hudson River birds. This work will also be used to help determine whether future studies will be performed, and if so, to help in their design.

4.0 METHODS

The Trustees have developed the study described in the attached work plan entitled, "Eastern Bluebird Egg Studies 2009" (Appendix A) to evaluate whether avian species in the vicinity of the Hudson River are injured due to exposure to PCBs. The attached work plan includes information regarding the experimental design, Quality Assurance/Quality Control, and Standard Operating Procedures that will be used in the study. The Trustees have developed the design described in Appendix A for work in 2009 to evaluate the effects of exposure of Eastern bluebirds to PCBs, through environmental exposure in the field. Sections 4.1 through 4.3 below summarize the work described in Appendix A.

4.1 COLLECTION AND ASSESSMENT OF EASTERN BLUEBIRD EGGS FROM PWRC AND THE UPPER HUDSON RIVER

Eastern bluebird eggs will be collected from the PWRC and Upper Hudson River for analysis to determine if there are differences in the eggs between the two sites that can be attributed to PCB contamination. A maximum of 15 eggs will be collected from the Upper Hudson River and 20 eggs will be collected from PWRC for incubation, hatching and sampling. Additionally, ten eggs will be collected for contaminant analysis from PWRC, and ten eggs will also be collected from the Upper Hudson River for contaminant analysis.

4.2 ENDPOINTS AND STATISTICAL ANALYSES

The following endpoints in Eastern bluebird eggs from PWRC and/or the Upper Hudson River will be assessed in this study:

- Embryo mortality
- Deformities
- Heart and respiratory rate
- Body and organ (heart, lung, liver and bursa) weights
- Heart histology
- CYP450 enzyme induction (liver)
- Genetic sex

These endpoints and the associated statistical analyses are described in greater detail in Appendix A.

Eggs may also be analyzed for chemical analytes that may include congener-specific PCBs, including the non-*ortho* congeners, polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polybrominated diphenyl ethers (PBDEs), organochlorine pesticides, and metals, as determined appropriate by the Trustees. Any analytical chemistry data will be validated as specified in the Analytical QA Plan (Hudson River Natural Resource Trustees 2005d).

5.0 QUALITY ASSURANCE/QUALITY CONTROL

This study is being conducted in accordance with the Quality Assurance Management Plan for the Hudson River NRDA (Hudson River Natural Resources Trustees, 2005d).

Strict chain-of-custody procedures will be used throughout the study. All samples collected under this Study Plan will be maintained under chain-of-custody upon collection, and through processing, storage and shipment to the testing laboratory, analytical laboratory or archive facility.

Analysis will be by appropriate methods approved by the Trustees. As noted above, chemical analytes may include congener-specific PCBs, including the non-*ortho* congeners, PCDDs, PCDFs, PBDEs, organochlorine pesticides, and metals, as determined appropriate by the Trustees.

In order to minimize analytical costs, and reduce the overall cost associated with the project, the Trustees may conduct the chemical or other analyses in stages, using initial work to inform subsequent decisions regarding which analyses to conduct on which samples.

The laboratories performing analytical work will be contracted to follow the Trustees' Analytical Quality Assurance Plan for the Hudson River NRDA (Hudson River Natural Resource Trustees 2005d). Laboratories will provide fully documented data packages which will enable data validation to be performed based on the criteria provided in the Analytical Quality Assurance Plan for the Hudson River NRDA, applicable laboratory Standard Operating Procedures, and relevant U.S. Environmental Protection Agency guidelines (USEPA 1999).

Quality assurance and quality control are described in greater detail in Appendix A.

6.0 SPECIAL PROVISIONS

All collection of eggs and any tissues, as well as bird handling, will be conducted under permits from USFWS and appropriate State agencies, and according to appropriate Animal Care and Use Committee approved protocols.

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APPENDIX A

FINAL WORK PLAN: EASTERN BLUEBIRD EGG STUDIES 2009

**WORK PLAN HR NRDA 017:
EASTERN BLUEBIRD EGG STUDIES 2009**

HUDSON RIVER NATURAL RESOURCE DAMAGE ASSESSMENT

11th of June 2009

Principal Investigator

Co-Principal Investigator

Quality Assurance Coordinator

Investigation Team Acknowledgement of Work Plan Review And Compliance

By my signature, I acknowledge that I have read this Work Plan and understand it, and will comply with it in performing this work.

Name (printed): _____ Name (printed): _____
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1 INTRODUCTION & OBJECTIVES

1.1 PREVIOUS WORK

In 2007 and 2008 we conducted dose finding experiments on small numbers of eastern bluebirds from Patuxent Wildlife Research Center (PWRC). Based on previous PCB avian egg injection studies using the spotted sandpiper (SPSA) mix, eastern bluebird eggs at PWRC were injected with either 25 μ g/g egg or 100 μ g/g egg using 0.4 μ l per gram of egg. However, both of these doses resulted in 100% mortality of treated eggs. Vehicle was not expected to play a role in this mortality as 100% of corn oil treated eggs hatched. In 2008, the dose was reduced in an attempt to determine a working range for further experiments. Eggs were injected with 2.94 μ g of SPSA mix per gram of egg (2.94 μ g/g egg using 0.1 μ l) per gram of egg. There were no significant differences in mortality between untreated, vehicle and both PCB doses. Sample sizes in 2008 were n=6-12, which was an increase from n=2-4 in 2007, however, due to low numbers of eastern bluebird nests we were not able to obtain samples sizes close to the 20-40 that were available for tree swallow egg injections. In 2008 we also collected eastern bluebird eggs in the Upper Hudson River (n=12) area to compare primary endpoints. There were no statistically significant differences in body or organ weight indices between field sites in response; however, this could in part be due to low sample sizes.

1.2 PROPOSAL SUMMARY

This year we would like to assess the effects of PCBs in environmentally exposed eastern bluebird eggs with a focus on heart anatomy and histology. Eastern bluebird eggs collected from the Upper Hudson River, Remnant 3, will be hatched in the lab and compared to hatchlings from the uncontaminated PWRC site. In the 2008 tree swallow field season there was a statistically significant effect of PCB 77 on heart index in the highest treatment group (WP014 report). PCB 77 is one of the components of both the SPSA PCB mix and the tree swallow PCB mix, and has been measured in avian eggs from the contaminated site.

A smaller study was conducted in eastern bluebirds last year. No differences were detected in eastern bluebird heart indices, potentially due to low sample sizes. It is thought that eastern bluebirds are more sensitive to activation of the aryl hydrocarbon receptor by dioxin-like compounds than tree swallows (Kennedy pers. comm.). Head *et al.* (2008) showed that the aryl hydrocarbon receptor amino acid sequence in eastern bluebirds is indicative of an intermediate sensitivity while tree swallows fall in a less sensitive group. The study this year will provide the sample sizes for eastern bluebirds with the statistical power to ascertain if there are differences in heart size and morphology following environmental PCB exposure.

2 WORK PLAN

2.1 STUDY SPECIES AND SITES

Eastern bluebird (*Sialia sialis*)

Untreated eastern bluebird eggs (n=45) will be collected under appropriate permits from Patuxent Wildlife Research Center (PWRC), MD and Upper Hudson River, NY.

Studies will be conducted under approved animal care protocols by the Animal Care and Use Committee and the PWRC Animal Use Committee. Federal and State collection permits will be obtained by Kathryn Jahn of USFWS.

Based upon available information, PWRC is a historically uncontaminated site. Concentrations of PCBs and other contaminants have been low or non-detectable. Yorks (1999) found an average of 0.7 ± 0.25 SD (N=6) $\mu\text{g/g}$ PCBs in eggs collected in 1995.

2.2 EGG COLLECTION

Eastern bluebird eggs will be collected from both the Upper Hudson River (maximum of 15 for incubation and 10 for contaminant analysis) and PWRC (maximum of 20 for incubation and 10 for contaminant analysis) in order to compare the effects of environmental PCB exposure on hatchlings. A slightly higher number of eggs for incubation will be collected from PWRC to ensure that we have sufficient samples to validate heart histology in this species.

2.3 ENDPOINTS

The literature indicates potentially adverse effects associated with several measurement endpoints following exposure to PCBs. This study will focus on selected endpoints related to cardiac anatomy and histology. We propose to measure liver EROD as an indicator of PCB exposure. This will provide an additional individual measure of exposure to which the heart analyses can be compared.

Contaminant analysis will be conducted under a separate work plan.

2.3.1 Deformities

Deformities are associated with PCB exposure in birds (Ludwig et al. 1996, Hoffman et al. 1998, Lavoie and Grasman 2007). Photographs will be taken of each embryo or hatchling that is scored for deformities. Any deformities/irregularities in eastern bluebird hearts will be noted.

2.3.2 Heart and respiratory rate

Once eastern bluebirds have hatched they will have their heart rate and respiration monitored for one minute prior to sacrifice. This will provide us with both a baseline for cardiovascular function in eastern bluebirds and enable us to ascertain if there are problems in exposed birds. Increased heart rate and respiration could be indicative of insufficient oxygenation of blood and tissue, while decreased or irregular heart rate could be indicative of a structural defect.

2.3.3 Body and organ (heart, lung, liver and bursa) weights

Organ weights can be affected by PCBs in chickens. Body weight at hatch is generally not affected by *in ovo* PCB exposure (Lavoie & Grasman 2007). However, body weights and organ weights are important cofactors for understanding other endpoints, e.g., body weight may explain unusually small organ weights and organ weights may explain outliers in other analyses. Organ weights will be collected for the liver, lung, heart and bursa. While not all of these are strictly related to cardiovascular output, they provide a valuable addition to the information already obtained from 2007 and 2008. Recently Kransler *et al.* (2008) showed that pre-natal exposure of rat pups to 2,3,7,8- tetrachlorodibenzo-p-dioxin slowed lung development and resulted in smaller lungs. This is thought to result from activation of the AhR (aryl hydrocarbon receptor) in lung tissue. Lung weight will be determined in exposed bluebirds.

2.3.4 Heart histology

Recent literature (Dewitt *et al.* 2006) demonstrates an association between PCBs and heart deformities in passerine birds. Heart tissues will be collected and preserved as part of this study. Procedures for staining, sectioning and analysis have been developed for hatchling tree swallow hearts. A subset of samples from PWRC will be used to verify the analyses in eastern bluebird hearts. Methods will be modified accordingly.

2.3.5 CYP450 enzyme induction (liver)

PCBs have been reported to increase the content or activity of several enzymes in birds, including P450 isozymes (Hoffman *et al.*, 1996). For example, planar PCBs strongly induce the P450 isozyme CYP1A [measured by increases in aryl hydrocarbon hydroxylase (AHH) or ethoxyresorufin-O-deethylase (EROD) activity]. Analysis of P450 isozyme CYP1A in liver tissue will be conducted under a separate work plan with separate SOPs.

EROD activity will be measured in these hatchlings rather than determination of PCB content of eggs from the two field sites in order to ensure that we have an indicator of differential PCB exposure between sites. Data obtained from hatchling tree swallow samples collected from PWRC and UHR in 2006 and 2007 showed that there were site specific differences and yearly variations in EROD activation. Measurement of EROD activity in 2009 samples will allow us to continue monitoring differences in exposure levels between the two sites.

2.3.6 Genetic sex

Blood samples for genetic sexing will be collected for this study, and genotyping will be analyzed. Gender is a possible cofactor in statistical analysis; furthermore, genotypic sex will confirm gender that cannot be determined from gonadal morphology if there are morphological changes such as intersex gonads.

3 EXPERIMENTAL DESIGN

All data collected will be recorded and handled according to SOP HR #001.

3.1 EASTERN BLUEBIRDS

Hatchlings will be handled according to Animal Care and Use Committee guidelines and under permits from USFWS. Untreated eggs will be collected from two sites, the Patuxent Wildlife Research Center (PWRC), MD and the Upper Hudson River (UHR), NY. PWRC hatchlings will be used as control subjects to determine the effects of PCB exposure on the heart.

3.1.1 Contaminant Analysis

Ten eggs will be collected for contaminant analysis from both PWRC and the Upper Hudson River. Contaminant analysis will be conducted under a separate work plan and will follow the requirements of the Hudson River Analytical QA Plan.

One egg will be collected from each nest after the fourth egg has been laid. A minimum of three eggs are required in each nest in order for nesting and parental behaviors to continue. However, if additional eggs in the nest do not develop these eggs will be collected at the time of hatching of the other eggs. These eggs can potentially be used for determination of intra-clutch determination in PCB levels.

Eggs for contaminant analysis will be identified by the same egg code sequence used for the individual site, e.g. 400-EABL-2009. Eggs from PWRC are defined by the egg code 01-199, while Upper Hudson River eggs are defined by codes 400 upwards. Eastern bluebird eggs weigh greater than 2.0g, so only a single egg is required for individual contaminant analysis

3.1.2 Egg Collections

Nests will be monitored for initiation of egg laying, clutch completion and initiation of incubation at PWRC and the Upper Hudson River, Remnant 3. Dates of these events will be noted accordingly (DOC CONT #021). We will follow the collection practices of Dr. Chris Custer (USGS) for tree swallows as eastern bluebirds have a similar egg laying rate. Once egg laying has begun monitored nests will be observed daily for eggs, which are laid at one day intervals. When the nest is complete (4-6 eggs) a date for collection will be set at approximately 9-11 days later. Our lab has shown excellent hatching success with eastern bluebirds, so it is not necessary to ensure that eggs are all collected at exactly the same stage of development. As long as they undergo more than two-thirds of their development in the field we have been able to obtain >70% hatching success. This flexibility of collection will allow us to reduce the amount of time spent in the field collecting eggs.

A maximum of 15 eggs will be collected from the Upper Hudson River and 20 eggs will be collected from PWRC according to SOP HR #030 for incubation, hatching and sampling. Expected date of collection for appropriate nests will be recorded (DOC CONT #023). Only a single egg will be removed from each nest containing four or more eggs. Where possible (nests where five eggs are laid), eggs will be removed for both hatching and contaminant analysis.

3.2 EGG INCUBATION

Eggs will be candled at least once in the field during incubation to ensure embryonic development is occurring. Candling will also take place at the time of collection from the nest and upon receipt in the laboratory. Dead embryos within a nest and approximate stage of development will be noted.

Eggs will be collected from nests at days 9-11 following the initiation of incubation. They will be transferred to the laboratory (DOC CONT #029) and placed in the incubator set at 37.0-38.0°C (98.5-100.5°F) and 30-40% humidity. To prevent damage and avoid jarring the eggs will be incubated on their sides in specially designed slots made of soft organza fabric glued to a standard pheasant egg rack. They are turned twice daily by 180°, with hourly 120° shifts in rack position. Minimum and maximum temperature and humidity are monitored and recorded twice daily (SOP HR #021, DOC CONT #007).

Twenty four hours prior to the expected hatch day the eggs are placed in plastic racks with gauze or tissue lining. No further positional changes are made after this time.

3.3 EGG HATCHING AND SAMPLING

Any eggs that fail to hatch will be opened and condition of the embryo noted. Deformities will be scored for presence or absence of crossed bill, shortened upper bill, missing or deformed eyes, edema of the neck and head area, incomplete ossification of skull (brain not enclosed in skull), gastroschisis in post stage 45 embryos, malformed or clubbed feet, asymmetrical body form, malposition in the egg, and any other abnormal appearances shall be noted on the data sheet (DOC CONT #018). Photographs of deformed and normal embryos and hatchlings will be taken for reference.

Embryos from eggs collected in the field and incubated in the lab will be dissected within 8 hours of hatching (DOC CONT #015). Heart and respiratory rate will be recorded immediately before sacrifice (DOC CONT #035). Animals will be sacrificed by cervical dislocation according to SOP #029.

Samples from each eastern bluebird hatchling or egg will be identified by a unique code ("sample I.D.") encompassing the egg code, species, and year, e.g. 01-EABL-2009 for a eastern bluebird collected in 2009 from PWRC. Each tissue that is collected will be labeled with the complete sample I.D. such as (01-EABL-2009) and the name of the type of sample: serum, liver, bursa, heart or thyroid. Blood spots will be collected on sample cards provided by the contracted laboratory, and labeled with the sample ID.

3.4 BIOLOGICAL TISSUE ANALYSES

3.4.1 Histological:

Heart tissue will be preserved in appropriate fixatives according to the appropriate SOP (External SOP #001 and #002). Slides will be labeled and well organized for retrieval and review.

3.4.2 Gender genotyping

Gender genotyping will be performed on blood collected on cards using polymerase chain reaction (PCR) techniques. SOPs and resulting data will be reviewed for adherence to QA/QC requirements.

3.4.3 Livers

Liver tissue will be snap frozen in liquid nitrogen before being transferred to the -80°C freezer. Tissue will be used for the measurement of cytochrome P450 activity in liver microsomes by EROD assay according to WP011.

SOP HR #001: Recording and Handling of Data for Avian Injection Studies

SOP HR #021: Monitoring and Recording Temperature and Humidity in Egg Incubators

SOP HR #029: Necropsy of Altricial Hatchlings

SOP HR #030: Field Collection of Avian Eggs for Contaminant Analysis and Hatching

External SOP #001: Fixation, Dehydration and Embedding of Large Heart Specimens

External SOP #002: Hematoxylin and Eosin Staining for Morphology

DOC CONT #007: Incubator Record Sheet

DOC CONT #015: Necropsy Sampling Sheet

DOC CONT #018: Deformity Score Sheet

DOC CONT #021: Avian egg injection nesting checking data sheet

DOC CONT #023: Avian Egg Field Collection Schedule

DOC CONT #028: Egg Incubation Data Sheet

DOC CONT #029: Egg Collection Data Sheet

DOC CONT #035: Hatchling Heart and Respiratory Rate

3.5 STATISTICAL ANALYSES

Data will be analyzed following examination of normality and proceeding with parametric ANOVAs or non-parametric tests, and regressions as appropriate. Mortality data will be analyzed with Fisher Exact Probability test and probit analysis for determining median lethal doses. When necessary, further analyses would be used to understand the significance of dose-responses and non-monotonic trends. If the predictions warrant the use of one-tailed tests, these tests will be used with consultation with our statistician. Additional tests may include bootstrap techniques if data are not normally distributed and sample sizes are low.

The Principal Investigators (PIs) plan to conduct the following comparisons. Null (HO) and alternative (HA) hypotheses are presented below. “PCBs” and “exposed to” refer to environmental PCB exposure for eggs and birds from the Upper Hudson River. “Controls” refers to eggs and birds from the reference sites. “Birds” represents any life stage for which an endpoint is measured.

3.5.1 Deformities:

Compare occurrence and severity of deformities between PCB exposed embryos and unexposed embryos.

General Hypotheses

HO: The occurrence and severity of deformities are equal in control and PCB exposed embryos

HA: The occurrence and severity of deformities are increased in PCB exposed embryos compared to controls

Statistical tests

Fisher Exact probability tests and probit analysis will be used for determining significant increases in deformities and for determining median effect concentrations.

3.5.2 Heart and Respiratory Rates

Compare the heart and respiratory rates of PCB exposed birds to unexposed birds.

General Hypotheses

HO: Heart and respiratory rates in PCB exposed birds are not different than controls

HA: Heart and respiratory rates in PCB exposed birds are different compared to controls

Statistical tests

Data will be examined for normality and homogeneity of variances as necessary for the tests used. If assumptions of normality and homogeneity of variances are met, histological indices of morphology will be compared across treatment groups and sex, and possible interactions examined, using 2-way ANOVA with appropriate post hoc comparisons. Alternately, data transformations will be used as required or appropriate non-parametric tests shall be used. Furthermore, regression analyses with estimates of confidence intervals or dose-response statistics such as the Jonckheere test will be used to evaluate dose related effects.

3.5.3 Organ Weights

Compare organ (heart, liver and bursa) weights of PCB exposed birds with unexposed birds.

General Hypotheses

HO: Organ weights in PCB exposed birds are not different than controls

HA: Heart and liver weights in PCB exposed birds are higher compared to controls

HA: Bursa and lung weight in PCB exposed birds is lower compared to controls.

Statistical tests

Data will be examined for normality and homogeneity of variances as necessary for the tests used. If assumptions of normality and homogeneity of variances are met, organ weights will be compared across treatment groups and sex, and possible interactions examined, using 2-way ANOVA with appropriate post hoc comparisons. Alternately, data transformations will be used as required or appropriate non-parametric tests shall be used. Furthermore, regression analyses with estimates of confidence intervals or dose-response statistics such as the Jonckheere test shall be used to evaluate dose related effects.

3.5.4 Heart Histology

Compare the histology of PCB exposed birds with that of unexposed birds.

General Hypotheses

HO: Heart histology of PCB exposed birds are not different than controls

HA: Heart histology in PCB exposed birds shows there are differences in heart size, shape and wall thickness.

Statistical tests

Data will be examined for normality and homogeneity of variances as necessary for the tests used. If assumptions of normality and homogeneity of variances are met, organ weights will be compared across treatment groups and sex, and possible interactions examined, using 2-way ANOVA with appropriate post hoc comparisons. Alternately, data transformations will be used as required or appropriate non-parametric tests shall be used. Furthermore, regression analyses with estimates of confidence intervals or dose-response statistics such as the Jonckheere test shall be used to evaluate dose related effects.

3.5.5 EROD

Compare liver EROD activity of PCB exposed birds with unexposed birds.

General Hypotheses

HO: Liver EROD activity in PCB exposed birds is not different than controls

HA: Liver EROD activity in PCB exposed birds is increased compared to controls

Statistical tests

Data will be examined for normality and homogeneity of variances as necessary for the tests used. If assumptions of normality and homogeneity of variances are met, organ weights will be

compared across treatment groups and sex, and possible interactions examined, using 2-way ANOVA with appropriate post hoc comparisons. Alternately, data transformations will be used as required or appropriate non-parametric tests shall be used. Furthermore, regression analyses with estimates of confidence intervals or dose-response statistics such as the Jonckheere test shall be used to evaluate dose related effects.

These hypotheses and statistical tests may be revised, or not performed by the PIs based on data collected. Further, the PIs may test other hypotheses and conduct additional statistical tests not noted above.

4 QUALITY ASSURANCE/QUALITY CONTROL

4.1 DATA QUALITY OBJECTIVES, INDICATORS, AND ASSESSMENT

4.1.1 Overview

This study is being conducted in accordance with the Quality Assurance Management Plan for the Trustees' Hudson River NRDA. As described in the plan, four general elements of quality assurance/quality control (QA/QC) must be addressed for each data collection effort:

- Project Management
- Data Generation and Acquisition
- Assessment and Oversight
- Data Validation and Usability

This section describes the Quality Assurance Plan (QAP) for the avian egg injection study, based on these four general elements. The objectives of the study are outlined in Section 1 of this Work Plan. To achieve these objectives, the following requirements must be met:

- All samples, from the initial eggs through embryos, hatchlings, dead or infertile eggs, necropsy samples, and egg products must be identified and stored following documented procedures to insure proper identification and handling.
- All procedures for assessment of biological impacts, including egg injections, necropsy, and biological tissue analyses, must be performed following documented procedures to ensure consistent, comparable data.
- PCB mixture preparation and egg contaminant levels: The laboratories performing chemical contaminant testing will follow the requirements of the Hudson River NRDA Analytical QA Plan. This effort is not part of the current work plan and will be funded separately.

4.1.2 Project Management

The study team is organized based on tasks and levels of responsibility to ensure good communication between all personnel. The Assessment Manager (Kathryn Jahn, USFWS) has overall project oversight responsibility and provides direction to the Quality Assurance Coordinator. The Assessment Manager also provides direction to the Principal Investigator and Co-Principal Investigator via the Project Coordinator. The Project Coordinator is responsible for ensuring that adequate coordination and communication occurs amongst the Assessment Manager, Quality Assurance Coordinator, Principal Investigator or Co-Principal Investigator. The Principal Investigator and Co-Principal Investigator are responsible for the project's design and implementation and provide guidance and technical expertise as needed to the study team and statistician. They will also work with the Project Coordinator and Quality Assurance Coordinator to ensure that the study is consistent with the overall QA objectives of the NRDA.

The work plan was developed to provide detailed and explicit instructions for the research staff to follow in collecting the study data. The plan has been reviewed, commented on, and approved by key parties to the study. Reliance on a detailed, explicit, and fully reviewed plan ensures that:

- Study objectives, methods, procedures, and details are documented.
- Data are collected in a systematic and consistent way throughout the study.
- Each member of the study team adheres to the requirements of the plan. In particular, the Principal Investigator and Co-Principal Investigator must ensure that their research staff adheres to the plan. Each team member is required to sign a statement that they have read the plan and understand it.

Events may arise during this study that requires changes to the procedures documented in the work plan. Deviations from the work plan will be documented in writing, with a detailed explanation of the reasons for these deviations. Predetermined deviations from the plan will be conducted only after the approval of the Principal Investigator or Co-Principal Investigator.

4.2 DATA GENERATION AND ACQUISITION

4.2.1 Data Quality Objectives

Data developed in this study must meet standards of precision, accuracy, completeness, and comparability, and be consistent with sound scientific methodology appropriate to the data quality objectives (DQOs).

4.2.1.1 Precision

The degree of mutual agreement will be tested among individual measurements of the same property under similar prescribed conditions, such as replicate measurements of the same sample. Precision is concerned with the “closeness” of the results. For this study, repeated independent measurements will be performed to assess the precision of several biological assays. Precision will be expressed as the relative standard deviation (RSD) between these replicate measurements on a single sample, and for the hormone assays, will be expressed as Coefficient of Variation.

4.2.1.2 Accuracy

The degree of agreement of a measurement with an accepted reference value and may be expressed as the difference between the two measured values or as a percentage of the reference value. For this study, evaluation of accuracy will be performed using a positive control sample or reference standard as specified in the SOP for each biological end point.

4.2.1.3 Completeness

Defined for this study as the percentage of the planned data collections compared to data actually collected within the work plan specifications. Because there is uncertainty due to the variables in number and viability of available eggs and hatchlings, the assessment of completeness achieved will be assessed in two ways. First, completeness will be assessed by comparing planned sampling versus samples collected at the end of the study. Secondly, the DQO for completeness

of data analysis is 95%, which pertains to no more than 5% of the data points collected are to be rejected as unreliable.

4.2.1.4 Comparability

Defined as the measure of confidence with which results from this study may be compared to another similar data set. For this study, evaluation of comparability will be performed using external reference standards or an internal standard prepared from a serum pool extract or a standard prepared within our laboratory, aliquoted and frozen into individual units for utilization within each assay as an internal quality control measure. These comparisons will also take into consideration inter-assay variability due to reagent differences. For example, antibodies used in hormone assays may differ in the forms of their cross reactivity with closely related hormones thereby providing differing absolute concentrations.

4.2.2 Study Documentation

All study procedures and results will be documented on data sheets, which will be placed in binders and retained for review. To the extent possible, information will be recorded on pre-formatted data sheets. The use of pre-formatted data sheets is a QA/QC measure designed to ensure that all necessary and relevant information is recorded for each sample and each sampling activity

- serve as checklists for the Principal Investigator, Co-Principal Investigator and their staff to help ensure completeness of the data collection effort
- assist the research staff by making data recording more efficient
- minimize the problem of illegible or hard-to-follow notebook entries

The researcher performing each procedure will be responsible for recording information on data forms.

Data entries will be made in waterproof ink, and corrections will be made with a single line through the error accompanied by the correction date and corrector's initials. Each completed data sheet will be reviewed, corrected (if necessary), and initialed by the Principal Investigator, Co-Principal Investigator, or their designee. Following completion of the study, data sheet originals will be retained.

4.2.3 Sample Identification Procedures

Strict sample identification procedures will be used throughout the study. The sample identification procedure will begin when an egg is collected. Each egg will be identified by a unique egg code.

The four-letter code of EABL will be used for eastern bluebirds. Each egg will be assigned a unique egg code as follows: Series of numbers 1-199 for Patuxent Wildlife Research Center, and 400 and higher for Hudson River. Samples from each egg/embryo will be identified by a sample ID encompassing the egg code, species, and year, e.g. 01-EABL-2009. Sampling of embryos and hatchlings will include body weight, organ weights, and collection of tissue.

The sample identification described above will be recorded on all data sheets used to document all procedures. This identification along with tissue type will be transferred to all other sample types originating from the egg, including hatchlings (live and sacrificed), and necropsy samples.

The sample ID will be used to uniquely identify all samples, either on a label or written directly on the container. The code will be recorded using either waterproof marker or laser printed clear plastic labels that withstand -80°C. If applicable, the label should also include the type of sample and date of collection and researcher's initials.

4.3 ASSESSMENT AND OVERSIGHT

The QA management plan specifies that studies that generate data will be audited to ensure that the project-specific plans are being properly implemented. Several mechanisms for internal audits of the data generation process will be used for the avian egg injection study. These mechanisms include:

- A project management structure that defines clear lines of responsibility and ensures communication between researchers and trustees. Clear responsibilities and communication can serve as a means of providing internal audits of the study as it proceeds.
- A requirement that laboratory notebooks and data forms be completed daily and be reviewed weekly by the Principal Investigator or Co-Principal Investigator.
- The use of pre-formatted data sheets that serve as a checklist for study procedures and assay results.

The Quality Assurance Coordinator or designee will conduct an audit of the procedures and documentation of the study.

4.4 DATA VALIDATION AND USABILITY

This study employs documented, repeatable procedures to perform the experiments and assays required to generate the data for this study. The work plan has been reviewed for the adequacy of the design and proposed methodology. The original data sheets and other study records will be maintained and archived for a minimum of eight years. Disposal of these records will require the approval of the Assessment Manager. Findings from this study can be reviewed against the data sheets to ensure that the data presented in the reports represent complete and accurate information. Chemistry contaminant data will be validated as specified in the Analytical QA Plan.

The Principal Investigator or Co-Principal Investigator will perform oversight of all egg injections and data collection for measurement endpoints. They will validate that Project Scientists and Technicians are correctly following the standard operating procedures and correctly documenting the results.

Data analysis will be performed using JMP version 7, SAS Institute Inc and SAS programming but not be limited to these statistical programs. All numeric data presented in reports will

contain basic statistical properties and uncertainty. The robustness of each parameter studied will be presented.

4.5 CHAIN OF CUSTODY PROCEDURES

Chain of Custody (COC) procedures will be used during the field sample collection and transfer to the laboratories for incubation or analysis. The purpose of COC is to assure the integrity of each sample and be able to clearly identify who was responsible for the sample at each step. The COC procedure will begin when an egg is collected from the nest. That collection is documented on field data forms (Avian Egg Collection Data Sheets), which clearly identify the team member(s) responsible, as well as the date and time. The egg collection forms will clearly identify to whom the sample was delivered for further processing, and will also include the date and time.

The immediate team members are personally responsible for the care and custody of the samples that are in their possession. A sample is in custody of the immediate team member if any of the following occur:

- The sample is in the individual's physical possession;
- The sample is within view after being in possession;
- The sample is in a locked or sealed container that prevents tampering after being in possession; or,
- The sample is in a designated secure area.

When the samples are packed in coolers or other containers for shipment to the laboratory or storage facility, completed COC records will accompany the samples. The COC form will contain the following information:

- 1) Project name;
- 2) Sample identification (unique for each sample);
- 3) Sample matrix (e.g., egg contents, liver) which may be part of the sample ID;
- 4) Name and signature of individual relinquishing custody;
- 5) Name and signature of individual accepting custody;
- 6) Sample shipping date and mode.

Other information such as date of sample collection, collection location, and jar sizes may be on the COC form or on accompanying documentation.

An original COC record for the samples in that cooler will accompany each shipping container. All sections of the COC form will be completed. Indication of the number of coolers per shipment (e.g., 1 of 3) will be listed on the form if more than 1 container is shipped. Once the

form is completely filled out, it will be placed securely inside the cooler (in a plastic sealable bag to keep it dry). Field personnel will maintain a copy of the COC to keep with the air bill. The cooler will be sealed with custody seals or the containers inside the cooler may be sealed with custody seals. Custody seals are used to detect unauthorized tampering with samples after sample collection until the time of use or analysis. Signed and dated gummed paper seals may be used for this purpose. The seals will be attached so that they must be broken to open the shipping container. Each cooler will be sturdy and well sealed with strapping or other tape. All samples will be kept in locked locations or with custody seals at all times until shipped.

An air bill, Federal Express shipping label, etc. can be used to document the transfer of a sample from the field team to an intermediate storage location, the analytical laboratory, or archive freezer.

Coolers or other containers containing samples will be opened at the analytical laboratories or archiving facility only by a person authorized to receive the samples. The containers will first be inspected for integrity of the chain of custody seals or other signs of tampering. The receipt of each sample in the coolers or containers will be verified on the COC forms. The signed COC forms will be photocopied, and the photocopy will be mailed to the sending party. Samples will be stored in a secure area according to procedures documented for each analytical facility.

5 PERSONNEL

Principle Investigator

The Principal Investigator (PI) is a neuroendocrinologist with over twenty five years of experience studying avian neuroendocrinology and reproduction. The PI will oversee all aspects of the studies.

Co-Principal Investigator

The Co-Principal Investigator is an endocrinologist with 16 years experience in studying reproductive and stress physiology in amphibians, birds and mammals (including humans). The Co-PI also has 8 years experience with project management in GLP compliant laboratories for both pre-clinical and clinical research, as well as having extensive field. The Co-PI will work closely with the PI on all aspects of the study, plan logistics, data collection, data analysis and will coauthor publications.

Research Technician

The Research Technician has many years of experience in avian biology and has worked with the PI for more than a decade. The Research Technician is familiar with all aspects of both field and laboratory based egg injection studies and will be involved with all aspects of these studies, including ordering materials and general coordination of laboratory tasks.

Support Staff

The PI and Co-PI will work with a variety of support staff throughout the project.

The full names, contact information, written signature and written initials of all individuals working on this project shall be maintained in the project file.

6 LITERATURE CONSULTED

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- Lavoie ET and Grasman KA 2007. *Effects of in ovo exposure to PCBs 126 and 77 on mortality, deformities and post-hatch immune function in chickens.* *J Tox Env Health A*, 70: 547-558.
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- Yorks AL. 1999. *Effects of polychlorinated biphenyls (PCBs) on reproduction, physiological processes and biomarkers in tree swallows (Tachycineta bicolor).* Dissertation University of Maryland.

7 STANDARD OPERATING PROCEDURES

SOP HR #001: Recording and Handling Data for Avian Egg Injection Study

This protocol describes procedures for recording and handling data in this laboratory.

Procedure

- 1) Blank data sheets are available in electronic format on the lab server in the "Lab Protocols" folder.
- 2) Data entry:
 - Entries will be made in ink.
 - All blank cells in the sheets should be filled with data, or marked with "NA". Large areas left blank (such as the bottom part of a partially-filled sheet) should be crossed out.
 - Any changes will be made by crossing through the error with a single line, and initialing and dating the change.
- 3) After hard copies of data sheets are filled out they must be reviewed by the PI or Co-PI then stored in the project notebook in the PI's office in a locked filing cabinet.
- 4) Data should be input as soon as possible, after collection, into electronic files, (Excel or JMP) and files stored on the PI's computer.
- 5) Back up copies should be made to a CD after any additions or changes to files are made. A back-up copy of data on CD shall be stored at the homes of the PI and Co-PI.
- 6) Any deviations from the protocols will be written out in detail by the investigator and added to the project notebook.

SOP HR #021: Monitoring and Recording Temperature and Humidity in Egg Incubators

This protocol describes procedures for monitoring and recording temperature and humidity in both the Georgia Quail Farms (GQF) and Kuhl incubators. Monitoring must be undertaken twice per day (am and pm) when the incubators are in use, and a week prior to incubation of any eggs. All personnel must be trained in the use of the equipment and SOP's before beginning.

Procedure

- 1) All information is recorded on the 'INCUBATOR RECORD SHEET' (DOC CONT #007).
- 2) Additional sheets can be found in the 'DOCUMENT CONTROL' binder. This binder should not be removed from its location.
- 3) One sheet must be used per incubator. First label the sheet with the appropriate incubator name and study. Attach sheet to a clipboard. The sheet must remain on the clipboard until completed, at which time it must be reviewed by the PI or Co-PI, then filed with the other raw data from the appropriate study.
- 4) A copy should be made to file as part of the 'INCUBATOR USE LOG'.
- 5) Data entry:
 - Entries must be made in ink.
 - Any changes will be made by crossing through the error with a single line, and initialing and dating the change.
 - Dates should be written as DAY/MONTH/YEAR. Example: 17 Jul 07
 - Temperature must be recorded in degrees Celsius (°C)
 - Time should be recorded as a 24 hour clock. Example: 2.15pm is 1415 h
- 6) WET BULB temperature: saturate the cotton sock in the top level of the incubator with water, then place one end in the orange lidded Schott bottle filled with water inside the incubator, and the other end on the dial thermometer on the side of the incubator. Allow to equilibrate for 5 min with the incubator doors sealed, then read. Record the measurement. Remove the sock and recap the bottle.
- 7) There are two 'Traceable Humidity/Temperature Pens' in each incubator. One at the top and one at the bottom. MIN/MAX recordings of temperature and humidity must be collected twice daily from each.
- 8) MIN/MAX TEMPERATURES: Ensure that the display shows degrees Celsius. The temperature control buttons are located on the front of the 'Traceable Humidity/Temperature Pen'. Press the 'THERMO MIN' button once to read the minimum temperature since the last measurement. Record the temperature. Press it a second time to return to current temperature. Press the 'THERMO MAX' button once to read the maximum temperature since the last measurement. Record the temperature. Press the 'THERMO MAX' to return to current temperatures.

- 9) Reset the temperatures by pressing 'THERMO MIN', then 'THERMO RESET', followed by 'THERMO MIN' to return to current temperature. Do the same for resetting 'THERMO MAX'.
- 10) MIN/MAX HUMIDITY: The humidity control buttons are located on the front of the 'Traceable Humidity/Temperature Pen'. Press the 'HYGRO MIN' button to read the lowest humidity since the last reading. Record. Press again to return to current % humidity. Press the 'HYGRO MAX' button to read the highest humidity since the last recording. Record. Press again to return to current % humidity.
- 11) To reset humidity press 'HYGRO MIN' and 'HYGRO MAX' simultaneously.
- 12) If 'HL' or 'LL' is flashing on the display this means the humidity in the incubator is beyond the limits of the hygrometer and there may be a fault in either the hygrometer or the incubator.
- 13) WATER LEVEL: record the height of the water in the bucket on top of the incubator to the nearest centimeter. If the water level is low, fill the bucket and note on the re-fill level.
- 14) Finally, initial the details.
- 15) Any deviations from the protocols will be written out in detail by the investigator and added to the project notebook.

SOP HR # 029: Necropsy of Altricial Hatchlings

This protocol outlines appropriate dissection techniques and sample storage conditions for tissue obtained from small hatchling altricial birds such as the eastern bluebird and tree swallow.

Procedure

- 1) Record time necropsy is initiated and completed. Record all data on appropriate data sheet.
- 2) Weigh the hatchling to the nearest 0.01 g on the Mettler PG503-S scale.
- 3) Kill the hatchling by cervical dislocation and decapitate with scissors. Collect a blood spot onto a clean piece of card, gauze or cotton swab labeled with the sample ID. Store each sample individually in a sealed paper envelope. Trunk blood can also be collected into a microcentrifuge tubes or glass culture tube for steroid analysis, if required by the work plan. This blood is allowed to stand at room temperature for at least 15min, then centrifuged for 15min at 2500g at 4°C. The serum can then be collected and stored at -80°C until assay.
- 4) Dissect out the brain and weigh to the nearest 0.01g. Freeze on dry ice if applicable to the work plan being used
- 5) Quickly dissect out the heart (trying to remove it before it has stopped beating), weigh it and preserve in appropriate fixative following External SOP # 001: Briefly these procedures are as follows: trim the heart of blood vessels in a standard manner from sample to sample, being careful not to remove any heart muscle. Rapidly immerse the heart in ice cold potassium chloride (25 mM) until it stops beating (watch closely) then rinse quickly with cold PBS. Dab excess liquid from the heart, weigh it (to the nearest 0.01mg using the Mettler MT5 balance) and then preserve it in cold 4% paraformaldehyde for 24-72 hours.
- 6) Dissect the liver, without the gall bladder, and weigh the liver (to the nearest 0.01mg using the Mettler MT5 balance). Cut the liver into three approximately equal parts and flash freeze them in liquid nitrogen for biochemical analysis.
- 7) Remove the spleen, yolk and gastro-intestinal tract and weigh to the nearest 0.01mg if applicable to the work plan. Yolk can then be frozen for steroid and/or PCB analysis if applicable to the work plan. Spleen and GI tract can be discarded according to appropriate procedures.
- 8) Remove both right and left thyroid at the same time. The thyroid is located at the caudal point of the thymus just anterior to the heart. Thyroid is within the thorax, ventral to and bound by fascia to the carotid artery; however if the head has been removed the tissue holding it in position will have shifted, so the thyroids may be in a more anterior position. Place thyroids together in a 1.5 mL micro-centrifuge tube, and freeze thyroids on dry ice (thyroids are too often too small to get an accurate weight in hatchling tree swallows).
- 9) Remove the bursa, weigh it (to the nearest 0.01mg using the Mettler MT5 balance) and fix it in 4% paraformaldehyde fixative using a minimum of fifteen parts fixative to one part tissue volume.

- 10) Identify the gonads to determine gender. Males have two circular shaped testes. Females have one left ovary. Dissect and store at -80°C if appropriate to the study. In tree swallows and eastern bluebirds these organs are too small to weigh.
- 11) The adrenal glands are located just anterior to the gonads. If required by the work plan dissect these out and also store at -80°C . These organs are also too small to weigh.
- 12) Remove lungs and weigh. Freeze on dry ice for drying if necessary according to the appropriate work plan.
- 13) If required by the work plan remove one of the legs and store at -80°C for oxidative damage assessment in muscle tissue.
- 14) Discard remainder of carcass appropriately.

Long term storage:

Store frozen tissues at -80°C . Store fixed tissue at room temperature or at 4°C as appropriate.

Equipment Needed:

Scales sensitive to 0.00001 grams

(Mettler MT5)

Scales (510 - 0.001 g) Mettler Toledo PG503-S

Dissecting scissors and forceps

Swabs and envelopes for blood collection

Dry Ice

Cryovials

1.5 mL microcentrifuge tubes

25 mM potassium chloride

4% paraformaldehyde

Liquid nitrogen

Labor: ideally a minimum of four people participate to ensure rapid dissection and storage of tissues

Data Sheets

“Hatchling Sampling Data Sheet”

SOP HR #030: Field Collection Of Avian Eggs for Contaminant Analysis and Hatching

Introduction

Tree swallow eggs from a PCB-contaminated location will be collected late in incubation and incubated to hatching. A subsample of eggs from the PCB-contaminated location will be selected for contaminants analysis. Hatchlings can be compared to those collected from uncontaminated sites.

Materials and Equipment

Scientific collecting permits

Field notebook, writing instruments (pencils/pens/permanent markers)

Padded egg collection boxes (hard-sided container, e.g., Tupperware or tackle box, with padding such as sawdust or holofill)

DOC CONT #029: Avian Egg Collection Data Sheets

Procedures

- 1) Collected eggs should be whole and not cracked. Embryos for hatching at the laboratory should be viable and developing.
- 2) Eggs for contaminant analysis can be collected either at the completion of the clutch or at the end of incubation, depending on the analysis to take place. Consult the appropriate work plan for that season to determine what is appropriate.
- 3) For tree swallows and eastern bluebirds, the following approach should be used: Incubation doesn't start until the clutch is complete. Monitor nests every two to three days. Birds generally lay eggs at one day intervals with a maximum clutch size of about 5-7 eggs for tree swallows and 4-6 eggs for eastern bluebirds. However, incubation can be interrupted if bad weather occurs immediately following or prior to completion of a nest. If this occurs check and candle the eggs daily until the clutch is complete and begins development. Tree swallow eggs can remain unincubated in cooler weather for up to a week.
- 4) When a nest is 2-5 days pre-hatch (based on when the clutch was completed and incubation began), that is, at approximately day 10, collect one egg from the nest for hatching at the laboratory. If applicable collect another egg(s) for contaminant analysis.
- 5) For each egg collected, complete the appropriate information on the Avian Egg Collection Datasheet. Maintain separate Avian Egg Collection Datasheets for eggs to be transported to the laboratory and for eggs to be analyzed for contaminants.
- 6) Place eggs in individually numbered compartments (one for each egg or eggs from each clutch). A list of the egg codes associated with each compartment will be placed inside the container. A fishing tackle box with compartments lined with sawdust or holofill is ideal – all eggs should be treated the same. Place this box in a hard-sided container with sufficient padding. Transport to the processing laboratory in a hard container avoiding temperature extremes and jostling.

- 7) For eggs that are going to be analyzed for contaminants and not incubated: Refrigerate eggs until opened, no longer than 48 hours. Processing of eggs for contaminants analysis will be completed on a daily basis as much as practical. Follow Standard Operating Procedure for Removal of Avian Egg Contents for Contaminants Analysis, Hudson River NRDA, compositing the 2 eggs from each nest in one jar. Archive samples at NYSDEC laboratory within two weeks of collection.

- 8) For eggs that are going to be incubated: Transport promptly to the laboratory. Prompt transport under appropriate conditions is essential. Use of a “Koolatron” to maintain a proper temperature of eggs during transport is recommended. A hot water bottle can be substituted if a Koolatron is not practical or malfunctions. Maintain a temperature of about 90 to 95°F (32-35°C), unless the transport time is going to be 8 hours or more, in which case a temperature as close as possible to 99.5°F (37.5°C) should be maintained. Complete chain of custody transfer of samples from field collection crew to the laboratory crew on Egg Collection Data Sheet.

External SOP #001: Fixation, Dehydration and Embedding of Large Heart Specimens

(acceptable for adult rodent hearts and most adult and hatchling avian species)

- 1) Dissect the heart and place in ice-cold 25 mM KCl until it stops beating. Rinse thoroughly with 1x PBS. You may find that cutting the very tip of the apex off of the left ventricle will help to completely flush the blood out of the heart cavities.
- 2) Immerse the specimen in a large (10x more than amount of tissue) volume of ice-cold 10% neutral-buffered formalin.
- 3) Allow tissue to fix overnight @ 4°C. The specimen should not remain in fixative for longer than 24 hrs.
- 4) The following day remove fixative and add a similar volume of 1x PBS for 30 min @ 4°C, repeat. This will remove excess fixative from the tissue.
- 5) Immerse the specimen in 5% sucrose overnight @ 4°C. It is acceptable to leave the specimens at 4°C over the weekend, but no longer than 3 days. This step will enhance penetration of the wax or OTC media.
- 6) Remove the tissue from the vial and place in a labeled cassette immersed in 50% ETOH to be transferred to the dehydrator. (We use an automatic dehydrator and embedder. This system dehydrates through 50%, 70%, 80% 95%, 100%, 100% for 1 hr each.
- 7) Immerse tissue in 1:1 Xylene: EtOH mixture for 1x 30min at room temperature.
- 8) Immerse tissue in 100% xylene for 1x15min at 58°C (in oven).
- 9) Immerse tissue in 1:1 xylene: paraplast for 1hr at 58 °C (in oven).
- 10) Immerse tissue in paraplast for 3x 30min at 58°C (in oven).
- 11) Place two Pasteur pipette volumes of paraffin wax (~3ml) in tear away mold. Heat spatula w/ Bunsen burner, scoop tissue from vial and place in mold. Orient tissue in mold using a probe that has been heated w/ the Bunsen burner. Place 1-2ml additional volumes of paraffin wax on top of tissue being careful not to disrupt tissue orientation. Let paraffin solidify for a min of 12hr.

Note: Steps 7-9 should not be lengthened as prolonged exposure of tissues to xylene will make them brittle.

External SOP #002: Hematoxylin and Eosin Staining for Morphology

- 1) Dewax slides in Hemo-D 3x3min, pour used solution back into bottle for reuse (~10x reuse).
- 2) Rehydrate slides in 100%, 95%, 70% EtOH for 3min each, pour solution back into bottle for reuse (~10x reuse).
- 3) Rehydrate in 1xPBS solution for 3min.
- 4) Stain in Gill's Hematoxylin (Fisher CS400-ID) for 2-5 seconds, pour used stain back into bottle for reuse.
- 5) Rinse for 3min in running tap water.
- 6) Stain in 0.5% Eosin for 6min (Fisher E-511 Eosin Y; 2.5g in 500 ml 1xPBS. Autoclave. Store in refrigerator 4°C)
- 7) Rinse for 30 seconds in running tap water
- 8) Dehydrate in 70%, 100% EtOH for 30 seconds each, pour solution back into bottle for reuse.
- 9) Place in Hemo-D solution for 3min; slides should be removed for coverslipping one at a time, as to prevent drying out.
- 10) Coverslip w/ Cytoseal, removing slides one at a time. No air bubbles should be present under slide.

Notes: After using Hemo-D and EtOH ~10x, dispose of it and use fresh.
Hematoxylin stains nuclei, Eosin stains cytoplasm.

DOC CONT #015: Hatchling Necropsy Sampling Sheet:

Study: _____ Date: _____

Species: _____

Egg Code: _____ Collection site: _____

Treatment: _____

Concentration: _____ Injection site: _____

Sample	Collection
Body weight (g)	
Blood (Y/N?)	
Liver (mg)	
Heart (mg)	
Left thyroid (mg)	
Right thyroid (mg)	
Bursa (mg)	
Yolk (mg)	
Brain (mg)	
Gender (M/F or indeterminate)	
Left testis (mg)	
Right testis (mg)	
Ovary + uterus (mg)	
Adrenals (mg)	
GI tract (mg)	
Feces (mg)	
Spleen (mg)	
Lung (mg)	
Leg muscle (mg)	

Dissector: _____ Recorder: _____

Name/Initials

Name/Initials

Reviewer: _____ Date: _____

DOC CONT #029: EGG COLLECTION DATA SHEET

Work Plan: _____

Study Site: _____

Page ____ of ____

Use a new sheet daily.

Collector: _____

Data Recorder _____

Egg Code	Location ¹	Date Collected ²	Embryonic Day	Time Collected ³	Eggs Warm Yes or No	Egg Destination ⁴ and Comments
	Signature					Signature
			Name			

1 Sub-site and nest box #; 2 In MM/DD/YEAR format; 3 In 24-hour format; 4 Contaminant Analysis (CA), Archive (AR) or Incubation & Hatch (I&H)
 Custody of samples listed above transferred from field collection crew to laboratory crew as follows:

Relinquished by: _____
 Signature Print Name Company/Title Date Time

Received by: _____
 Signature Print Name Company/Title Date Time

Data Sheet checked by: _____ Date: _____
 Name/Initials

