

**STUDY PLAN FOR
AVIAN INJURY STUDY
YEAR 3 (2008)**

**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

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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 also determined that it was appropriate to conduct an avian egg injection study and began such a study in 2006. 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. 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. Analysis of data from these studies is ongoing.

The Trustees proposed work for Year 3 of the avian injury study, releasing a Draft Avian Injury Study Plan for Year 3 (2008), dated March 17, 2008, for public review and comment, in accordance with the Hudson River NRDA Plan. All comments received on the Draft Avian Injury Study Plan have been considered by the Trustees in preparing this Final Study Plan. Where warranted, the Trustees incorporated these public comments on the Draft Avian Injury Study Plan into this document to produce this Final Avian Injury Study Plan. A Responsiveness Summary, noting public comments and the Trustees' response to those comments, will be provided by the Trustees in the near future.

The work for 2008 entails: (1) continuation of the egg injection studies conducted on tree swallows (*Tachycineta bicolor*), American kestrels (*Falco sparverius*) and Eastern bluebirds (*Sialia sialis*) in 2006 and/or 2007, along with a pilot study of injection of a PCB mixture into eggs of Eastern screech owl (*Otus asio*), and (2) a comparison of endpoints in tree swallow and Eastern bluebird eggs collected at Upper Hudson River sites with eggs collected from control sites at the Patuxent Wildlife Research Center (PWRC).

Samples will be collected for potential assessment of the following endpoints in tree swallow and Eastern bluebird eggs from PWRC and the Upper Hudson River:

- Embryo mortality
- Deformities
- Body and organ weights (heart, liver and bursa)
- Bursa histology
- Heart histology
- Thyroid gland T4 content
- CYP450 enzyme induction (liver)
- Oxidative stress (liver)
- Genetic sex
- Gene expression

Samples will also be collected from American kestrels and Eastern screech owls from PWRC for assessment of these endpoints.

Embryomortality, deformities and body organ weights will be assessed in all birds. The Trustees will then determine whether to proceed with assessment of the other endpoints noted above, specifically: bursa histology, heart histology, gene expression, oxidative stress (liver), CYP450 enzyme induction (liver), thyroid gland T4 content, and genetic sex.

The Draft Avian Injury Study Plan noted that these endpoints that were proposed for assessment in Year 3 had been studied in Years 1 and 2 and the work plan regarding such had been peer reviewed at that time. In the interest of efficiency and to not unnecessarily increase the cost of the NRDA, the Trustees determined that Year 3 peer review of these same injury endpoints would be limited in scope to new and/or otherwise relevant information regarding them that was not reviewed earlier. The Trustees subsequently identified no new or otherwise relevant information regarding those endpoints that was not reviewed earlier, and thus no additional formal peer review of the Draft Avian Injury Study Plan was conducted.

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. In addition, birds may be exposed to PCBs by consuming food items 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 also determined that it was appropriate to conduct an avian egg injection study and began such a study in 2006 pursuant to study plans (Hudson River Natural Resource Trustees 2006 and 2007a) that were, as appropriate pursuant to the Hudson River NRDA Plan (Hudson River Natural Resource Trustees 2002), subject to peer review and public review and comment.

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 determined it was appropriate to conduct a second year of avian egg injection work (Hudson River Natural Resource Trustees 2007b). 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 (*Sialia sialis*). Analysis of data from the Year 1 and Year 2 studies is ongoing.

The Trustees now plan to conduct a third year of avian injury work as described in this Final Study Plan. The work for 2008 entails: (1) continuation of the egg injection studies conducted on tree swallows (*Tachycineta bicolor*), American kestrels (*Falco sparverius*) and Eastern bluebirds (*Sialia sialis*) in 2006 and/or 2007, along with a pilot study of injection of a PCB mixture into eggs of Eastern screech owl (*Otus asio*), and (2) a comparison of endpoints in tree swallow and Eastern bluebird eggs collected at the Patuxent Wildlife Research Center (PWRC) and Upper Hudson River sites, with eggs collected at PWRC being used as natural controls for PCB contamination.

Samples will be collected for potential assessment of the following endpoints in tree swallow and Eastern bluebird eggs from PWRC and the Upper Hudson River:

- Embryo mortality
- Deformities
- Body and organ weights (heart, liver and bursa)
- Bursa histology

- Heart histology
- Thyroid gland thyroxine (T4) content
- CYP450 enzyme induction (liver)
- Oxidative Stress (liver)
- Genetic sex
- Gene expression

Samples will also be collected from American kestrels and Eastern screech owls from PWRC for potential assessment of these endpoints.

Embryomortality, deformities and body organ weights will be assessed in all birds. The Trustees will then determine whether to proceed with assessment of the other endpoints noted above, specifically: bursa histology, heart histology, gene expression, oxidative stress (liver), CYP450 enzyme induction (liver), thyroid gland T4 content, and genetic sex.

The Draft Avian Injury Study Plan noted that these endpoints that were proposed for assessment in Year 3 had been studied in Years 1 and 2 and the work plan regarding such had been peer reviewed at that time. In the interest of efficiency and to not unnecessarily increase the cost of the NRDA, the Trustees determined that Year 3 peer review of these same injury endpoints would be limited in scope to new and/or otherwise relevant information regarding them that was not reviewed earlier. The Trustees subsequently identified no new or otherwise relevant information regarding those endpoints that was not reviewed earlier, and thus no additional formal peer review of the Draft Avian Injury Study Plan was conducted.

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.

2.0 INTRODUCTION

This Final Study Plan is for Year 3 (2008) of an avian egg injection and field study.

The primary aim of this study is to integrate data collected in both the lab and field to gain a better appreciation of the impact of PCBs on free-living avian species. This study plan will be carried out in two parts, the first is the *in ovo* lethality aspect, which will be a continuation of the injection studies conducted on tree swallow (*Tachycineta bicolor*), American kestrel (*Falco sparverius*) and Eastern bluebird (*Sialia sialis*) eggs in 2006 and 2007 (Hudson River Natural Resource Trustees 2007a, 2007b) with a pilot study of injection of a PCB mixture into eggs of Eastern screech owl (*Otus asio*). The second part is a comparison of endpoints in tree swallow and Eastern bluebird eggs collected at the Patuxent Wildlife Research Center (PWRC) and Upper Hudson River sites, with eggs collected at PWRC being used as natural controls for PCB contamination.

Specific endpoints to be addressed in the study are discussed in section 4.3. Egg injection and collection studies for all species will use the same endpoints.

3.0 PURPOSE AND OBJECTIVE

The Trustees will conduct laboratory and/or field studies of tree swallows, Eastern bluebirds, American kestrels and Eastern screech owls to evaluate whether specific avian species in the vicinity of the Hudson River are injured due to exposure to PCBs.

This study 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 studies described below for work in 2008 to evaluate the effects of exposure of tree swallows, American kestrels, Eastern bluebirds and Eastern screech owls to PCBs, through exposure via avian egg injection or through environmental exposure in the field.

The attached work plans entitled, “American Kestrel and Eastern Screech Owl Egg Injection Studies 2008” (Appendix A) and “Tree Swallow and Eastern Bluebird Egg Injection Studies 2008” (Appendix B) describe the avian investigation that the Trustees will implement to evaluate whether specific avian species in the vicinity of the Hudson River are injured due to exposure to PCBs. The attached work plans include 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 designs described in Appendix A and B for work in 2008 to evaluate the effects of exposure of tree swallows, American kestrels, and Eastern bluebirds to PCBs, through exposure via avian egg injection or through environmental exposure in the field, and to evaluate, in a pilot study, the effects of exposure of Eastern screech owl to PCBs through exposure via avian egg injection. Sections 4.1 through 4.3 below summarize the work described in Appendices A and B.

4.1 EGG INJECTION STUDY WITH TREE SWALLOW, AMERICAN KESTREL, EASTERN BLUEBIRD AND EASTERN SCREECH OWL EGGS

Tree swallow, American kestrel, Eastern bluebird and Eastern screech owl eggs at PWRC will be injected *in situ* with either PCB 77 (for tree swallows) or with a mixture of PCB congeners that mimics the spectrum of congeners found in avian eggs in the Upper Hudson River (for American kestrels, Eastern bluebirds and Eastern screech owls). The PCB congener mixture to be injected is the 58-congener PCB mixture described in the Trustees’ revised study plan for 2006 work (Hudson River Natural Resource Trustees 2007a); that 58-congener PCB mixture contains PCB congeners in proportions equivalent to those of the congener composition of spotted sandpiper eggs from the Hudson River. The study will be supplemented with additional tree swallow eggs from an upstate New York colony (Cobleskill Reservoir, New York), to be injected *in situ* with PCB 77.

For the tree swallows and Eastern bluebird, the eggs will be naturally incubated for about the first two-thirds of incubation by the parents. This should provide excellent hatching success when eggs are brought to the lab for artificial incubation in the last one-third of incubation.

For American kestrels and Eastern screech owls, the eggs will be entirely incubated in the laboratory.

These data will provide a median lethal dose for the field levels of PCB congeners found in tree swallow, American kestrel, Eastern bluebird and Eastern screech owl eggs and allow assessment of the consequences of exposure to PCBs. Samples from tree swallow, American kestrel, Eastern bluebird and Eastern screech owl eggs will be collected for potential assessment of the endpoints identified in Section 4.3.

Appendices A and B provide additional details regarding the egg injection studies of American kestrels and screech owls, and tree swallows and Eastern bluebird, respectively.

4.2 COLLECTION AND ASSESSMENT OF TREE SWALLOW AND EASTERN BLUEBIRD EGGS FROM PWRC AND THE UPPER HUDSON RIVER

Tree swallow and 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. In this instance there will be no experimental manipulations of the eggs — endpoints will relate directly to environmental conditions. Samples from tree swallow and Eastern bluebird eggs will be collected for potential assessment of the endpoints identified in Section 4.3.

4.3 ENDPOINTS AND STATISTICAL ANALYSES

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

- Embryo mortality
- Deformities
- Body and organ weights (heart, liver and bursa)
- Bursa histology
- Heart histology
- Thyroid gland T4 content
- CYP450 enzyme induction (liver)
- Oxidative stress (liver)
- Genetic sex
- Gene expression

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

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 Appendices A and B.

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

AMERICAN KESTREL AND SCREECH OWL EGG INJECTION STUDIES 2008

**AMERICAN KESTREL AND SCREECH OWL EGG
INJECTION STUDIES 2008**

HUDSON RIVER NATURAL RESOURCE DAMAGE ASSESSMENT

15th of May, 2008

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): _____

Signature: _____ Signature: _____

Date: _____ Date: _____

Title: _____ Title: _____

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1 INTRODUCTION & OBJECTIVES

1.1 PREVIOUS WORK

The work plan presented here is a continuation of experiments with American kestrel (*Falco sparvensus*) eggs conducted in the 2006 (Hudson River Natural Resource Trustees 2006a) and a small pilot study on Eastern Screech owl (*Megascops asio*) eggs.

In 2006 American kestrel eggs were injected with 24 or 98ug/g of PCB mixture ('sandpiper' mixture), which resulted in high mortality. However, the sample size was low and lethality of both doses was greater than 70%, indicating that lower doses will be necessary to determine the median lethal dose and endpoints of interest in this species when affected by PCB exposure.

1.2 OBJECTIVES

1) To further assess the effects of PCB exposure on *in ovo* development of American kestrels. American kestrel eggs (approximately 40) are available this year from Patuxent Wildlife Research Center (PWRC) and will be injected with the PCB mixture under laboratory conditions. In addition, anatomical, biochemical and histological endpoints will be evaluated in tissues from the exposed hatchlings.

2) Conduct a pilot study on the effects of the PCB mixture by injecting a limited number of eastern screech owl eggs obtained from PWRC.

1.3 PROPOSAL SUMMARY

American kestrel and screech owl eggs will be obtained from the PWRC breeding facility for injection studies. In 2006 American kestrel eggs were successfully incubated, but the concentrations of PCB mix injected resulted in high mortality, indicating that the levels were beyond the median lethal dose. This year we will use lower concentrations to determine what the effects of *in ovo* PCB exposure are in this species. Screech owls have not yet been studied in these experimental protocols, but are thought to be among potentially more sensitive species that may experience effects of PCB bioaccumulation. Concentrations of the PCB to be injected into these eggs will be the same as those used for the American kestrels. Tissues collected from surviving hatchlings will also be used to validate biochemistry and histology assays for these species.

2 WORK PLAN

2.1 STUDY SPECIES AND SITES

American kestrel (*Falco sparvensis*)

Eastern screech owl (*Megascops asio*)

These eggs will be obtained from the PWRC animal colony.

Animal care protocols will be provided by the relevant Animal Care and Use Committees.

Based upon available information (Yorks 1999), PWRC is a historically uncontaminated site. Concentrations of PCBs and other contaminants have been low or non-detectable.

Eastern screech owl eggs collected in the Hudson River in 2002 and 2003 had levels of PCBs ranging from 1-8 μ g/g egg (Hudson River Natural Resource Trustees 2005).

2.2 EGG INJECTIONS

American kestrel and eastern screech owls eggs will be injected with 'sandpiper' PCB mix (please see Hudson River Natural Resource Trustees 2006b for detail) and incubated in the laboratory. In 2006 American kestrel eggs injected with PCB mix at 24 and 98 μ g/g had more than 70% mortality in response to both doses. This year we will reduce the doses to 2.5 and 25 μ g/g.

Currently we have no data on the lethal doses of PCBs in eastern screech owls. The 2005 Hudson River Natural Resource Trustees Report showed that contaminant levels in eastern screech owl eggs ranged from 744 to 8010ppb. To study both lethality and the endpoints of interested listed in section 2.4, we will use 2.5 and 25.0 μ g/g of PCB mixture found in the sandpipers.

All eggs will be hatched in the laboratory. Hatchlings will be necropsied within 24h of hatch and tissue collected for analysis.

2.3 PCB MIXTURES

PCB mixtures will be provided in our EG-1 vehicle to both the American kestrel and eastern screech owl eggs.

2.4 ENDPOINTS

The literature indicates potentially adverse effects associated with these measures following exposure to PCBs.

2.4.1 Embryo mortality

Mortality due to incubation or injection protocol will be minimized, as much as possible, through consistent monitoring of moisture loss and injection of low volumes using aseptic technique. American kestrel eggs have been successfully incubated in the laboratory and it is expected that eastern screech owls will require similar conditions. As such it should be possible to determine mortality due to PCB load.

2.4.2 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.

2.4.3 Body and organ weights

Organ weights can be affected by PCBs in chickens. Body weight at hatch is generally not affected by *in ovo* PCB exposure (Lavoie and 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, heart and bursa.

2.4.4 Bursa Weight and Histology

Decreases in bursa weight and altered cellular morphology are strongly associated with PCB toxicity in chickens (Fox and Grasman 1999; Lavoie and Grasman 2007). Studies in quail have shown similar effects with exposure to other xenobiotics. Impacts on the bursa during B-cell development could result in reduced immunological fitness as nestlings and adults.

2.4.5 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. Histological and other analyses of heart samples will be conducted under a separate work plan with separate SOPs.

2.4.6 Thyroid gland: Thyroxine content

Thyroid hormone balance is impacted by PCB exposure (McNabb and Fox 2003). A decrease in thyroxine reserve as reflected by thyroxine concentration in the gland at time of hatch could be detrimental to growth and survival because thyroid hormone plays a role in thermoregulation and metabolism. The former is especially critical for altricial species, which hatch without thermoregulatory control. Analysis of thyroid gland thyroxine content will be conducted under a separate work plan with separate SOPs.

2.4.7 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.*, 1996a). 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.

2.4.8 Oxidative Stress (liver)

In Hoffman *et al.* (1996b), for American kestrels there were some associations between oxidative stress (ox-red glutathione ratio) and increasing PCB 126. Liver tissues will be collected and preserved as part of this study. Analysis of oxidative stress markers in liver tissue will be conducted under a separate work plan with separate SOPs.

2.4.9 Genetic sex

Blood samples for genetic sexing will be collected for this study, and genotyping will be analyzed by DDC Veterinary, Fairfield, Ohio. 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. Gonads will also be weighed as an indication of phenotype.

3 EXPERIMENTAL DESIGN

3.1 EGG COLLECTION AND INCUBATION

Eggs collected from the managed PWRC colony will be used for injection of the 'sandpiper' PCB mix. Eggs will be transferred to the processing laboratory to begin incubation and will be injected after 5 days. This is equivalent to approximately ED3 in the Japanese quail. This procedure will be followed as it has been highly successful for injection and incubation of American kestrel eggs at PWRC.

Reproduction in eastern screech owls is described in the Gehlbach (1995) species account. Pairs will often reuse nest sites in consecutive years. Two to eight (average 3-5) white eggs are laid every two days and incubation begins after laying the first egg. Eggs are approximately 15-20g in weight. The incubation period ranges from 26 to 34 (average 27) days. Females do most of the incubating with some male assistance and food can be stockpiled during early incubation. They are single brooded, but may re-nest if the first clutch is lost. Eastern screech owls hatch in semi-altricial 2, that is, they are downy but incapable of opening their eyes.

Eggs will be assigned to treatment groups on the day of injection. The four-letter code of AMKE will be used for American kestrel samples and ESOW will be used for eastern screech owl samples. Each egg will be assigned a unique egg code using a series of numbers 01-40, species, and year, e.g. 01-AMKE-2008. (see sections 3.4 and 4.2.3).

American kestrel eggs will be injected and incubated according to Standard Operating Procedure (SOP) HR #023. This SOP was approved for use in the 2006 work plan.

Currently there is no SOP for incubation of eastern screech owl eggs. Following consultation with individuals who have successfully incubated eggs of numerous bird species in the laboratory SOP HR #023 will be used as the basis for incubation of eastern screech owls. **For screech owl eggs, there are two modifications to this SOP:**

- First, in section 6 of SOP HR #023 the humidity of the incubator for the eastern screech owl eggs will be modified to 40% relative humidity.
- Second, eggs will be candled and weighed twice weekly to determine moisture loss (DOC CONT #012), rather than 3 times per week as described for American kestrel eggs (DOC CONT #016). During incubation eggs should lose approximately 15% of their weight, or at a rate of 0.5% per day (SOP HR #023).

The injection protocol component of the eastern screech owl experimentation will be identical to that described in SOP HR #023 for the American kestrel.

At time of candling, any dead eggs (first week of development) or those that did not develop will be removed and the egg contents will be archived (according to approved protocol SOP HR #025) using the appropriate data sheet (DOC CONT #019). Any dead embryos (second half of incubation) will be evaluated for stage of development and deformities; abnormal embryos will be photographed, preserved, and archived. Type of deformity will be recorded (DOC CONT #018)

Incubator temperature and humidity will be monitored twice daily according to approved SOP HR 021 and the information will be recorded on the appropriate data sheets (DOC CONT #007).

Standard Operating Procedure (SOP's) and Data Sheets Used:

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

SOP HR #023: Egg Injection and Incubation Procedure for American Kestrel Eggs.

SOP HR #025: Removal of Avian Egg Contents for Contaminants Analysis

DOC CONT #007: Incubator Record Sheet

DOC CONT #012: Eastern Screech Owl Egg Moisture Loss

DOC CONT #016: American Kestrel Egg Moisture Loss

DOC CONT #018: Deformity Score Sheet

DOC CONT #019: Avian Egg Processing Data Sheet

3.2 EGG INJECTIONS

American kestrel and eastern screech owl eggs will be dosed through the air cell at 0.1µl/g egg of the 'sandpiper' PCB mixture in vehicle (Table 1). The mixture must be warmed to approximately 30°C prior to injection.

Treatment and injection volumes will be recorded on DOC CONT #013 and #017.

Table 1: Egg injection numbers and dosages for American kestrel and eastern screech owl eggs collected from PWRC.

Treatment (µg/g PCBs)	# Eggs	Predicted Lethality (%)	Resulting Sample Size
American kestrels			
Untreated	6-8	20	5-6
Vehicle	6-8	20	5-6
2.5	7-10	25-30	5-6
25.0	9-14	40-50	5-6
totals	25-40		20-24
Eastern screech owls			
Untreated	6	20	4-5
Vehicle	6	20	4-5
2.5	8	~25	4-6
25.0	11	30-50	4-6
totals	25		16-22

Standard Operating Procedure (SOP's) and Data Sheets Used:

SOP HR #023: Egg Injection and Incubation Procedure for American Kestrel Eggs.

DOC CONT #013: Eastern Screech Owl egg treatment and incubation

DOC CONT #017: American Kestrel egg treatment and incubation

3.3 DOSING SOLUTIONS

The injection vehicle has PCBs added according to the publicly released Hudson River Natural Resource Trustees report (2006b). Appropriate concentrations of the PCB mixture solutions will be provided by U.S. Geological Survey's Columbia Environmental Research Center. The preparation and storage of the 58-congener mixture has been fully described in the Hudson River Natural Resource Trustees report (2006b).

3.4 EGG HATCHING AND TISSUE SAMPLING

Any eggs that fail to hatch will be opened and condition of the embryo noted. Deformities will be scored on the appropriate data sheet (DOC CONT #018). Briefly, they are 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. Photographs of deformed and normal embryos and hatchlings will be taken for reference.

Embryos will be dissected within 24h of hatching according to approved SOP HR #004. Data will be recorded on Hatchling Necropsy Sampling Sheets (DOC CONT #015) Samples from each hatchling or egg will be identified by a unique code (“sample ID”) encompassing the egg code, species, and year, e.g. 01-AMKE-2008 for a American kestrel collected in 2008. Each tissue that is collected will be labeled with the complete sample I.D. such as (01-AMKE-2008) and the name of the type of tissue: liver, bursa, heart or thyroid. Blood for genetic sexing will be collected on sample cards provided by the contracted laboratory, and labeled with the sample ID.

Standard Operating Procedure (SOP’s) and Data Sheets Used:

SOP HR #004: Necropsy of Hatchling Birds

DOC CONT #015: Hatchling Necropsy Sampling Sheet

DOC CONT #018: Deformity Score Sheet

3.5 BIOLOGICAL TISSUE ANALYSES

3.5.1 *Histological:*

Bursa and heart tissue will be preserved in appropriate fixatives. Bursa tissues will be embedded, sectioned and stained by standard methods according to the approved SOP HR 015. Slides will be labeled and well organized for retrieval and review. The SOP for the heart histology will be described and conducted under separate Work Plans.

3.5.2 *Gender genotyping*

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

3.5.3 *Thyroid glands*

Thyroid glands from each hatchling will be collected and stored at -80° C in a microcentrifuge tube. Analysis of thyroid gland thyroxine content will be conducted under a separate work plan that will be based on the approved 2007 work plan.

3.5.4 Livers

Livers will be divided into two (2) samples stored frozen in separate cryovials. One sample will be prepared and used according to the workplan for the measurement of cytochrome P450 activity in liver microsomes by EROD assay. This workplan has been approved for EROD measurement in hatchling tree swallows and the same procedures will be used for hatchling eastern screech owls and American kestrel tissues. The second liver sample will be used for measurement of oxidative stress markers. The procedures for these measurements will be described and conducted under a separate work plan.

3.6 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 the PCB mixture for eggs injected or natural PCB exposure for birds from the Upper Hudson River. “Controls” refers to either uninjected/vehicle injected eggs in the egg injection study or eggs and birds from the reference sites for the field study. “Birds” represents any life stage for which an endpoint is measured.

3.6.1 Embryo survival or hatchability

Compare the embryo survival or hatchability of eggs exposed to PCBs with eggs that are not exposed to PCBs.

General Hypotheses

HO: Hatchability of eggs injected with the PCBs is equal to the hatchability of control eggs

HA: Hatchability of eggs injected with the PCBs is less than the hatchability of control eggs in a dose response manner

Statistical tests

Fisher Exact probability tests and probit analysis will be used for determining significant decreases in survival or hatchability and for determining median lethal doses.

3.6.2 Deformities

Compare the 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.

3.6.3 Histology

Compare the histology of bursa and heart of PCB exposed birds to unexposed birds

General Hypotheses

HO: Bursa and heart morphology in PCB exposed birds are not different than controls

HA: Bursa and heart morphology in PCB exposed birds are different compared to controls and are proportionally related to the dose of treatment

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.

3.6.4 T4 content of thyroid gland

Compare the thyroxine (T4) content of thyroid glands from PCB exposed birds to that of unexposed birds.

General Hypotheses

HO: Thyroid hormone (T4) content of thyroid glands in PCB exposed birds is not different than controls

HA: Thyroid hormone (T4) content of thyroid glands in PCB exposed birds differs from controls and is proportionally related to the dose of treatment.

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, T4 concentrations 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.

3.6.5 EROD activity

Compare the 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 and is proportionally related to the dose of treatment

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.

3.6.6 Oxidative Stress

Compare oxidative stress in liver samples from PCB exposed birds to that of unexposed birds

General Hypotheses

HO: Oxidative stress level in PCB exposed birds is not different than controls

HA: Oxidative stress level in PCB exposed birds is higher than controls and is proportionally related to the dose of treatment.

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, oxidative stress indicators 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.

3.6.7 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 and are proportionally related to the dose of treatment

HA: Bursa weight in PCB exposed birds is lower compared to controls and is proportionally related to the dose of treatment

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.

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 (PI) 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 may require 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 determined among individual measurements 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 AMKE will be used for American kestrel samples and ESOW will be used for eastern screech owl samples. Each egg will be assigned a unique egg code as follows: Series of numbers 001-040. Samples from each egg/embryo will be identified by a sample ID encompassing the egg code, species, and year, e.g. 01-AMKE-2008. 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 a waterproof marker. 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.

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 IN version 5, release 5.1, 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 (approved SOP HR 026 and DOC CONT #020) 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 PI is a neuroendocrinologist with thirty years of experience studying avian neuroendocrinology and reproduction. The PI will oversee all aspects of the studies.

Co-Principal Investigator

The Co-PI is an endocrinologist with 15 years experience in studying reproductive and stress physiology in amphibians, birds and mammals (including humans). The Co-PI also has 7 years experience with project management in GLP compliant laboratories for both pre-clinical and clinical research, as well as having extensive field experience. 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.

Scientific Consultant

The Scientific Consultant is an avian toxicologist with experience in egg injection studies and immune and endocrine disruption studies in birds. The Scientific Consultant will participate in data analysis, quality assurance and will co-author 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. The Research Technician will be heavily involved with all aspects of these studies, including ordering materials and general coordination of laboratory tasks.

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

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- Tullett, S.G. (1990). Science and the art of incubation. *Poult. Sci.* 69: 1-15.
- 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

NOTE: All SOP's and data sheets shown below have been approved for use in previous studies unless otherwise stated.

SOP HR #001: Recording and Handling Data

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.
 - 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.
 - Data recorder will date and initial each sheet; the sheets will contain documentation such that each individual performing the injections/measurements can be identified.
- 2) 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 Co-PI's office.
- 3) Data should be input as soon as possible, after collection, into electronic files (Excel or JMP) and files stored on the PI's or Co-PI's computers. Data entry must be 100% verified against the hard copy by someone other than the person who performed the initial data entry.
- 4) 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 will be made weekly and will be stored at the homes of the PI or Co-PI.

Any deviations from the protocols will be written out in detail by the Principal Investigator and added to the project notebook.

SOP HR #023: Egg Injection and Incubation Procedure for

American Kestrel (*Falco sparverius*)

This protocol outlines procedures for incubating eggs and injecting chemicals into the eggs of American kestrels. The purpose of which is to mimic maternal deposition of chemicals into the egg and determine toxicity toward the embryo.

Smallwood and Bird (2002) in *Birds of North America* describe the eggs, their incubation and hatching of American kestrel chicks and is summarized herein:

Eggs are approximately 34 x 28 mm and 10 to 18 g in weight. Egg color is variable from white to cream to yellowish to light red-brown with blotches and mottling of varying shades but especially brown shades. Eggs are generally not glossy. Incubation length for American kestrel eggs averages 27 to 29 days in captivity but approximately 30 days in the wild. “Apparently relatively cold-hardy...Captive-produced eggs hatched successfully in an incubator that shut down twice due to power failures to the point of ice forming on added water.” Kestrels are considered semi-altricial. An embryo takes approximately 48-52 hours to hatch from start of pipping, the female assists the chick out of the shell. Hatchling’s skin is pinkish and covered in sparse white down, bill, cere and talons are white-pink and legs and yellowish. Belly is prominently protruding and nearly naked. Hatchling is able to raise head, open its bill and ‘peep’.

Egg Collection from Patuxent NWRC Kestrel Colony

- 1) Collect eggs between 8 and 9:30 am (during feeding to minimize disturbance).
- 2) Have rubber gloves on to collect the eggs. Have a pair of leather gloves on hand in case you have to push aside a female kestrel.
- 3) Label eggs in pencil at the pointed end. If a nest box is #660, then label the egg 660-1, or 660-2 etc depending on egg order.
- 4) Place the eggs in a cushioned container for transport back to the laboratory.

Incubation Procedures

- 1) Upon receipt of the eggs at the laboratory, examine them noting any evidence of damage or embryonic development (by candling). Note on the coding sheet the source, nest number, egg number for the clutch etc. Wash eggs in a 40°C 1% betadine solution, and then rinse in 40°C water. Submerge eggs for less than 5 seconds in the betadine solution, and lightly scrub the cuticle off with hands and dry with a paper towel (wash one egg at a time as a pencil label often rubs off).
- 2) Re-label the egg with its number if it has washed off.

- 3) Weigh eggs to the nearest one hundredth of a gram and note weight. Eggs collected from the Patuxent colony in thus far in 2006 weigh between 13 and 18 grams (personal communication with Moira McKernan).
- 4) Hold eggs in cold storage (13°C) for not more than 4 days (Pisenti et al. 2001).
- 5) Warm eggs by leaving them at room temperature for one hour.
- 6) Place eggs on their sides in the Kuhl incubator in Kuhl brand pheasant egg racks. Incubate the eggs at 99.5°F and 55-65% humidity (84°F wet bulb). In addition to the hourly turning (60°) of the eggs done automatically by the incubator, turn the eggs 180° twice a day at 9 am and 5 pm +/- 1 hr. Draw an O and an X on opposite sides of the egg. At the morning time point turn the egg so that the O is showing and at the afternoon time point turn the egg so that the X is showing. This step provides additional turning that may be necessary for wild bird eggs.
- 7) Check moisture loss by weighing the eggs on Monday, Wednesday and Friday of each week and adjust the humidity appropriately to ensure correct moisture loss with egg mass loss averaging between 9 and 14% over entire incubation period. For an average 14 g kestrel egg over a 28 day incubation, moisture loss should average 2 grams or approximately 0.5% or 0.07 g per day to achieve a 14% weight loss over 28 days.

Steps number 6 and 7 are adapted from the methods of Pisenti et al. (2001) and personal communication with staff at Patuxent NWRC. Pisenti et al. (2001) described a 9% egg mass loss for embryos surviving to hatch and staff at Patuxent NWRC adjusted humidity as needed to attain a 14% mass loss with good hatchability using a Kuhl brand incubator.

- 8) On approximately days seven, twelve and twenty-four of incubation, candle the eggs and remove infertile and dead eggs. Open eggs containing dead embryos and stage the embryo based on the guide in Pisenti et al. (2001). Note the stage of the embryo and any deformities on the egg treatment log.
- 9) On embryonic day 24, transfer eggs to a 'hatcher' incubator (99.5°F and 70-75% humidity) or separate tray in the same incubator with each egg placed in its own compartment fashioned from plastic mesh.
- 10) Necropsy within 24 hours after hatch.

Injection Procedures

- 1) Five days into incubation (expected equivalent age to a 3 day old quail embryo, i.e. 18% of incubation based on a 28 day incubation for a kestrel egg, candle eggs and remove infertile or dead eggs if possible to see through the egg shell. Retain any infertile and dead eggs for contaminant analysis if warranted.
- 2) Assign the eggs to treatment groups with consideration of number of eggs available, number of eggs from the same clutch, and optimal number of treatment groups.

Weigh each egg to the nearest one hundredth of a gram. Calculate and record the volume of dosing solution to be added to each egg. Round the volume to the nearest 0.01 μL .

- 3) Make injections into the egg as follows, allowing the eggs to be outside the incubator for not more than 30 minutes:
 - a. Wipe the blunt (air cell) end of the egg with 70% ethanol.
 - b. Gently make a hole in the egg with the Dremel drill.
 - c. Inject the vehicle or PCB mixture solution, 0.1 $\mu\text{L/g}$ egg into the air cell, with a micro-pipettor and extended tip.
 - d. Seal the hole with paraffin.
- 4) Place eggs back into the incubator on their sides. Randomly place treatment groups in the egg racks. Avoid placing eggs in the very top, very bottom, very back and very front of the incubator.

Equipment Needed

Rubber and leather gloves

Foam filled case for egg transport

Betadine

Incubators: Natureform NMC2000 or GQF Sportsman 1502 or Kuhl

Pheasant egg trays

Light for candling

Ethanol and tissue or alcohol wipes

Dremel drill with fine point attachment

Paraffin and tool to apply it to eggs

Heating block

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

Rainin Pipettman with extended tips: one tip per egg

Data Sheets

DOC CONT #017: American kestrel Egg Treatment and Incubation Log

DOC CONT #016: American kestrel Egg Moisture Loss Data Sheet

DOC CONT #018: Deformity Score Sheet.

Literature Consulted

Smallwood, J. A., and D. M. Bird. 2002. American Kestrel (*Falco sparverius*). In *The Birds of North America*, No. 602 (A. Poole and F. Gill, eds.). The Birds of North America, Inc., Philadelphia, PA.

Pisenti JM, Santolo GM, Yamamoto JT, Morzenti AA. 2001 Embryonic development of the American Kestrel (*Falco sparverius*): External criteria for staging. *J. Raptor Res.* 35:194-206.

DOC CONT #012: EASTERN SCREECH OWL EGG MOISTURE/WEIGHT LOSS

(ALL ENTRIES MUST BE MADE IN INK)

INCUBATOR: _____

STUDY: _____

EGG ID	QC	ED1	ED4	ED8	ED11	ED15	ED18	ED22	ED25
	date								
	mass								
	initials								
	date								
	mass								
	initials								
	date								
	mass								
	initials								
	date								
	mass								
	initials								

RECORD DATE AS DD/MM/YEAR

Reviewed by: _____ Date: _____

DOC CONT #016: AMERICAN KESTREL EGG MOISTURE/WEIGHT LOSS

(ALL ENTRIES MUST BE MADE IN INK)

INCUBATOR: _____

STUDY: _____

EGG ID	QC	ED1	ED4	ED7	ED10	ED13	ED16	ED19	ED22	ED25
	date									
	mass									
	initials									
	date									
	mass									
	initials									
	date									
	mass									
	initials									
	date									
	mass									
	initials									

RECORD DATE AS DD/MM/YEAR

Reviewed by: _____ Date: _____

SOP HR #004: Necropsy of Hatchling Birds

Hatchling birds are maintained in the incubator in which they hatch for 18-24 hours before necropsy to allow complete drying of feathers. Birds are sampled as close to 24 hours after hatch as possible. This protocol outlines appropriate dissection techniques and sample storage conditions for several tissues including:

- Blood for serum
- feces for steroid analysis
- Brain
- Liver for CYP450 or chemical analysis
- Yolk or gastrointestinal tract for chemical analysis
- Thymus and Bursa for mass and histology
- Thyroid for thyroid hormone radioimmunoassay
- Gonads for histology or biochemical analysis

Procedure

- 1) Bring 10 to 20 hatchlings at a time to the necropsy room in a small box and keep in the box on a warm surface such as a heating plate on a low setting.
- 2) Weigh the hatchling.
- 3) Kill the hatchling by cervical dislocation and decapitate with scissors. Immediately collect trunk blood into a 12 x 75 mm glass tube. Set tube aside allowing blood to clot for serum collection.
- 4) Immediately remove the brain from the head, intact, and drop it directly into dry ice powder. After at least one minute on dry ice, fold the brain into a cold piece of aluminum foil and keep temporarily on dry ice.
- 5) If appropriate, dissect away the remaining yolk sac, weigh it, and place it in a chemically clean glass container and keep on wet ice.
- 6) Dissect the liver, remove the gall bladder and weigh the liver. Place the liver in a cryovial, or mince it and divide the tissue between multiple cryovials, and flash freeze it in liquid nitrogen for CYP450 analysis. Store a portion of liver for further analysis.
- 7) Dissect each lobe of the thymus from the neck and remove each thyroid at the same time. The thyroid is located at the caudal point of the thymus just anterior to the heart. Weigh all four organs individually in their storage vials to prevent drying on weigh paper. Freeze thyroids on dry ice.

- 8) Remove the bursa, weigh it and place it in a 1.5 mL microcentrifuge tube in Bouin's fixative.
- 9) Identify the gonads to determine gender. Males have two kidney shaped testicles. Females have one left ovary. Remove gonads intact on a portion of the carcass's back and fix in 10% buffered formalin or other appropriate fixative or freeze.
- 10) Discard carcass appropriately.

DOC CONT # 015: Hatchling Necropsy Sampling Sheet:

Study: _____ **Date:** _____

Species: _____

Egg Code: _____ **Collection site:** _____

Treatment: _____

Concentration: _____ **Injection site:** _____

Sample	Collection
body weight (g)	
blood (volume estimate)	
liver (mg)	
heart (mg)	
left thyroid (mg)	
right thyroid (mg)	
bursa (mg)	
thymus piece	
brain (mg)	
gender (M/F or indeterminate)	
left testis (mg)	
right testis (mg)	
ovary + uterus (mg)	
adrenals	
GI tract (mg)	
feces (mg)	
spleen (mg)	
leg muscle g)	

Dissector: _____ **Recorder:** _____
Name Name

Reviewer: _____ **Date:** _____
Name and signature

SOP HR #015: Histological Analysis Of Japanese Quail Tissue:

Bursa of Fabricius

This protocol describes the necessary steps in storage and preparation of tissues for histological analysis.

Procedure

When collected, bursas are first stored in Bouin's solution. Before dehydration, the bursas can be brought to 70% ethanol (ETOH) and stored in this solution almost indefinitely. To bring to 70% ETOH, put the bursas that are stored in Bouin's solution into 3 changes of 50% ETOH for 6 hours each. After the last 6 hours, the bursas can be placed into the 70% ETOH solution.

1. Dehydration:

70% ETOH → 85% ETOH – 1 hour

85% ETOH → 95% ETOH – 40 min

95% ETOH → 95% ETOH – 40 min

95% ETOH → 100% ETOH – 1 hr or overnight

2. Clearing:

100% ETOH → 1:1 xylene:100% ETOH – 1 hr

1:1 xylene:100% ETOH → 100% xylene – 1 hr

100% xylene → 100% xylene – 1 hr

100% xylene → 100% xylene – 1 hr

*note – xylene can be substituted with chloroform

For steps 1 & 2, a glass container is preferable. Small plastic snap-cap vials are also suitable when working with small tissues, however, xylene should not remain in plastic containers for long, otherwise they may disintegrate. The tiny perforations in standard cassettes, are too large to prevent small tissues such as hatchling bursae from escaping.

3. Embedding:

100% xylene → paraplast – 40 min

paraplast → paraplast – 1 hr

paraplast → paraplast – 1 hr

Pour new paraplast into boat with tissue in an ice bath, or embed your tissue into new paraplast using an embedding machine.

*note – oven should not exceed 56/60°C

4. Cut tissue in 10 – 12.5 um sections. Place 3 sections on each of 3 slides (total of 9 sections).

5. Staining Procedure:

Xylene	2 min
Xylene	2 min
100% ETOH	1 min
100% ETOH	1 min
95% ETOH	1 min
95% ETOH	1 min
tap water (non-running)	10 min
Mayer's hematoxylin	15 min
Lukewarm running tap water	20 min
Eosin	2 min
95% ETOH	2 min
95% ETOH	2 min
100% ETOH	2 min
100% ETOH	2 min
100% ETOH	2 min
Xylene	2 min
Xylene	2 min
Xylene	2 min

Let remaining xylene run off slide, then coverslip immediately to prevent dessication of the tissue.

6. Endpoints

Record a digital image of each section used.

For bursa analysis, measure number of follicles per section, number of vacuoles, thickness of epithelial layer, and follicle size. Other qualitative aspects to also consider with each section is arrangement of bursal buds and arrangement of epithelial layers.

Equipment Needed

Oven

Image Analysis Equipment

Staining Jars

References

Pait, T. 1999. Effects of endocrine disrupting compounds on the fish *Fundulus heteroclitus*.

PhD Dissertation, University of Maryland, College Park, MD.

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

- 11) All information is recorded on the 'INCUBATOR RECORD SHEET' (DOC CONT #007).
- 12) Additional sheets can be found in the 'DOCUMENT CONTROL' binder. This binder should not be removed from Rm 3115 of the Animal and Avian Sciences building.
- 13) 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, Scientific Consultant or the Co-PI, then filed with the other raw data from the appropriate study.
- 14) A copy should be made to file as part of the 'INCUBATOR USE LOG'.
- 15) 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
- 16) 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.
- 17) 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.

- 18) 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.
- 19) 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'.
- 20) 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.
- 21) To reset humidity press 'HYGRO MIN' and 'HYGRO MAX' simultaneously.
- 22) 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.
- 23) 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.
- 24) Finally, initial the details.
- 25) Any deviations from the protocols will be written out in detail by the investigator and added to the project notebook.

DOC CONT #007: INCUBATOR RECORD SHEET (ALL ENTRIES MUST BE MADE IN INK)

INCUBATOR: _____

STUDY: _____

DATE (dd/mon/yr)	TIME (24h clock)	TEMP (°C)				HUMIDITY (%)				Water level	Initials	
		WET BULB	TOP		BOTTOM		TOP		BOTTOM			
			MIN	MAX	MIN	MAX	MIN	MAX	MIN			MAX

Reviewed by: _____ Date: .

SOP HR #025: Removal of Avian Egg Contents for Contaminants Analysis

Introduction

Avian eggs are a common sample for contaminants analysis. An accurate analysis depends upon getting the egg contents from the shell to a clean sample jar without introducing other sources of contamination. This protocol, which has been developed and refined by many researchers over the decades, was written for those who have minimal experience. Your first egg should be a practice egg. *It is suggested that all personnel practice on several quail eggs to improve technique. Chicken eggs may be used if quail eggs are not available.*

Laboratory Materials And Equipment

- Avian Egg Processing Data Sheets
- paper or other towels
- green scrubby or sponge
- Acculab V-200 balance, weighs to nearest 0.01 gm
- calipers
- Chemically-clean jars, 1 per sample
 - Make sure they are cleaned for the contaminants you are sampling, e.g., I-Chem pesticide/PCBs Series 200 or 300.
 - Size: 4 oz.
- chemically-clean stainless steel scalpel blades (No. 21 or No. 22 with No. 4 handles work well)
- chemically-clean forceps
- aluminum foil sheets (approximately 30 x 30 cm square), 1 per egg
- sharps container for used blades or disposable scalpels

Laboratory Procedures

- 1) Fill out the Avian Egg Processing Data Sheet; use one data sheet per egg.
- 2) If debris is present, rinse egg in cool water while gently scrubbing with green scrubby or sponge. Do not soak the egg.
- 3) Dry and weigh whole egg to the nearest .01 g

- 4) Transfer egg contents to chemically-clean jar using the following procedure:
- I. Use nitrile gloves for this part of the procedure. Avoid letting contents run over your hands into the sample jar. Note that the two eggs collected from a nest will both be placed in one jar for analysis.
 - II. Create a catch basin out of the aluminum foil by turning edges up and securing the corners. This will catch egg contents in case they spill over the edge of the jar. Use a separate piece of foil for each sample. The foil also is a clean place to place your instruments when they are not in use.
 - III. Weigh the clean empty jar with lid on, and note this tare weight on data sheet.
 - IV. Place jar in center of aluminum foil, and loosen the lid.
 - V. Score equator with serrated blade or scalpel blade. Use a new, chemically-clean scalpel blade for each egg. This part takes practice. Cradle the egg in one hand (don't squeeze too tightly!) and gently score while rotating the egg. Many light strokes are preferable to a fewer deeper strokes, increasing the evenness of the score and decreasing the possibility of eggshells not separating cleanly or of punching through the shell. Continue to work on your score until you see the membrane, which usually appears gray underneath the white of the eggshell. When you see the first bit of membrane, remove the lid from the jar so that it will be ready as soon as you need it. Avoid getting shell dust, or anything else besides the egg contents, in the jar. Try to expose the membrane evenly around the entire egg. Often the score line can be used to help pick the egg shell apart using forceps.
 - VI. Place the egg over the jar and cut through membranes with the scalpel. For large eggs a new scalpel blade may be used at this point to reduce the potential for cross contamination and since the blade may become dull during the cutting process. The scalpel can also be used to finish scoring down to the membranes. Pour contents into jar, or use the scalpel to gently scrape if that is necessary. Small stainless steel scoops may also be used to help remove the contents. Use forceps to remove any shell fragments from the jar. Cover the jar.
 - VII. For swallows, hold the egg vertically with air cell end up. Using scissors cut the top of the eggshell just below the air cell. Pour contents into the jar, and use a pipet to gently collect egg contents that don't freely flow out. Use forceps to remove any shell fragments from the jar. Avoid getting shell dust, or anything else besides the egg contents, in the jar.
 - VIII. Save the egg shell and associated membranes separately in a second I-chem jar labeled with the same sample ID as for the egg contents and note 'egg shell' on the jar.
 - IX. The target for the minimum weight of egg tissue is 4 grams for analysis. It may be possible to analyze smaller samples ranging from 1 – 2 grams. Analysis of these

samples may result in a lower ability to detect contaminants due to the lack of mass. An effort must be made to maximize the amount of each sample that is usable. The weight of each sample should be made in the laboratory during egg processing using the following procedure:

- a) Place a small jar on a balance that reads to at least 1 milligram and that has been appropriately calibrated.
- b) Tare the jar or record the jar weight if the balance cannot be tared.
- c) Open the egg, according to the procedures referenced above and empty the contents into the jar.
- d) Record the weight, to the nearest .01g, of the egg contents if the balance was tared. If the balance was not tared, then record the weight for the egg contents and the jar, then subtract the previously recorded weight of the jar. Record the weight of the egg contents in the field notebook and on the jar label.
- e) If egg is developed, estimate age of embryo. Documentation of embryo development is very limited (Powell *et al.* 1998; Bird *et al.* 1984), therefore, documenting this phase of the egg processing is important. Note amount of decay or anything else pertinent to your study, and examine for deformities, particularly bill deformities such as crossed bills or lack of jaws, but also lack of skull bones, club feet, rotated ankles, or dwarfed appendages (Gilbertson *et al.* 1991).
- f) Repeat these procedures for any other eggs that need to be added to the sample jar.

Do not touch or move the jar between steps b. and d. above. It is preferable to add the egg contents to the jar while the jar is still on the balance, immediately after taring the jar.

- 5) Place label on jar. Place clear tape over the label to keep it from getting wet.
- 6) Prepare Chain of Custody records and maintain egg samples under chain of custody.
- 7) Freeze samples. Ship under Chain of Custody (see attached COC form) overnight on dry ice to the sample archive or analytical laboratory.

Literature Consulted

Bird, D.M, J. Gautier, and V. Montpetit. 1984. *Embryonic growth of American kestrels*. Auk 101:392-396.

Gilbertson, M., T. Kubiak, J. Ludwig, G. Fox. 1991. *Great Lakes embryo mortality, edema, and deformities syndrome (GLEMEDS) in colonial fish-eating birds: similarity to chick-edema disease*. J. Toxicol. Environ. Health 33:455-520.

Powell, D.C., R.J. Aulerich, R.J. Balander, K.L. Stromborg, and S.J. Bursian. 1998. *A photographic guide to the development of double-crested cormorant embryos*. Colonial Waterbirds 21(3):348-355.

These egg-processing guidelines were developed by the U.S. Fish and Wildlife Service and modified for the project based on consultation with the author of these guidelines and on conversations with the Quality Assurance Coordinator for this project.

DOC CONT #019: Avian Egg Processing Data Sheet

Processor(s):Name _____ Name _____

Signature _____ Signature _____

Date Processed: _____

Egg Code: _____

Sample ID: _____

Jar lot number _____ **Balance within limits?** Yes OR No

Whole Egg Weight (g): _____

Contents weight:

Weight of jar (g) : _____

Weight of jar + contents (g): _____

Weight of contents (g): _____

Membrane location: ___ with embryo OR ___ with eggshell

Contents condition (embryo development ¹, state of decay, etc.) and other comments:

Other comments: _____

Contaminants disposition (catalog number and date submitted, etc):

¹ None, 1/4, 1/2, 3/4 , full term

Data Sheet checked by: _____ Date: _____

Name/Initials

SOP HR #026 Procedures for NRDA Sample Archive Shipment

Chain-of-Custody (COC)/Transfer Record

1. You may use the original Chain-of-Custody if has at least one set of “Relinquished By” and “Received By” lines remaining.
2. If the original COC is not available, a new COC or Transfer Record (form is attached to these instructions) shall be prepared. List each sample or inventoried container (e.g., rack of vials, etc.) on a separate line, identifying with original field ID, laboratory ID and description or other identifier. (A Transfer Record is attached to this procedure).
3. Record on the COC, as appropriate:
 - Volume or quantity of sample, if available
 - Comments – apparent preservation problems or custody concerns
4. Cross check all sample identifiers from container to COC before packaging the samples.
5. Sign and date the “Relinquished By Signature” block on the COC. Make a copy for your records. Place the COC in a ziplock bag and tape it to the inside lid of the appropriate cooler.

Packaging/Shipping

1. Sample shipments are best made early in the week. Do not ship samples on Friday unless specific arrangements have been made with the courier and Receiver for Saturday delivery.
2. Wrap or package each item, as appropriate and place in cooler/package. Bubble wrap is a good cushion. **Dry ice or other coolants are not cushioning material, because the jars will become loose as the ice melts or evaporates.**
3. Place coolant, e.g., dry ice, wet ice or frozen gel packs (see below for dry ice info) in the cooler *so that the contents will remain at temperature for a minimum of 48 hours.*

For Dry Ice:

- **You must use < 5 kg/package**
 - **The drain plug on the cooler must be taped open for ventilation of carbon dioxide (CO₂) gas that occurs when the dry ice vaporizes**
 - **Indicate that dry ice has been used as a coolant on the shipping documents, however, when less than 5 kg is used as a coolant, dry ice is not considered a “Dangerous Good”.**
4. Seal the lid shut. Wrap duct/shipping tape around either end of the cooler (three times) to ensure a tight seal.

5. Place a minimum of two COC seals on the cooler in such a manner that if the container was opened, the seals would have to be broken.
6. Sign and date the COC seals, which are placed on the outside of the cooler. The same person who signed the COC record should do this.
7. Place clear shipping tape over the COC seals.
8. Adhere the appropriate address label on the top, outside surface of the cooler with clear shipping tape.
9. Fill out appropriate shipping documents:

Coolers are to be sent by Federal Express Priority One-Day service or a comparable, traceable service.

The cooler/package should be sent to:

"Receiving Contact Name" at:

**NOAA Building 32
7600 Sand Point Way NE
Seattle, WA USA 98115-0070**

The contact phone number on the airbill should be Receiving Contact's number

10. Fax or email a Notification of Shipment Form (attached to this procedure) to Receiver. Receiver will:
 - Coordinate receipt with NOAA Shipping and Receiving Department, on the day of arrival
 - Sign the COC in the "Received By Signature" block
 - Make sure that the cooler/package(s) are placed into the archive freezer at NOAA ARD West (Bldg. 32).

APPENDIX B

TREE SWALLOW AND EASTERN BLUEBIRD EGG INJECTION STUDIES 2008

**TREE SWALLOW AND EASTERN BLUEBIRD
EGG INJECTION STUDIES 2008**

HUDSON RIVER NATURAL RESOURCE DAMAGE ASSESSMENT

June 13, 2008

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): _____

Signature: _____ Signature: _____

Date: _____ Date: _____

Title: _____ Title: _____

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1 INTRODUCTION & OBJECTIVES

1.1 PREVIOUS WORK

The work plan presented here is a continuation of experiments with tree swallows (*Tachycineta bicolor*) conducted in 2006 and 2007, and a pilot Eastern Bluebird (*Sialia sialis*) study conducted in the 2007 breeding season.

Previously, the majority of the field work and egg injections have concentrated on tree swallows due to their availability and high tolerance of human intervention at their nest sites. In 2006, tree swallow eggs were collected from two sites with low polychlorinated biphenyl (PCB) contamination: Patuxent Research Refuge (PRR), Maryland and Great Sacandaga Lake, New York. These eggs were injected with PCB 126 in two separate experiments and incubated in the laboratory. In 2007 eggs collected from PRR and Cobleskill, New York (a further low PCB contamination site) were injected in the field with a PCB mixture that is environmentally relevant to tree swallows in the Upper Hudson River (referred to as the 'tree swallow' mixture - see Hudson River Natural Resource Trustees 2007a for detail). Two thirds of egg incubation was conducted in the field, with the eggs being hatched in the laboratory. Mortality and hatchability of the embryos were monitored and median lethal doses were estimated. Hatchlings were dissected and tissues were analyzed for a variety of histological and biochemical endpoints.

In addition, in both years, tree swallow eggs naturally exposed to PCBs in the Upper Hudson River were collected mid-incubation and hatched in the laboratory. These hatchlings were also dissected for the same endpoints used in the experimental groups. Preliminary results from these experiments suggest potential adverse effects from PCB exposure and the same potentially responsive endpoints are proposed for the 2008 study. These egg injection studies could provide strong data for determining avian injury in that they encompass both single PCB congener and environmentally relevant PCB mixture exposure for tree swallows.

In addition to the tree swallow work, a pilot study was conducted in 2007 on eastern bluebird eggs. Eggs were injected with 25 or 100ug/g of 'sandpiper' PCB mix (please see Hudson River Natural Resource Trustees 2006 for detail). None of the eggs that received either of the doses of PCB hatched, and while this could be attributable to low sample size, it could also be the result of greater sensitivity of this species. This year we will expand the eastern bluebird study to encompass the same environmental variables and injection protocols as the tree swallows, but on a smaller scale.

1.2 OBJECTIVES

- 1) To further assess the effects of PCB exposure on *in ovo* development of tree swallows, and eastern bluebirds. To this end we will increase the sample size of eastern bluebird eggs injected with the PCB mixture that mimic profiles found in avian eggs from the Upper Hudson River ('sandpiper' mix). Eggs from Patuxent Research Refuge and a second low contamination site (Cobleskill Reservoir, NY) will be used to increase the overall power of the study, with the goal of approximately 200 fertile eggs.
- 2) To assess the effects of PCB 77 exposure on tree swallows. Both the 'sandpiper' and 'tree swallow' PCB mixtures are composed of 58 congeners, however, they differ in the

concentration of PCB 77 (see Hudson River Natural Resource Trustees 2006 and 2007a for detail). Thus far, we have investigated the effects of the ‘tree swallow’ mixture and PCB 126 on *in ovo* development of tree swallows. PCB 77, like PCB 126 is a coplanar PCB; however, there are species-specific differences in its toxicity and metabolism. Injections of PCB 77 alone will help to provide us with a better understanding of how these compounds affect developing tree swallow embryos.

3) Compare anatomical, histological and biochemical endpoints of naturally and experimentally exposed tree swallow and bluebird eggs. Eggs from both species will be obtained from the Upper Hudson River, Remnant 3 site late in incubation and hatched in the laboratory for sampling. This method was quite successful in 2007 and further collections this season will allow us to increase sample sizes.

1.3 PROPOSAL SUMMARY

To fully understand the effects of PCBs on *in ovo* development, comparisons need to be made using experimentally manipulated eggs and those naturally exposed to PCBs. Tree swallow and eastern bluebird eggs will be injected *in situ* at two low contamination areas, PRR and Cobleskill Reservoir, NY. These eggs will be incubated naturally for the majority of incubation by the parents. In 2006 and 2007 this was shown to provide excellent hatching success when eggs were brought to the laboratory for the last third of their incubations. Eggs incubated in the laboratory from embryonic days zero to two had lower hatching success than those left in the nest for the first two thirds of incubation. Eastern bluebird eggs will be injected with mixtures of PCB congeners that mimic the spectrum of congeners found in eggs in the Upper Hudson River. A congener mix has yet to be determined for the eastern bluebirds so we have chosen to use the ‘sandpiper’ PCB mix. Sandpipers feed primarily along the shallow edges of water courses, which have lower PCB concentrations than the deeper bodies of water. Eastern bluebirds forage in mixed open and wooded habitats on a variety of terrestrial insects and berries (up to 32%) whereas tree swallows consume flying insects (80%), including those newly emerging from waterways, resulting in greater PCB exposure. In the 2007 pilot study eastern bluebirds were shown to be more sensitive to PCB exposure *in ovo* than tree swallows, as such doses will need to be adjusted accordingly. Tree swallow eggs will be injected with PCB 77 as a further experimental series to determine the effects of PCBs on embryonic development.

Eggs will be collected from the Upper Hudson River for potential contaminant analysis. We will also continue studies in tree swallow and eastern bluebird hatchlings from the Upper Hudson River, Remnant 3 site. This will include collection of two to three eggs from each active nest. Three eggs will be collected at approximately day ten of incubation. One egg will be incubated in the laboratory until hatch at which time the chick will be sampled for biochemical and histological endpoints. The other two eggs will be analyzed for PCBs and will be representative of the PCB exposure of the chick that is sampled for biological analyses.

2 WORK PLAN

2.1 STUDY SPECIES AND SITES

Tree swallow (*Tachycineta bicolor*)
Eastern bluebird (*Sialia sialis*)

Both tree swallow and eastern bluebird eggs will be collected under permits from the Patuxent Research Refuge (U.S. Fish and Wildlife Service (FWS)), FWS (Migratory Birds), and Maryland Department of Natural Resources for the studies at Patuxent Research Refuge (PRR), MD; and from New York State Department of Environmental Conservation (NYSDEC) and FWS (Migratory Birds) for Cobleskill Reservoir, NY and Upper Hudson River, NY.

Animal care protocols prepared by the principal investigators (PIs) will be reviewed for approval by the relevant Animal Care and Use Committees. All work will be conducted according to the approved protocols. Federal and State collection permits will be obtained by Kathryn Jahn of USFWS and provided to both animal care committees.

Based upon available information, Patuxent Research Refuge 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 INJECTIONS

Based upon available information, Patuxent Research Refuge 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. Tree swallow and eastern bluebird eggs collected from Patuxent Research Refuge and Cobleskill Reservoir will be used in an egg injection study to determine the effects of PCBs on *in ovo* development.

During the 2007 eastern bluebird pilot study it was found that both the 25 and 100 $\mu\text{g/g}$ doses of the 'sandpiper' PCB mix resulted in 100% mortality of the eastern bluebird embryos. This year dosages will be modified (2.5 and 25.0 $\mu\text{g/g}$) for further determination of the lethal range. However, there were very few eggs available for this pilot study. Therefore, the lower dose (25 $\mu\text{g/g}$) will serve as the high dose for the study this year.

Dosages (please see Hudson River Natural Resource Trustees 2006 for detail) of PCB 77 for tree swallow egg injections will be based on the solubility of PCB 77 in the vehicle and the volume to be delivered. Previously, volumes of 0.4 $\mu\text{l/g}$ of egg were injected into tree swallow eggs. This year it has been possible to obtain an electronic micropipette (0.2-10.0 μl ; Hamilton) that will allow accurate delivery (2.5% precision) of 0.2 μl , thus allowing us to decrease the injection volume and subsequent injection mortality.

All eggs will be hatched in the laboratory. Hatchlings will be necropsied within 24h of hatch and tissue collected for analysis.

2.3 EGG COLLECTION

Both tree swallow and eastern bluebird eggs and embryos collected from the Upper Hudson River will be used to evaluate the PCB exposure and biochemical effects of the PCB exposure in tree swallows and eastern bluebirds. Tissues from the egg injection study will also be evaluated for PCB-associated effects on biochemistry.

Bluebird eggs will also be collected from the Cobleskill Reservoir as an uncontaminated comparator. Currently, there are insufficient numbers of bluebirds nesting within this site to make it a viable option for an injection study, so collections of untreated controls will be carried out.

2.4 PCBs

PCB 77 in a vehicle (CO-1) will be used for tree swallows egg injections and a 58-congener PCB mix ('sandpiper' mix) in a vehicle (EG-1) will be provided for eastern bluebird egg injections.

Appropriate concentrations of the PCB 77 or the PCB mixture will be provided by U.S. Geological Survey's Columbia Environmental Research Center. There will be two solutions of each provided so that the injection volumes will be the same into each egg.

2.5 ENDPOINTS

The literature indicates potentially adverse effects associated with these measures following exposure to PCBs. Of these, embryo mortality, deformities and organ weights will all be collected within 24 hours of hatch. Other endpoints will be analyzed as part of a separate work plan to be provided at a later point, however, tissue will be collected and stored appropriately as part of the current work plan.

2.5.1 Embryo mortality

Incubation in the laboratory is less successful than natural incubation. Therefore, this study will incorporate incubation in the field and movement of the eggs into the laboratory late in incubation. This will improve hatching success of untreated eggs and provide a basis for determining embryo mortality due to PCB injection.

2.5.2 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.

2.5.3 Body and organ (heart, 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, heart and bursa for statistical analysis.

2.5.4 Bursa Weight and Histology

Decreases in bursa weight and altered cellular morphology are strongly associated with PCB toxicity in chickens (Fox and Grasman 1999; Lavoie and Grasman 2007). Studies in quail have shown similar effects with exposure to other xenobiotics. Impacts on the bursa during B-cell development could result in reduced immunological fitness as nestlings and adults.

2.5.5 Gene Expression (microarrays and polymerase chain reaction)

Microarray analyses of Japanese quail (*Coturnix coturnix japonica*) have shown that there are several genes which are responsive to PCB exposure in this species. Many of these genes are highly conserved and so will be investigated further by PCR, particularly those related to oxidative damage, specific receptor activation, inflammation and immune function. Investigation of gene expression will be based on SOP #6.19 of the Revised Work Plan for Tree Swallow, American Kestrel and Chicken Egg Injection Studies for 2006 (Hudson River Natural Resource Trustees 2007b). An updated SOP or deviation will be written to document the specific parameters chosen for this study.

2.5.6 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. Histological and other analyses of heart samples will be conducted under a separate work plan with separate SOPs.

2.5.7 Thyroid gland: Thyroxine content

Thyroid hormone balance is impacted by PCB exposure (McNabb and Fox 2003). A decrease in thyroxine reserve as reflected by thyroxine concentration in the gland at time of hatch could be detrimental to growth and survival because thyroid hormone plays a role in thermoregulation and metabolism. The former is especially critical for altricial species, which hatch without thermoregulatory control. Analysis of thyroid gland thyroxine content will be conducted under a separate work plan with separate SOPs.

2.5.8 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., 1996a). 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.

2.5.9 Oxidative Stress (liver)

In Hoffman et al. (1996b), for American kestrels there were some associations between oxidative stress (ox-red glutathione ratio) and increasing PCB 126. Liver tissues will be collected as part of this study. Analysis of oxidative stress markers in liver tissue will be conducted under a separate work plan with separate SOPs.

2.5.10 Genetic sex

Blood samples for genetic sexing will be collected for this study, and genotyping will be analyzed by DDC Veterinary, Fairfield, Ohio. 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

3.1 TREE SWALLOWS AND EASTERN BLUEBIRDS

Eggs and tissues will be collected, and birds will be handled according to relevant Animal Care and Use Committee guidelines and under permits from USFWS. Eggs will be assigned to treatment groups (untreated, vehicle injected or PCB injected) on the day of injection. Assignment to treatment group will be made under guidance of our statistical consultant. Since we can only estimate the number of eggs available for the study, we will consider sample size, statistical power, sampling day, eggs per breeding pair and dose in determining treatment allocation. The goal will be to maximize the number of eggs per independent parent within each treatment group. We will prioritize treatment groups based on data from previous years where possible.

3.1.1 Patuxent Research Refuge and Cobleskill Injection Studies

This year identical protocols for injection and collection will be conducted at Patuxent Research Refuge (PRR) and Cobleskill Reservoir, NY for tree swallows. Eastern bluebird egg injections will only be conducted at PRR. Approximately 140 tree swallow eggs are expected to be available for injection at PRR (2-4 per nest from ≥ 50 nests), while approximately 40 eastern bluebird eggs (2-4 per nest from 20 nests) are expected. It is hoped that equivalent numbers of tree swallow eggs and nests will be available at Cobleskill. Each tree swallow egg from PRR will be identified with a specific numeric code from 1-199 written in soft pencil (8B) on the pointed end of the egg. Cobleskill tree swallow eggs will be numbered from 200-399. Eastern bluebird eggs are blue-green in color and readily discernible from tree swallow eggs, but they will be labeled as B1-50 at PWRC and B200-250 at Cobleskill to ensure that there is no confusion with samples following necropsy. The same protocol will be used for injecting and incubating eastern bluebird eggs as that for the tree swallows (SOP HR #027). Dosages and projected egg numbers from each site are shown in **Table 1**.

Eggs transferred from the Cobleskill Reservoir will be collected at the same time point (~day 10) as at PRR then stored in a Koolatron brand cooler set at temperature suitable to maintain the eggs for an 8 - 10 hour drive from New York to the processing laboratory.

Table 1: Tree swallow and eastern bluebird injection groups.

The numbers shown in the table represent egg requirements for each site for tree swallows and the requirements for PRR for eastern bluebirds. These are target numbers of eggs; final number will be adjusted depending on availability of eggs.

Treatment ($\mu\text{g/g}$ PCBs)	# Eggs Injected	Predicted Lethality (%)	Resulting Sample Size
Tree swallows (PCB 77)			
Untreated	30 (no injection)	20	25
Vehicle	30	20	25
1.0	36	30.5	25
10.0	45	44.4	25
totals	141		100
Eastern bluebirds ('sandpiper' mix)			
Untreated	8	20	6
Vehicle	8	20	6
2.5	10	25-30	6-8
25.0	14	40-50	6-8
totals	40		24-38

3.1.2 Egg Collections

Up to 20 tree swallow and 10 eastern bluebird eggs will also be collected for background contaminant exposure at PRR, Cobleskill and Remnant 3 of the Upper Hudson River. Eggs from PRR and Cobleskill will be assigned for contaminant analysis to verify the eggs are suitable as a 'clean' source. Eggs from the Upper Hudson River Remnant 3 will be used for contaminant analyses to determine the PCB profile of eggs of both species.

At a minimum, two eggs from each of five nests at each site will be collected, but potentially more eggs will be selected for contaminant analyses. A sample of two eggs per clutch is recommended for determining contaminant levels both in terms of accurate reflection of variation of contaminants within the clutch (Reynolds *et al.* 2004) and to ensure sufficient egg sample volume for analyses. Composited eggs for contaminant analysis will be identified by a combined egg code; for example, 100+101-TRES-2008 for tree swallows, B1+2-2008 for bluebirds. Bluebird eggs weigh about 3 gm (Gowaty *et al.*, 1998; Hudson River Natural Resource Trustees, 2005), which may be sufficient to allow analysis of individual eggs for selected contaminants, if required.

Because the number of eggs available from PRR may be limited, additional eggs will be assigned to PCB analysis from eggs that fail to develop from the un-injected control group. Eggs containing embryos that die early in development will be archived, along with the eggshells (in separate jars).

Nests will be monitored for initiation of egg laying, clutch completion and initiation of incubation. Tree swallow eggs will be injected at embryonic day (ED) 2.5 and later collected at ~ ED10. We will follow the collection practices of Dr. Chris Custer (USGS), in which the monitored nests will be observed daily for eggs, which are laid at one day intervals. When the fifth egg is laid, then two eggs will be injected at ED2.5 for later collection; if a clutch is six eggs, we will inject and later collect three eggs instead of two. In this way, the female should not

abandon the nest because three eggs will remain. In tree swallow eggs, PCB contamination appears not to be affected by egg order (personal communication Drs. Custer).

3.1.3 Embryos from Upper Hudson River

Up to 20 tree swallow and 10 eastern bluebird eggs will be collected from Remnant 3 of the Upper Hudson River for contaminant analysis. A further 10 eggs will be collected at approximately day 10 of incubation and transferred to the processing laboratory in the same way as the Cobleskill eggs. The eggs from Upper Hudson River will allow us to compare the effects of experimental manipulation of PCB exposure to environmental exposure.

Nests will be monitored at the Remnant 3 site for initiation and completion of egg clutch. Clutch size and date of initiation of incubation will be noted. Nests will be observed daily for eggs, which are laid at one-day intervals. At approximately day ten of incubation three eggs will be collected from each nest. Two of these eggs will be preserved for contaminant analysis and the third will be transported to the processing laboratory for incubation and hatch.

Samples collected from each tree swallow hatchling or tree swallow egg will be identified by a unique code (“sample ID”) encompassing the egg code, species and year, e.g. 400-TRES-2008 for a tree swallow collected in 2008. The series of numbers starting at 400 and higher will be used for eggs and embryos from Upper Hudson River, Remnant 3 site. Eastern bluebird eggs are blue-green in color and readily discernible from tree swallow eggs, but they will be labeled as B400-450 at Upper Hudson River, Remnant 3 to ensure that there is no confusion with samples following necropsy. Composited eggs for contaminant analysis will be identified by a combined egg code, for example, 400+401-TRES-2008.

SOPs:

SOP HR #004: Necropsy of Hatchling Birds

SOP HR #027: Egg Injection and Incubation Procedure for Tree Swallow Eggs at Patuxent Research Refuge and Cobleskill Reservoir: Nest Monitoring, Egg Injection, Egg Collection and Egg Incubation

SOP HR #028: Field Collection of Tree Swallow Eggs from Upper Hudson River, New York for Injury Assessment Hudson River NRDA

Note: Both SOPs HR #027 and 028 were developed specifically to tree swallow eggs, but will be applied identically to this pilot study for eastern bluebird eggs.

3.2 EGG INCUBATION AND INJECTION

Eggs from both species will be injected at a time point approximately 18% (ED2.5) of incubation which is specifically defined in SOP HR #027. Eggs will be candled during incubation at least once in the field after injection and upon receipt in the laboratory. At time of candling, any dead eggs (first week of development) will be removed and the egg contents will be archived. Any dead embryos (second half of incubation) will be evaluated for stage of development and deformities; abnormal embryos will be photographed, preserved, and archived.

Tree swallow eggs will be dosed through the air cell with 0.1µl/g of PCB 77 in CO-1 vehicle, while eastern bluebirds will be dosed with 0.1µl/g of ‘sandpiper’ PCB in EG-1 vehicle. Eggs will be sealed with medical adhesive or paraffin wax.

SOP HR #027 has been slightly modified this year to account for a more accurate electronic micropipette (Hamilton) and lower injection volumes. According to the certificate of analysis, the pipette delivers with a 2.75% accuracy at the lowest dose, compared to >10% for most manual micropipettes. However, this pipette only delivers in 0.1µl increments from 0.2µl – 10.0µl. As such eggs will be dosed to the nearest gram in weight. That is 0.2µl for tree swallows and 0.3µl for eastern bluebirds. Weights will still be taken to determine moisture loss during natural incubation.

3.3 DOSING SOLUTIONS

PCB 77 in CO-1 vehicle (tree swallows) and the PCB mixture in EG-1 vehicle (eastern bluebird) will be injected. The PCB mixture solutions will be provided by Columbia Environmental Research Center (CERC) Columbia, Missouri, at concentrations designed to the appropriate doses for each species at the required volumes. The doses used for the bluebird egg injections are the same as that used for American kestrels and eastern screech owls at 0.1µl/g egg of the ‘sandpiper’ PCB mixtures in EG-1 vehicle. The PCB mixture is prepared according to the protocol described in the publicly released Hudson River Natural Resource Trustees report (2006).

3.4 EGG HATCHING AND TISSUE 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: Deformity Score Sheet). 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 immediately after hatching. Samples from each tree swallow or eastern bluebird hatchling or egg will be identified by a unique code (“sample ID”) encompassing the egg code, species, and year, e.g. 001-TRES-2008 for a tree swallow collected in 2008. Each tissue that is collected will be labeled with the complete sample I.D. such as (001-TRES-2008) and the name of the type of tissue: liver, bursa, heart or thyroid. Blood will be collected on sample cards provided by the contracted laboratory, and labeled with the sample ID.

SOPs:

SOP HR # 004: Necropsy of Hatchling Birds

DOC CONT #015: Hatchling Necropsy data sheet

DOC CONT #018: Deformity Score Sheet

3.5 BIOLOGICAL TISSUE ANALYSES

3.5.1 *Histological:*

Bursa and heart tissue will be preserved in appropriate fixatives. Bursa tissues will be embedded, sectioned and stained by standard methods. Slides will be labeled and well organized for retrieval and review. The SOP for the heart histology will be described and conducted under separate Work Plans.

3.5.2 *Gender genotyping*

Will be performed on blood collected on cards using polymerase chain reaction (PCR) techniques at DDC Veterinary, Fairfield, Ohio. SOPs and resulting data will be reviewed for adherence to QA/QC requirements.

3.5.3 *Thyroid glands*

Thyroid glands from each hatchling will be collected and stored at -80° C in a microcentrifuge tube. Analysis of thyroid gland thyroxine content will be conducted under a separate work plan based on the 2007 approved work plan.

3.5.4 *Livers*

Livers will be divided into two parts for snap freezing. One vial will be used for the measurement of cytochrome P450 activity in liver microsomes by EROD assay. The second vial will be used for measurement of oxidative stress markers, including gene expression of markers of oxidative stress and toxicity markers. The procedures for these measurements will be described and conducted under separate Work Plans.

3.5.5 *Gene Expression*

Analysis of gene expression will be conducted on selected samples by PCR and possibly microarrays according to SOP #6.19 of the Revised Work Plan for Tree Swallow, American Kestrel and Chicken Egg Injection Studies for 2006 (Hudson River Natural Resource Trustees 2007b). The selection of genes for analysis will be based on ongoing analysis for genes responsive to embryonic PCB exposure.

3.6 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 (H₀) and alternative (H_A) hypotheses are presented below. “PCBs” and “exposed to” refer to the PCB mixture for eggs injected or natural PCB exposure for birds from the Upper Hudson River. “Controls” refers to either uninjected/vehicle injected eggs in the egg injection study or eggs and

birds from the reference sites for the field study. “Birds” represents any life stage for which an endpoint is measured.

3.6.1 Embryo Mortality

Compare the embryo survival or hatchability of eggs exposed to PCBs with eggs that are not exposed to PCBs.

General Hypotheses

HO: Hatchability of eggs injected with the PCBs is equal to the hatchability of control eggs

HA: Hatchability of eggs injected with the PCBs is less than the hatchability of control eggs in a dose response manner

Statistical tests

Fisher Exact probability tests and probit analysis will be used for determining significant decreases in survival or hatchability and for determining median lethal doses.

3.6.2 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.

3.6.3 Histology

Compare histology of bursa and heart of PCB exposed birds to unexposed birds.

General Hypotheses

HO: Bursa and heart morphology in PCB exposed birds are not different than controls

HA: Bursa and heart morphology in PCB exposed birds are different compared to controls and are proportionally related to the dose of treatment

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.

3.6.4 Thyroid Hormones

Compare thyroxine (T4) content of thyroid glands from PCB exposed birds to that of unexposed birds.

General Hypotheses

HO: Thyroid hormone (T4) content of thyroid glands in PCB exposed birds is not different than controls

HA: Thyroid hormone (T4) content of thyroid glands in PCB exposed birds differs from controls and is proportionally related to the dose of treatment

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, T4 concentrations 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.

3.6.5 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 and is proportionally related to the dose of treatment

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.

3.6.6 Oxidative Stress

Compare oxidative stress in liver samples from PCB exposed birds to that of unexposed birds.

General Hypotheses

HO: Oxidative stress level in PCB exposed birds is not different than controls

HA: Oxidative stress level in PCB exposed birds is higher than controls and is proportionally related to the dose of treatment.

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, oxidative stress indicators 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.

3.6.7 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 and are proportionally related to the dose of treatment

HA: Bursa weight in PCB exposed birds is lower compared to controls and is proportionally related to the dose of treatment

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.

3.6.8 Gene Expression

Compare expression of selected gene products with PCB exposure compared to unexposed birds.

General Hypotheses

HO: Expression of selected genes in PCB exposed birds does not differ from controls

HA: Gene expression in PCB exposed birds is higher compared to controls in proportion to the dose of treatment

HA: Gene expression in PCB exposed birds is lower compared to controls in proportion to the dose of treatment

Statistical tests

Data for expression of specific genes 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. 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 among individual measurements of the same property under similar prescribed conditions, such as for example replicated 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 TRES will be used for tree swallow samples. Each egg will be assigned a unique egg code as follows: Series of numbers 001-199 for Patuxent Research Refuge, 200-399 for Cobleskill Reservoir, NY and 400 and higher for Hudson River. Eastern bluebird eggs are blue-green in color and readily discernible from tree swallow eggs, but they will be labeled as B1-50 at PWRC, B200-250 at Cobleskill, and B400 and higher for Hudson River to ensure that there is no confusion with samples following necropsy. Samples collected from each egg/embryo will then be identified by a sample ID encompassing the egg code, species, and year, e.g. 1-TRES-2008. 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 waterproof ink. 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 IN version 5, release 5.1, 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

Principal Investigator

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

Co-Principal Investigator

The Co-PI is an endocrinologist with 15 years experience in studying reproductive and stress physiology in amphibians, birds and mammals (including humans). The Co-PI also has 7 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.

Scientific Consultant

The Scientific Consultant is an avian toxicologist with experience in egg injection studies and immune and endocrine disruption studies in birds. The Scientific Consultant will participate in data analysis, quality assurance and will co-author 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. The Research Technician will be heavily involved with all aspects of these studies, including ordering materials and general coordination of laboratory tasks.

Field Technician

The Field Technician has participated in many of the PI's prior studies, especially in the field portions. The Field Technician will work on sample collection and on other aspects of the study as required.

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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7 STANDARD OPERATING PROCEDURES

SOP HR #001: Recording and Handling Data

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.
 - 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.
 - Data recorder will date and initial each sheet; the sheets will contain documentation such that each individual performing the injections/measurements can be identified.
- 2) After hard copies of data sheets are filled out they must be reviewed by the PI or the Co-PI then stored in the project notebook in the Co-PI's office.
- 3) Data should be input as soon as possible, after collection, into electronic files (Excel or JMP) and files stored on the PI's or the Co-PI's computers. Data entry must be 100% verified against the hard copy by someone other than the person who performed the initial data entry.
- 4) 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 will be made weekly and will be stored at the homes of the PI or the Co-PI.

Any deviations from the protocols will be written out in detail by the Principal Investigator and added to the project notebook.

SOP HR #025: Removal of Avian Egg Contents for Contaminants Analysis

Introduction

Avian eggs are a common sample for contaminants analysis. An accurate analysis depends upon getting the egg contents from the shell to a clean sample jar without introducing other sources of contamination. This protocol, which has been developed and refined by many researchers over the decades, was written for those who have minimal experience. Your first egg should be a practice egg. *It is suggested that all personnel practice on several quail eggs to improve technique. Chicken eggs may be used if quail eggs are not available.*

Laboratory Materials And Equipment

- Avian Egg Processing Data Sheets
- paper or other towels
- green scrubby or sponge
- Acculab V-200 balance, weighs to nearest 0.01 gm
- calipers
- Chemically-clean jars, 1 per sample
 - Make sure they are cleaned for the contaminants you are sampling, e.g., I-Chem pesticide/PCBs Series 200 or 300.
 - Size: 4 oz.
- chemically-clean stainless steel scalpel blades (No. 21 or No. 22 with No. 4 handles work well)
- chemically-clean forceps
- aluminum foil sheets (approximately 30 x 30 cm square), 1 per egg
- sharps container for used blades or disposable scalpels

Laboratory Procedures

- 1) Fill out the Avian Egg Processing Data Sheet; use one data sheet per egg.
- 2) If debris is present, rinse egg in cool water while gently scrubbing with green scrubby or sponge. Do not soak the egg.
- 3) Dry and weigh whole egg to the nearest .01 g

- 4) Transfer egg contents to chemically-clean jar using the following procedure:
- I. Use nitrile gloves for this part of the procedure. Avoid letting contents run over your hands into the sample jar. Note that the two eggs collected from a nest will both be placed in one jar for analysis.
 - II. Create a catch basin out of the aluminum foil by turning edges up and securing the corners. This will catch egg contents in case they spill over the edge of the jar. Use a separate piece of foil for each sample. The foil also is a clean place to place your instruments when they are not in use.
 - III. Weigh the clean empty jar with lid on, and note this tare weight on data sheet.
 - IV. Place jar in center of aluminum foil, and loosen the lid.
 - V. Score equator with serrated blade or scalpel blade. Use a new, chemically-clean scalpel blade for each egg. This part takes practice. Cradle the egg in one hand (don't squeeze too tightly!) and gently score while rotating the egg. Many light strokes are preferable to a fewer deeper strokes, increasing the evenness of the score and decreasing the possibility of eggshells not separating cleanly or of punching through the shell. Continue to work on your score until you see the membrane, which usually appears gray underneath the white of the eggshell. When you see the first bit of membrane, remove the lid from the jar so that it will be ready as soon as you need it. Avoid getting shell dust, or anything else besides the egg contents, in the jar. Try to expose the membrane evenly around the entire egg. Often the score line can be used to help pick the egg shell apart using forceps.
 - VI. Place the egg over the jar and cut through membranes with the scalpel. For large eggs a new scalpel blade may be used at this point to reduce the potential for cross contamination and since the blade may become dull during the cutting process. The scalpel can also be used to finish scoring down to the membranes. Pour contents into jar, or use the scalpel to gently scrape if that is necessary. Small stainless steel scoops may also be used to help remove the contents. Use forceps to remove any shell fragments from the jar. Cover the jar.
 - VII. For swallows, hold the egg vertically with air cell end up. Using scissors cut the top of the eggshell just below the air cell. Pour contents into the jar, and use a pipet to gently collect egg contents that don't freely flow out. Use forceps to remove any shell fragments from the jar. Avoid getting shell dust, or anything else besides the egg contents, in the jar.
 - VIII. Save the egg shell and associated membranes separately in a second I-chem jar labeled with the same sample ID as for the egg contents and note 'egg shell' on the jar.
 - IX. The target for the minimum weight of egg tissue is 4 grams for analysis. It may be possible to analyze smaller samples ranging from 1 – 2 grams. Analysis of these

samples may result in a lower ability to detect contaminants due to the lack of mass. An effort must be made to maximize the amount of each sample that is usable. The weight of each sample should be made in the laboratory during egg processing using the following procedure:

- a) Place a small jar on a balance that reads to at least 1 milligram and that has been appropriately calibrated.
- b) Tare the jar or record the jar weight if the balance cannot be tared.
- c) Open the egg, according to the procedures referenced above and empty the contents into the jar.
- d) Record the weight, to the nearest .01g, of the egg contents if the balance was tared. If the balance was not tared, then record the weight for the egg contents and the jar, then subtract the previously recorded weight of the jar. Record the weight of the egg contents in the field notebook and on the jar label.
- e) If egg is developed, estimate age of embryo. Documentation of embryo development is very limited (Powell *et al.* 1998; Bird *et al.* 1984), therefore, documenting this phase of the egg processing is important. Note amount of decay or anything else pertinent to your study, and examine for deformities, particularly bill deformities such as crossed bills or lack of jaws, but also lack of skull bones, club feet, rotated ankles, or dwarfed appendages (Gilbertson *et al.* 1991).
- f) Repeat these procedures for any other eggs that need to be added to the sample jar.

Do not touch or move the jar between steps b. and d. above. It is preferable to add the egg contents to the jar while the jar is still on the balance, immediately after taring the jar.

- 5) Place label on jar. Place clear tape over the label to keep it from getting wet.
- 6) Prepare Chain of Custody records and maintain egg samples under chain of custody.
- 7) Freeze samples. Ship under Chain of Custody (see attached COC form) overnight on dry ice to the sample archive or analytical laboratory.

Literature Consulted

- Bird, D.M, J. Gautier, and V. Montpetit. 1984. *Embryonic growth of American kestrels*. Auk 101:392-396.
- Gilbertson, M., T. Kubiak, J. Ludwig, G. Fox. 1991. *Great Lakes embryo mortality, edema, and deformities syndrome (GLEMEDS) in colonial fish-eating birds: similarity to chick-edema disease*. J. Toxicol. Environ. Health 33:455-520.

Powell, D.C., R.J. Aulerich, R.J. Balander, K.L. Stromborg, and S.J. Bursian. 1998. *A photographic guide to the development of double-crested cormorant embryos*. Colonial Waterbirds 21(3):348-355.

These egg-processing guidelines were developed by the U.S. Fish and Wildlife Service and modified for the project based on consultation with the author of these guidelines and on conversations with the Quality Assurance Coordinator for this project.

Appendix A: Chemically-Clean Instruments for Collecting Contaminants Samples

To minimize cross-contamination when collecting biological samples for contaminants analysis, a primary requirement is use of chemically-clean instruments. These are made of appropriate materials (stainless steel or teflon) and rinsed with alcohol and solvents to remove contamination and organics. Once rinsed, the instruments should be treated as sterile instruments, e.g. not placed on unclean surfaces.

Because every laboratory situation is different, this document tells you what to do, but not how to do it. The chemicals used for rinsing are hazardous, so you should follow proper safety and laboratory protocols when using them. This includes proper personal protective equipment (lab coats, gloves specific to the chemical, eye protection), proper laboratory equipment and procedures (use of hood, proper storage and disposal methods), and knowledge of chemical hazards such as flammability, reactivity, and toxicity (MSDS required). If this is all new to you, enlist the help of a chemist to help you make the proper decisions and reduce your risks of exposure and accident.

For organics, rinse with a reagent grade isopropyl alcohol, air-dry, rinse with reagent-grade hexanes, and air-dry.

Rinsing should be done using glass pipettes or wash bottles (made of appropriate material for the rinsing agent). Glass funnels, wide enough to accommodate your instruments and foil sheets, are invaluable in directing the flow of used chemicals into disposal containers or waste jars. Use disposal containers that are the same as your source chemical containers (e.g. brown glass). Never rinse into or pour unused chemicals back into your source chemical bottle.

DOC CONT # 012: Avian Egg Processing Data Sheet

Processor(s): Name _____

Signature _____

Date Processed: _____

Egg Code: _____

Sample ID: _____

Jar lot number _____

Balance within limits? Yes OR No

Whole Egg Weight (g): _____

Contents weight:

Weight of jar (g) : _____

Weight of jar + contents (g): _____

Weight of contents (g): _____

Membrane location: ___ with embryo OR ___ with eggshell

Contents condition (embryo development ¹, state of decay, etc.) and other comments:

Other comments:

Contaminants disposition (catalog number and date submitted, etc):

¹ None, 1/4, 1/2, 3/4, full term

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

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 personal 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, the Scientific Consultant, or the 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 #026 Procedures for NRDA Sample Archive Shipment

Chain-of-Custody (COC)/Transfer Record

1. You may use the original Chain-of-Custody if has at least one set of “Relinquished By” and “Received By” lines remaining.
2. If the original COC is not available, a new COC or Transfer Record (form is attached to these instructions) shall be prepared. List each sample or inventoried container (e.g., rack of vials, etc.) on a separate line, identifying with original field ID, laboratory ID and description or other identifier. (A Transfer Record is attached to this procedure).
3. Record on the COC, as appropriate:
 - Volume or quantity of sample, if available
 - Comments – apparent preservation problems or custody concerns
4. Cross check all sample identifiers from container to COC before packaging the samples.
5. Sign and date the “Relinquished By Signature” block on the COC. Make a copy for your records. Place the COC in a ziploc bag and tape it to the inside lid of the appropriate cooler.

Packaging/Shipping

1. Sample shipments are best made early in the week. Do not ship samples on Friday unless specific arrangements have been made with the courier and Receiver for Saturday delivery.
2. Wrap or package each item, as appropriate and place in cooler/package. Bubble wrap is a good cushion. **Dry ice or other coolants are not cushioning material, because the jars will become loose as the ice melts or evaporates.**
3. Place coolant, e.g., dry ice, wet ice or frozen gel packs (see below for dry ice info) in the cooler *so that the contents will remain at temperature for a minimum of 48 hours.*

For Dry Ice:

- **You must use < 5 kg/package**
 - **The drain plug on the cooler must be taped open for ventilation of carbon dioxide (CO₂) gas that occurs when the dry ice vaporizes**
 - **Indicate that dry ice has been used as a coolant on the shipping documents, however, when less than 5 kg is used as a coolant, dry ice is not considered a “Dangerous Good”.**
4. Seal the lid shut. Wrap duct/shipping tape around either end of the cooler (three times) to ensure a tight seal.

5. Place a minimum of two COC seals on the cooler in such a manner that if the container was opened, the seals would have to be broken.
6. Sign and date the COC seals, which are placed on the outside of the cooler. The same person who signed the COC record should do this.
7. Place clear shipping tape over the COC seals.
8. Adhere the appropriate address label on the top, outside surface of the cooler with clear shipping tape.
9. Fill out appropriate shipping documents:
 - Coolers are to be sent by Federal Express Priority One-Day service or a comparable, traceable service.
 - The cooler/package should be sent to:
 - **"Receiving Contact Name"** at:
 - **NOAA Building 32**
 - **7600 Sand Point Way NE**
 - **Seattle, WA USA 98115-0070**
 - The contact phone number on the airbill should be Receiving Contact's number
10. Fax or email a Notification of Shipment Form (attached to this procedure) to Receiver. Receiver will:
 - Coordinate receipt with NOAA Shipping and Receiving Department, on the day of arrival
 - Sign the COC in the "Received By Signature" block
 - Make sure that the cooler/package(s) are placed into the archive freezer at NOAA ARD West (Bldg. 32).

SOP HR #027: Egg Injection and Incubation Procedure For Tree Swallow Eggs At Patuxent Research Refuge And Cobleskill Reservoir: Nest Monitoring, Egg Injection, Egg Collection And Laboratory Egg Incubation

Nest Checking Procedures:

- 1) In March, repair nest boxes and remove old nesting material.
- 2) Beginning in late April, check nest boxes for signs of nesting and egg laying.
- 3) Upon initiation of egg laying, record date of clutch initiation on data sheet and visit nestbox(es) at least every other day to determine completion of clutch and initiation of incubation. Note accordingly on the Nest Checking Data Sheet (HR #21) and during collection on the Egg Collection Sheet (HR#29).

4a) Patuxent:

During egg laying randomly select one nest at each of the four pond sites and collect two eggs per nest for PCB analysis: Refrigerate eggs until opened, no longer than 48 hrs. Processing of eggs for contaminants analysis will be completed on a daily basis as much as practical. Follow Standard Operating Procedure HR#025 for Removal of Avian Egg Contents for Contaminants Analysis, Hudson River NRDA.

4b) Cobleskill Reservoir:

During egg laying randomly select three nests and collect two eggs per nest for PCB analysis: Refrigerate eggs until opened, no longer than 48 hrs. Processing of eggs for contaminants analysis will be completed on a daily basis as much as practical. Follow Standard Operating Procedure HR#025 for Removal of Avian Egg Contents for Contaminants Analysis, Hudson River NRDA.

- 5) In nests containing at least five eggs: On day two of incubation (initiation of incubation = day zero), in the afternoon, select 3 eggs from the nest. Candle these eggs and determine the two (if any) that fit stage 1-2 of development as described in the table below, this will determine approximately a 2.5 day old embryo (~18% of incubation):

Developmental Stage	Appearance of Vascularization & Embryo
0	Nothing visible
1	Pale, faint vascularization, faint embryo (red spot)
2	Obvious embryonic spot and vascularization
3	Classic embryonic shape apparent, vascularization around ~ 1/3 diameter of egg
3+	Significant vascularization and defined embryo

6) If available eggs in the stage 1-2 range are limited, assign any stage 3 or 3+ eggs to the untreated group as appropriate.

7) If eggs meet stage 1-2 appearance, assign each of the eggs from a clutch to a treatment group with no assignment of multiple eggs in a clutch to the same treatment. The egg will be labeled gently with soft pencil with an egg I.D. number on the pointed end of the egg and an 'X' and 'O' marked on opposite sides of the egg. Inject the egg using the following procedure being sure to limit the time the egg is out of the nest:

Injection Procedures

1) Assign the eggs to treatment groups and weigh each egg to the nearest one tenth of a gram. Calculate and record the volume of dosing solution to be added to each egg (egg weight multiplied by 0.4). The volume will be rounded to the nearest 0.01 μL .

2) Make injections into the egg as follows, allowing the eggs to be outside the nest for not more than 30 minutes:

- a. Wipe the blunt (air cell) end of the egg with 70% ethanol.
- b. Gently make a hole in the egg with Dremel drill with a fine drill bit.
- c. Inject the dosing solution, 0.1 $\mu\text{L}/\text{g}$ egg into the air cell, with a micro-pipettor and extended tip.
- d. Seal the hole with tissue adhesive or paraffin wax.
- e. Allow the egg to sit pointed end down for at least 10 minutes.

3) Place eggs back into the nest.

4) Monitor the nests containing injected eggs while visiting adjacent nests during the nest monitoring period. Candle injected eggs at least once after injection and before collection to confirm they are developing post-injection.

5) On day ten of incubation collect the injected eggs from the nests and transport them to the laboratory for completion of incubation in an incubator; transport the eggs in individual compartments surrounded in saw dust and in a Koolatron incubator to maintain warmth.

Laboratory Incubation Procedures

1) Upon receipt of the eggs at the laboratory, examine them noting any evidence of damage or embryonic development (by candling) on data sheet HR#028.

2) Weigh eggs and record the weight on data sheet HR #028. Determine total weight (moisture) loss based on egg weight at injection.

3) Place eggs in an incubator in an egg rack adapted for tree swallow eggs, on their sides. Incubate at 99.5°F and humidity adjusted as needed to ensure correct moisture loss. Eggs will be turned hourly by automatic rotation in the incubator for a total of 60° every two hours. In addition turn eggs 180° by hand or using a scoopula twice per day (before 10 am and after 4 pm). Confirm turning of eggs by assigning the O to day-time orientation and the X to night-time orientation.

4) On day 12, candle the eggs and transfer each egg to its own hatching ‘nest’ (a weigh boat with a piece of fabric in the bottom such as quilt batting) and place in incubator without egg turner, 99.5°F and 70-80% humidity.

5) Upon hatching, sample tissues per necropsy protocol.

Deformity Scoring Procedures

Any eggs that fail to hatch should be opened and condition of the embryo noted on the Deformity Score Sheet (HR #018). Deformities should 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. Photographs of deformed and normal embryos will be taken for reference. Deformed embryos will be preserved in a liquid fixative such as 70% ethanol or 10% neutral buffered formalin. Original memory cards from the digital camera should be kept under Chain-of-Custody (attached).

Equipment Needed:

- Scientific collecting permits
- Incubators: Natureform NMC2000 and GQF Sportsman 1502
- Egg trays
- Light for candling
- Ethanol and tissue or alcohol wipes
- Dremel drill
- Hamilton syringes: one per treatment
- Paraffin and tool to apply it to eggs
- Heating block
- Scales (510 - 0.001 g) Mettler Toledo PG503-S
- Rainin Pipettman with extended tips: one tip per egg

Data Sheets

“Nest Checking”,

“Egg Collection”

“Egg Incubation”,

“Incubator Record”,

“Egg Treatment and Incubation Log”

“Deformity Score Sheet”

Literature Consulted

Robertson, R. J., Stutchbury, B. J., and R. R. Cohen. 1992. Tree Swallow. *In The Birds of North America, No. 11* (A. Poole, P. Stettenheim, and F. Gill, Eds.). Philadelphia: The Academy of Natural Sciences; Washington, DC: The American Ornithologists' Union.

Robertson et al. (1992) in *Birds of North America* describe the nests, eggs, incubation and the hatching of tree swallow chicks and summarized below:

Tree swallow nests are constructed before the laying period. The nest cup is built primarily of grasses, especially when located near fields. It can also be composed of mosses, small roots, sticks, aquatic vegetation, and other plant materials. Feathers are a distinguishing characteristic, usually contour feathers of other fowl, are added after the formation of the nest cup. They are oriented so that the quill is buried in the nest, and the ends of the feathers cover the eggs when the female is not incubating the eggs. Bluebird nests are similar in appearance to tree swallow nests when in a nest box but do not have feathers lining the nest. Eggs are 18.7 x 13.2 mm and 1.4 to 2.6 g in size with an average weight of 1.9 g. Approximately 14% of mass is lost between laying and the end of incubation. Egg color translucent and rosy pink at time of laying turning to pure white (without any markings) around the fourth day of incubation. Eggs become glossier during incubation. Incubation length for tree swallow eggs averages 14-15 days but ranges from 11 to 19 days. Female incubation rhythms have been reported as 11 minutes on the nest and 9 minutes off the nest. An embryo takes one to two hours to hatch from start of pipping and clutches hatch over a one to two day period, occasionally over three days. Hatchlings weigh 1.5 to 1.7 g, eyes are closed, skin is uniform pink and the gape is yellow. Hatchling is able to raise head to beg and position itself with the dorsal side up.

SOP HR #028: Field Collection Of Tree Swallow Eggs From Upper Hudson River, New York For Injury Assessment Hudson River NRDA

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.

Materials and Equipment

Field:

- 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)
- Avian Egg Collection Data Sheets

Procedures

Field:

- ❑ Collected eggs should be whole and not cracked.
- ❑ For tree swallows, the following approach should be used: Incubation of tree swallow eggs doesn't start until the clutch is complete. Monitor nests every two to three days. Tree swallows generally lay eggs at one day intervals with a maximum clutch size of about 5-7 eggs. When a nest is 2-5 days pre-hatch (based on when the clutch was completed and incubation began), collect three eggs from that nest -- one egg will be incubated at the processing laboratory and the other 2 sibling eggs will be subject to contents collection for PCB analysis. In order to facilitate transport of eggs to the processing laboratory, eggs for PCB analysis can be collected independently of those to be transported to the processing laboratory. Eggs should be collected from all active nests at Remnant 3.
- ❑ 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.
- ❑ 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.
- ❑ 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.

For eggs that are going to be incubated: Transport promptly to the processing 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 degrees F, unless the transport time is going to be 8 hours or more, in which case a temperature as close as possible to 99.5 degrees should be maintained. Complete chain of custody transfer of samples from field collection crew to processing laboratory crew.

SOP HR #004: Necropsy of Hatchling Birds

Hatchling birds are maintained in the incubator in which they hatch for 18-24 hours before necropsy to allow complete drying of feathers. Birds are sampled as close to 24 hours after hatch as possible. This protocol outlines appropriate dissection techniques and sample storage conditions for several tissues including:

- Blood for serum
- feces for steroid analysis
- Brain
- Liver for CYP450 or chemical analysis
- Yolk or gastrointestinal tract for chemical analysis
- Bursa for mass and histology
- Thyroid for thyroid hormone radioimmunoassay
- Gonads for histology or biochemical analysis

Procedure

- 1) Bring ten to twenty hatchlings at a time to the necropsy room in a small box and keep in the box on a warm surface such as a heating plate on a low setting.
- 2) Weigh the hatchling.
- 3) Kill the hatchling by cervical dislocation and decapitate with scissors. Immediately collect trunk blood into a 12x75 mm glass tube. Set tube aside allowing blood to clot for serum collection.
- 4) Immediately remove the brain from the head, intact, and drop it directly into dry ice powder. After at least one minute on dry ice, fold the brain into a cold piece of aluminum foil and keep temporarily on dry ice.
- 5) If appropriate, dissect away the remaining yolk sac, weigh it, and place it in a chemically clean glass container and keep on wet ice.
- 6) Dissect the liver, remove the gall bladder and weigh the liver. Place the liver in a cryovial, or mince it and divide the tissue between multiple vials, and flash freeze it in liquid nitrogen for CYP450 analysis.
- 7) Dissect each lobe of the thymus from the neck and remove each thyroid at the same time. The thyroid is located at the caudal point of the thymus just anterior to the heart. Weigh

thyroid individually in the storage vial to prevent drying on weigh paper. Freeze thyroids on dry ice.

- 8) Remove the bursa, weigh it and place it in a 1.5 mL microcentrifuge tube in Bouin's fixative.
- 9) Identify the gonads to determine gender. Males have two kidney shaped testicles. Females have one left ovary. Remove gonads intact on a portion of the carcass's back and fix in 10% buffered formalin or other appropriate fixative or freeze.
- 10) Discard carcass appropriately.

DOC CONT # 015: Hatchling Necropsy Sampling Sheet:

Study: _____ Date: _____

Species: _____

Egg Code: _____ Collection site: _____

Treatment: _____

Concentration: _____ Injection site: _____

Sample	Collection
body weight (g)	
blood (volume estimate)	
liver (mg)	
heart (mg)	
left thyroid (mg)	
right thyroid (mg)	
bursa (mg)	
thymus piece	
brain (mg)	
gender (M/F or indeterminate)	
left testis (mg)	
right testis (mg)	
ovary + uterus (mg)	
adrenals	
GI tract (mg)	
feces (mg)	
spleen (mg)	
leg muscle g)	

Dissector: _____ Recorder: _____
Name Name

Reviewer: _____ Date: _____
Name and signature

SOP HR #015: Histological Analysis Of Japanese Quail Tissue:

Bursa of Fabricius

This protocol describes the necessary steps in storage and preparation of tissues for histological analysis.

Procedure

When collected, bursas are first stored in Bouin's solution. Before dehydration, the bursas can be brought to 70% ethanol (ETOH) and stored in this solution almost indefinitely. To bring to 70% ETOH, put the bursas that are stored in Bouin's solution into 3 changes of 50% ETOH for 6 hours each. After the last 6 hours, the bursas can be placed into the 70% ETOH solution.

1. Dehydration:

70% ETOH → 85% ETOH – 1 hour

85% ETOH → 95% ETOH – 40 min

95% ETOH → 95% ETOH – 40 min

95% ETOH → 100% ETOH – 1 hr or overnight

2. Clearing:

100% ETOH → 1:1 xylene:100% ETOH – 1 hr

1:1 xylene:100% ETOH → 100% xylene – 1 hr

100% xylene → 100% xylene – 1 hr

100% xylene → 100% xylene – 1 hr

*note – xylene can be substituted with chloroform

For steps 1 & 2, a glass container is preferable. Small plastic snap-cap vials are also suitable when working with small tissues, however, xylene should not remain in plastic containers for long, otherwise they may disintegrate. The tiny perforations in standard cassettes, are too large to prevent small tissues such as hatchling bursae from escaping.

3. Embedding:

100% xylene → paraplast – 40 min

paraplast → paraplast – 1 hr

paraplast → paraplast – 1 hr

Pour new paraplast into boat with tissue in an ice bath, or embed your tissue into new paraplast using an embedding machine.

*note – oven should not exceed 56/60°C

4. Cut tissue in 10 – 12.5 um sections. Place 3 sections on each of 3 slides (total of 9 sections).

5. Staining Procedure:

Xylene	2 min
Xylene	2 min
100% ETOH	1 min
100% ETOH	1 min
95% ETOH	1 min
95% ETOH	1 min
tap water (non-running)	10 min
Mayer's hematoxylin	15 min
Lukewarm running tap water	20 min
Eosin	2 min
95% ETOH	2 min
95% ETOH	2 min
100% ETOH	2 min
100% ETOH	2 min
100% ETOH	2 min
Xylene	2 min
Xylene	2 min
Xylene	2 min

Let remaining xylene run off slide, then coverslip immediately to prevent dessication of the tissue.

6. Endpoints

Record a digital image of each section used.

For bursa analysis, measure number of follicles per section, number of vacuoles, thickness of epithelial layer, and follicle size. Other qualitative aspects to also consider with each section is arrangement of bursal buds and arrangement of epithelial layers.

Equipment Needed

- Oven
- Image Analysis Equipment
- Staining Jars

Quality Assurance Parameters and Procedures:

Sample Analysis: Multiple tissue sections per slide. Slides coded to obscure identifying marks and presented to reader in random order.

Duplicate (repeat) count of 10% of slides.

Criterion: reject results and repeat counts if the difference between counts from the replicate slides exceeds $2x \sqrt{(\text{highest count})}$.

Performance Evaluation: Independent recount of a previously counted set of slides.

Frequency: Once for every tissue type or every 10 sets of slides.

Criterion: Repeat counts of previous sets if the difference between repeated counts exceeds $3x \sqrt{(\text{highest count})}$.

References

Pait, T. 1999. *Effects of endocrine disrupting compounds on the fish Fundulus heteroclitus*.

PhD Dissertation, University of Maryland, College Park, MD.