STUDY PLAN FOR AVIAN EGG INJECTION STUDY

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

PUBLIC RELEASE VERSION*

FINAL

RELEASED MAY 12, 2006

REVISED JANUARY 31, 2007

Available from:
U.S. Department of Commerce
National Oceanic and Atmospheric Administration
Hudson River NRDA, Lead Administrative Trustee
Assessment and Restoration Division, N/ORR3
1305 East-West Highway, Rm 10219
Silver Spring, MD 20910-3281

Names of certain individuals and affiliations have been removed to maintain confidentiality

REVISION HISTORY
to

Study Plan for Avian Egg Injection Study

The following changes have been made since the original release of this document on May 12, 2006:

1. Appendix A was revised as described therein on p. 2 of 87.

2. Appendix B was replaced in its entirety.

3. All references to the "Final Work Plan for Tree Swallow, American Kestrel, and Chicken Egg Injection Studies" have been changed to the "Revised Work Plan for Tree Swallow, American Kestrel, and Chicken Egg Injection Study."

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.

In 2002, the Trustees conducted an avian egg exposure preliminary investigation for the Hudson River. That preliminary investigation revealed that of the eleven avian species tested, the highest PCB levels were found in belted kingfisher and spotted sandpiper.

Based on the results of avian investigations conducted by the Trustees, and considering factors such as the life histories of various Hudson River avian species, avian toxicology, and the goals of the NRDA, the Trustees determined that it was appropriate to conduct further investigations focused on avian species. Pursuant to that determination and to the Hudson River NRDA Plan, the Trustees conducted a study of belted kingfisher, spotted sandpiper and tree swallow in 2004 and 2005. The Trustees further proposed conducting an avian egg injection study.

A Draft Study Plan for the egg injection work was peer reviewed and made available to the public for review and comment. All comments received on the Draft Study Plan, as part of the peer and public review process, have been considered. The Trustees evaluated peer and public comments and, where warranted, incorporated these comments in the Draft Study Plan to produce the Final Study Plan. In the remaining instances, public comments on the Draft Study Plan have been addressed by letters to the commenters, acknowledging receipt of comments and providing an initial response and noting that a more detailed Responsiveness Summary will be provided by the Trustees in the near future.

The Trustees will conduct an avian egg injection study of tree swallow and American kestrel in 2006 to evaluate whether specific avian species in the vicinity of the Hudson River are injured due to exposure to PCBs. Additionally, egg injection experiments will be conducted using chicken eggs to provide a point of reference for impacts observed in other species and in relation to effects levels identified in the toxicology literature for PCBs, dioxins, and other chemicals. Work on the species studied in 2006 or other species may continue in 2007.

The objective of the investigation is to evaluate the toxicity and adverse effects of embryonic exposure of multiple avian species to dose ranges of PCB 126 or a PCB mixture. This study will be used to evaluate whether avian reproduction and/or development is affected as a result of exposure to PCBs from the Hudson River.

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.

TABLE OF CONTENTS

1.0 BACKGROUND

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

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

In 2002, the Trustees conducted an avian egg exposure preliminary investigation for the Hudson River. The investigation entailed collection of eggs, and subsequent analysis for PCBs, from six primary species (belted kingfisher (*Ceryle alcyon*), American robin (*Turdus migratorius*), Eastern phoebe (*Sayornis phoebe*), spotted sandpiper (*Actitis macularia*), red winged blackbird (*Agelaius phoenicius*), and American woodcock (*Scolopax minor*)) and from five additional species (Eastern screech owl (*Otus asio*), common grackle (*Quiscalus quiscula*), northern rough-winged swallow (*Stelgidopteryx serripennis*), barn swallow (*Hirundo rustica*), and Eastern bluebird (*Sialia sialis*)) based on the opportunities for survey team members to locate the nests of these species. The geographic scope of the 2002 avian egg investigation was the Hudson River and its floodplains, from Hudson Falls to Lower Schodack Island, New York.

That preliminary investigation was undertaken by the Trustees to assist in determining the extent to which avian species in the Hudson River are contaminated with PCBs, to determine if additional pathway and injury assessment studies focused on avian species should be conducted as part of the Hudson River NRDA, and for potential use in the design of future studies to assess the health of Hudson River birds. The Trustees noted in the Hudson River NRDA Plan that, based on the results of the bird egg study, the Trustees would determine whether injury determination and quantification studies were warranted.

That preliminary investigation revealed that of the eleven avian species tested, the highest PCB levels were found in belted kingfisher and spotted sandpiper (Hudson River Natural Resource Trustees, 2003). Spotted sandpiper eggs contained a mean of 15 parts per million (ppm) PCBs (as total homologues, fresh weight basis). Of the eleven species tested, spotted sandpiper eggs exhibited the highest individual egg concentration of PCBs (56 ppm) as well as the highest average PCB concentration (15 ppm). Of the eleven species tested, belted kingfisher eggs exhibited the second highest individual egg concentration of PCBs (43 ppm).

Based on the results of avian investigations conducted by the Trustees, including the tree swallow (*Tachycineta bicolor*) work (McCarty and Secord 1999a, 1999b, Secord *et al.* 1999) and the 2002 avian egg preliminary investigation (Hudson River Natural Resource Trustees 2003), and input from a panel of avian experts, and considering factors such as the life histories of various Hudson River avian species, avian toxicology, and the goals of the NRDA, the Trustees determined that it was appropriate to conduct further investigations focused on avian species, and initially on belted kingfisher, spotted sandpiper, and tree swallow, to be started in the year 2004.

Pursuant to that determination and to the Hudson River NRDA Plan, the Trustees released in 2004 a "Study Plan for Year 2004 Avian Investigations for the Hudson River - Final, Public Release Version," dated June 15, 2004 (Hudson River Natural Resource Trustees, 2004). That Avian Injury Study Plan described the activities that constituted the Trustees' planned approach to conducting investigations of injury to avian species as part of the Hudson River NRDA.

As noted in the Avian Injury Study Plan, the Trustees planned to assess the following potential injuries to birds: reduced avian reproduction and overt external malformations. The Trustees planned to: (1) assess the relationship between contaminant concentrations in nest sample eggs and parameters of nest reproduction by application of appropriate statistical analysis of data to determine whether reproductive success of spotted sandpipers, tree swallows and belted kingfishers nesting on the Hudson River is negatively affected by PCB exposure; (2) assess the incidence of gross deformities in embryos or hatchlings; (3) assess organic contaminant accumulation rates in belted kingfisher chicks on the Hudson River; and (4) initiate an avian egg injection pilot study in 2004.

Pursuant to the Final Avian Injury Study Plan and a May 4, 2005 Modification to that Study Plan (Hudson River Natural Resource Trustees, 2005a), the U.S. Geological Survey (USGS) conducted a study of belted kingfisher, spotted sandpiper and tree swallow in 2004 and 2005. The USGS study was directed at items (1), (2) and (3) above. Trustee review of the data and results from the USGS study is ongoing.

Regarding item (4) above, the Trustees' Final Avian Injury Study Plan proposed a "pilot" study, a preliminary investigation focused on incubation of eggs of Hudson River avian species in 2004 with injection of PCBs into eggs of avian species of interest in a subsequent year. The Trustees subsequently determined that, considering preliminary work done by the Trustees and the literature on avian egg injection studies, it was not necessary to conduct a separate incubation-focused pilot study prior to initiating an avian egg injection study.

A Draft Study Plan for an avian egg injection experiment (Hudson River Natural Resource Trustees 2006) was developed, and that Draft Study Plan was peer reviewed and made available to the public for review and comment. All comments received on the Draft Study Plan, as part of the peer and public review process, have been considered. The Trustees evaluated peer and public comments and, where warranted, incorporated these comments in the Draft Study Plan to produce a Final Study Plan. In the remaining instances, public comments on the Draft Study Plan have been addressed by letters to the commenters, acknowledging receipt of comments and providing an initial response and noting that a more detailed Responsiveness Summary will be provided by the Trustees in the near future.

2.0 INTRODUCTION

Avian egg injection is a well-established technique to assess the effects of contaminants on a developing avian embryo (Allred and Strange 1977, Blankenship et al. 2003, Blomqvist et al. 2006, Boily et al. 2003, Bruggeman et al. 2003, Brunström 1986, Brunström et al. 1999, Brunström 1990, Brunström 1988, Brunström and Andersson 1988, de Roode et al. 2000, DeWitt et al. 2005a, DeWitt et al. 2005b, Drake et al. 2006, Fernie et al. 2005, Fox and Grasman 1999, Goff et al. 2005, Gould et al. 1997, Grasman and Whitacre 2001, Halldin et al. 2002, Heinz et al. 2006, Hoffman et al. 1998, Ivnitski et al. 2001, Janz and Bellward 1996, Jin et al. 2001, Katynski et al. 2004, Lim et al. 2005, Meneely and Wuttenbach 1989, Murvoll and Skaare 2005, Nosek et al. 1993, Ottinger et al. 2005, Powell et al. 1996a, Powell et al. 1996b, Powell et al. 1997a, Powell et al. 1997b, Powell et al. 1998, Stanton et al. 2003, U.S. Environmental Protection Agency 2001, Walker and Catron 2000, Walker et al. 1997, Wilhelms et al. 2006, Zhang et al. 2002, and Zhao et al. 1997).

To conduct an avian egg injection experiment, eggs are collected and brought into a laboratory where they are injected with the substance being tested. In avian egg injection experiments, various doses of a contaminant of concern (for example, PCBs in a vehicle or carrier solution) are typically injected into the yolk sac (for example, Hoffman et al. 1998), air cell (for example, Brunström and Andersson 1988, Fox and Grasman 1999), or albumen (for example, Nosek et al. 1993) of eggs. The eggs are then incubated in a laboratory and their development monitored. Measurement endpoints may include embryomortality, malformations, and hatching success. Measurement endpoints may also extend to hatchlings, for which chick growth and development, for example, may be measured.

Results reported in the literature of injecting contaminants, such as PCBs, into avian eggs include embryomortality and malformation. Death, including embryomortality, for example, and physical deformation, such as external malformation, skeletal deformities, and organ and soft tissue malformation, are injuries pursuant to the regulations written by the U.S. Department of the Interior contained in Title 43 of the Code of Federal Regulations Part 11, Natural Resource Damage Assessment (the "DOI NRDA Regulations"), and would be relevant to determining injury as part of the NRDA.

3.0 PURPOSE AND OBJECTIVE

The Trustees will conduct an avian egg injection study of tree swallow and American kestrel in 2006 to evaluate whether specific avian species in the vicinity of the Hudson River are injured due to exposure to PCBs. Additionally, egg injection experiments will be conducted using chicken eggs to provide a point of reference for impacts observed in other species and in relation to effects levels identified in the toxicology literature for PCBs, dioxins, and other chemicals. Work on the species studied in 2006 or other species may continue in 2007.

The objective of the investigation is to evaluate the toxicity and adverse effects of embryonic exposure of multiple avian species to dose ranges of PCB 126 or a PCB mixture. The PCB mixture is made up of individual PCB congeners and fits a similar profile to the mixture of PCBs occurring in the eggs of birds nesting in the Upper Hudson River. This study will be used to evaluate whether avian reproduction and/or development 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 at the embryonic level. 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

4.1 *Year 1 (2006) Avian Egg Injection Study of Tree Swallow, American Kestrel, and Chicken*

The attached work plan entitled, "Revised Work Plan for Tree Swallow, American Kestrel, and Chicken Egg Injection Studies" (Appendix A) describes the avian egg injection 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 plan includes information regarding the experimental design, Quality Assurance/Quality Control, and Standard Operating Procedures that will be used in the study.

In Year 1 (2006), the Trustees will focus on injection of test PCBs and incubation methods for eggs from chosen species. If injection and incubation methods are successful, tissues will be collected for analysis, providing initial datasets for these species. Year 1 (2006) work will focus on those species with eggs that are more easily obtainable than others.

In 2006, work will be conducted on tree swallow and American kestrel (*Falco sparverius*). These species have been selected because they represent different positions in the ecosystem, breed in the Hudson River basin, and may be sensitive to PCB exposure. Eggs of American kestrel will be obtained from Patuxent Wildlife Research Center, Maryland. Eggs of tree swallows will be obtained from Patuxent National Wildlife Refuge, Maryland, from a breeding colony on Great Sacandaga Lake, New York, and from the Hudson River, New York. Additionally, egg injection experiments will be conducted using chicken (*Gallus domesticus*) eggs to provide a point of reference for impacts observed in other species and in relation to effects levels identified in the toxicology literature for PCBs, dioxins and other chemicals. Work will begin in Spring 2006.

Trials will be conducted using select PCBs administered early in embryonic development. As described in the "Revised Work Plan for Tree Swallow, American Kestrel, and Chicken Egg Injection Studies," eggs will be injected with PCBs (PCB 126 or a PCB mixture reflective of chemical exposures in the Hudson River region). A vehicle control and an untreated group of eggs will be included. The PCBs to be injected into the eggs have been selected by the Trustees based on existing contaminants data from Hudson River biota and other relevant factors. Appendix B provides information on the PCB congener mixture to be used in the egg injections.

There will be a separate experiment conducted for each species. The timing of each experiment will depend on the availability of eggs for that species. In the case of chickens, for example, eggs will be available over much of the year, so those experiments will be scheduled around the work with the other species.

The "Revised Work Plan for Tree Swallow, American Kestrel, and Chicken Egg Injection Studies" (Appendix A) notes the endpoints to be assessed and the methods that will be used.

4.2 *Year 2 (2007) Avian Egg Injection Study*

Year 1 work focuses on injection of test PCBs and incubation methods for eggs from tree swallow, American kestrel and chicken. These studies are projected to continue into a second year to allow further development of injection and incubation protocols for eggs from wild species and, in some cases, to produce larger sample sizes. Work on the species from 2006 or other species may be conducted in 2007. Work in 2007 will be conducted pursuant to a Study Plan Amendment for Year 2007.

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, 2005b).

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. Analytes 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.

In order to minimize analytical costs, and reduce the overall cost associated with the project, the Trustees may conduct the chemical 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 2005b). 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 the U.S. Environmental Protection Agency guidelines (1999).

6.0 SPECIAL PROVISIONS

Any necessary collection permits, such as those from New York State or Maryland where eggs will be collected, or from the U.S. Fish and Wildlife Service, will be obtained.

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HUDSON RIVER

STUDY PLAN FOR AVIAN EGG INJECTION STUDY

Appendix A

Revised Work Plan for Tree Swallow, American Kestrel, and Chicken Egg Injection Studies

HUDSON RIVER
Study Plan for Avian Egg Injection Study

REVISED WORK PLAN

FOR

TREE SWALLOW,
AMERICAN KESTREL,
AND CHICKEN

EGG INJECTION STUDIES

HUDSON RIVER NATURAL RESOURCE DAMAGE ASSESSMENT

Released: May 12, 2006
Revised: June and October 2006

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Principal Investigator

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Co-Principal Investigator

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Quality Assurance Coordinator

REVISION HISTORY
to

Final Work Plan for Tree Swallow, American Kestrel, and Chicken Egg Injection Studies, Hudson River Natural Resource Damage Assessment dated May 12, 2006

The following changes have been made since the original release of this document on May 12, 2006:

1. Section 2.1 entitled "Animals" has been replaced in its entirety.

2. Section 2.4.3 entitled "Dosing Solutions" has been replaced in its entirety.

3. Section 2.4.4 entitled "Egg Hatching and Tissue Sampling" has been replaced in its entirety.

4. Section 3.2.3 entitled "Sample Identification Procedures" has been replace in its entirety.

5. The "Avian Egg Collection Data Sheet – Hudson River Avian Egg Injection Study" located in Section 6.2 has been replaced in its entirety.

6. "Laboratory Procedure" 8.e. found in Section 6.3 entitled "SOP: Removal of Avian Egg Contents for Contaminants Analysis Hudson River NRDA" has been replaced in its entirety.

7. The second bullet in "Procedures" found in Section 6.21 entitled "Field Collection of Tree Swallow Eggs from Upper Hudson River, New York for Injury Assessment Hudson River NRDA" has been replaced in its entirety.

8. The "Avian Egg Collection Data Sheet – Hudson River Avian Egg Injection Study – Upper Hudson River" located in Section 6.21 has been replaced in its entirety.

9. In Section 6.6, the injection volume of 0.4 μL/g egg was changed to 0.1μL/g egg. This change was made because chicken eggs are large, and because many of the published egg injection studies using chicken eggs have used 0.1 μL/g egg for injection volume (Henshel et al. 1997, Fox and Grasman 1999, Grasman and Whitacre 2001, Dewitt et al. 2005). The use of an injection volume of 0.1 μL/g egg for this experiment will allow us to better compare results to the literature.

10. The following references that are associated with the revisions listed above were added to Section 5: Barron et al., 1995; Brunstrom and Lund, 1988; Brunstrom and Reutergardh, 1986; DeWitt et al., 2005; Fox and Grasman, 1999; Grasman and Whitacre, 2001; Henshel et al., 1997; U.S. EPA, 2001.

INVESTIGATION TEAM ACKNOWLEDGEMENT OF WORK PLAN REVIEW AND COMPLIANCE

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

Name (printed): ________________________	Name (printed): __________________________
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ABBREVIATIONS

ED	**Embryonic Day**
EIA	**Enzyme Immuno Assay**
EROD	**Ethoxyresorufin-o-dealkylase**
GnRH-I	**Gonadotropin Releasing Hormone-I**
HPLC	**High Pressure Liquid Chromatography**
PCB	**Polychlorinated Biphenyl**
RIA	**Radioimmunoassay**
ELISA	**Enzyme Linked ImmunoSorbent Assay**

TABLE OF CONTENTS

1. INTRODUCTION

This work plan is for assessment of the biological impacts of selected chemicals of concern for the Hudson River assessment area, using an embryo bioassay on sentinel avian species for this region.

These experiments are projected over two years to allow development of injection and incubation protocols for eggs from wild species and to produce statistically useful sample sizes. This work plan defines work to be done in year one with three species for which we have identified egg sources: American kestrel (*Falco sparverius*), tree swallow (*Tachycineta bicolor*) and domestic chicken (*Gallus domesticus*). In addition to ready egg sources being available, the two wild species have been chosen because they represent a range of positions in the ecosystem and individuals from each species can be found for some or all of the year in the Hudson River basin. An experiment with chickens will be conducted to round out a species sensitivity comparison.

> Our objective is to use egg injections as a method for evaluating the toxicity of PCB 126 and an environmentally realistic mixture of PCB congeners on the embryos of kestrels, tree swallows and chickens.

Based on data from other studies on the consequences of endocrine disrupting chemical exposure in birds, we expect sensitive endpoints to include 1) viability—embryo survival, pipping, hatching success, gross abnormalities, 2) gonadal and thyroid gland morphology, 3) endocrine measures—including hormone levels, 4) status of neural systems important in metabolic and reproductive axes function—hypothalamic neurotransmitters, aromatase enzyme, and gonadotropin releasing hormone (GnRH-I), 5) Cytochrome P450 enzyme activity and 6) Endocrine Disrupting Chemical (EDC) induced abnormalities, such as gonadal, biochemical and hormonal end points that are evident in the hatchling. Therefore, we will include these measurement end points in an embryo testing protocol that we have developed in other studies. These trials will be conducted by administering the PCB solutions early in embryonic development (before gonadal differentiation). Samples will be taken at hatch for measurement of selected end points known to be steroid responsive and sexually dimorphic. It is expected that endocrine active substances will affect these end points, thereby making treated individuals less sexually dimorphic.

2. WORK PLAN

2.1 Animals

We will receive eggs from two species in the 2006 breeding season: American kestrel (*Falco sparverius*) and tree swallow (*Tachycineta bicolor*). Kestrel eggs will be from the captive colony maintained at Patuxent National Wildlife Refuge (NWR)/Research Center. Eggs from tree swallows will be collected from nest boxes located on the Patuxent National Wildlife Refuge and from Great Sacandaga Lake in New York state. In addition, tree swallow eggs at mid-late incubation will be collected from nest boxes located on the Upper Hudson River for hatching in the lab and collection of tissues but without any egg injection treatments. Chicken eggs will be acquired in the Summer and(or) Fall from a commercial supplier. Protocols for egg incubation and tissue sampling will be reviewed by the animal care and use committees of the organizations involved in the study.

2.2 Treatments

This experiment will evaluate the effects of exposure to a dose range of PCB 126 in tree swallows and a PCB mixture in kestrels and chickens. The PCBs will be injected into eggs early in incubation, and tissues will be sampled from hatchlings. The time of egg injection will be at approximately 18% of incubation.

2.3 End Points

- Embryo survival/embryo mortality
- Gross morphology—including abnormalities
- Organ weights for liver, heart, brain, thyroid, thymus, bursa, spleen, and gonads
- Gonad histology and morphology of the reproductive tract
- Biochemical analyses
  - Liver CYP4501A
  - Brain monoamines/aromatase/GnRH-I (gonadotropin releasing hormone-I)
  - Thyroid hormones: T3 and T4 in thyroid gland; serum samples will be archived for T3 and T4 analysis if deemed necessary
  - Estradiol and androgen concentrations in fecal samples
- Bursal morphology
- Cardiac histology*
- Microarray analysis of RNA from liver and brain samples

* Hearts will be dissected and preserved for histological analysis.

Tissues will be collected for all biochemistries and histology but analyses will be made as deemed appropriate based on sample sizes.

2.4 Experimental Design

2.4.1 Egg Collection

Eggs will be collected under permits from Kathryn Jahn, 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, the statistician will provide guidance on treatment group assignment with consideration of sample size, statistical power, sampling day, eggs per breeding pair, and estimated median lethal dose. The goal will be to maximize the number of eggs per independent parent within each treatment group and to attain data demonstrating a normal distribution. We will prioritize treatment groups based on data from other avian studies and we will evaluate which treatment groups can be eliminated to maximize sample size and dose response. Since we expect to obtain hundreds of eggs from tree swallows, but only two dozen kestrel eggs we shall achieve a broader number of treatments for tree swallows and only a control and two or three PCB exposed groups for kestrels. A subset of tree swallow eggs will be assigned to contaminants analysis to verify the eggs are suitable as a 'clean' source. At a minimum, 2 eggs from each of three nests but potentially as many as 2 eggs from each of 10 nests will be kept for contaminants analyses. Because the number of eggs collected from PWRC may be limited, additional eggs will be assigned to contaminant analysis from eggs that fail to develop from the un-injected control group. Since the sources of kestrel eggs are birds that are fed a controlled diet, and because the number of eggs we will attain is limited, we will not submit fresh eggs for contaminants analysis but will retain yolks from hatchlings for analyses of experimental exposure (and background contamination from untreated eggs). Furthermore, we will keep infertile eggs and eggs containing dead embryos for contaminant analyses from the tree swallows and kestrels; this will substantially increase the amount of eggs available for analyses. There are also historical data available for Patuxent tree swallow contaminant burden available for consideration.

Tree swallow eggs will be collected from nests that will be monitored daily. 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 are collected; if another egg is laid on the following day, it is possible to also collect that egg. In this way, the female should not abandon the nest.

We will also sample tissues from fifteen or more tree swallow embryos collected from the Upper Hudson River area.

The kestrels are housed in pairs at Patuxent Wildlife Center; pairs are monitored daily. Egg collection will be conducted according to husbandry practices for these colonies.

Standard Operating Procedure (SOP): "Field Collection of Avian Eggs for Avian Egg Injection Study Hudson River NRDA", "Removal of Avian Egg Contents for Contaminants Analysis Hudson River NRDA"," **Field Collection of Tree Swallow Eggs from Greater Sacandaga Lake, New York for Avian Egg Injection Study"** , "**Field Collection of Tree Swallow Eggs from Upper Hudson River, New York for Injury Assessment Hudson River NRDA".**
Data Sheet: "Avian Egg Collection Data Sheet", "Avian Egg Processing Data Sheet"

2.4.2 Egg Incubation and Injection

Eggs will be injected at a time point approximately 18% of incubation and is specifically defined in the SOP. Eggs will be candled during incubation at time points approximately equivalent to 18% (i.e., before injection), 24% (one day post-injection) 41%, and 82% of incubation and specifically defined in the SOPs. At time of candling, any dead or infertile eggs will be removed and any dead embryos evaluated for stage of development and deformities.

SOP: "Egg Injection and Incubation Procedure for Tree Swallow (*Tachycineta bicolor*) Eggs", "Egg Injection and Incubation Procedure for American Kestrel (*Falco sparverius*) Eggs".
Data Sheets: **"**Incubator Record", "Log of checking of eggs", "Egg Moisture Loss".

2.4.3 Dosing Solutions

PCB 126 solutions were prepared in corn oil, Sigma C8267, and provided by the Columbia Environmental Research Center (CERC) Columbia, Missouri. Concentrations of the solutions provided by CERC are 0 (corn oil only), 5.9 ng/uL, 20 ng/uL, 60 ng/uL, 110 ng/uL, 500 ng/uL, 2,000 ng/uL, 5,000 ng/uL, and 10,000 ng/uL. We will inject these solutions at 0.4 µL/gram of egg and therefore the actual egg doses will be 0 ng/g, 2.4 ng/g, 8 ng/g, 24 ng/g, 44 ng/g, 200 ng/g, 800 ng/g, 2,000 ng/g and 4,000 ng/g.

The PCB mixture solutions prepared in corn oil (Sigma-Fisher Scientific) will be provided by CERC at concentrations of 0 (corn oil), 8 µg/uL, 15 µg/uL, 31 µg/uL, 62 µg/uL, 123 µg/uL and 246 µg/uL. We will inject these solutions at 0.4 µL/gram of egg and therefore the actual egg doses will be 3 µg/g, 6 µg/g, 12 µg/g, 25 µg/g, 49 µg/g, and 98 µg/g.

Since chickens are more sensitive to dioxin-like compounds relative to other tested birds (Brunstrom and Reutergårdh 1986, Brunstrom and Lund 1988, Barron et al. 1995, Brunstrom and Halldin 1998, USEPA 2001), the dose range for the PCB mixture will be adjusted appropriately to include lower doses than those used in the American kestrel experiment. The following dosing solutions will be prepared by CERC: 0 (corn oil), 0.25 µg/uL, 0.74 µg/uL, 2.2 µg/uL, 6.7 µg/uL, 20 µg/uL and 60 µg/uL. For our injection procedure, 0.1 µL of solution per gram of egg will be injected yielding actual egg doses of 0, 0.025 µg/g, 0.074 µg/g, 0.22 µg/g, 0.67 µg/g, 2 µg/g, and 6 µg/g.

SOP: "Egg Injection and Incubation Procedure for Tree Swallow (*Tachycineta bicolor*) Eggs", "Egg Injection and Incubation Procedure for American Kestrel (*Falco sparverius*) Eggs", "Egg Injection Procedure for chicken (*Gallus domesticus*) Eggs".
Data Sheets: "Egg treatment and incubation log", "Log of checking of eggs"

2.4.4 Egg Hatching and Tissue Sampling

Initially, embryos will be sampled at 90% of incubation to maximize the number of viable embryos for tissue collection. If viability at 90% of incubation is high, we may monitor hatchability in subsequent sets of eggs. 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, unusually small or large eyes, edema of the neck and head area, incomplete ossification of skull (brain not enclosed in skull), gastroschisis in post stage 45 embryos (if incubation is taken to hatch), malformed or clubbed feet, asymmetrical body form, mal-position in the egg, and any other abnormal appearances shall be noted on the data sheet.

Chickens will be sampled within 24 hours of hatching.

SOP: "Necropsy of Hatchling Birds"
Data Sheet: "Deformity Score Sheet", "Hatchling Sampling Data Sheet"

2.4.5 Biological Tissue Analysis

Gonads and bursa of Fabricius will be analyzed for all birds by histological assessment. Slides will be labeled and well organized for retrieval and review. Liver tissue will be analyzed for EROD activity as a biomarker of planar PCB exposure. Because the amount of tissue will be limited, we will prioritize the analyses for the hypothalami and will perform aromatase enzyme in all individuals, then monoamines and GnRH-I when possible, i.e., when tissue samples are large enough. Please note that the monoamines and GnRH-I are analyzed in the same extract. Hypothalamic monoamines, GnRH-I and aromatase activity will be measured from the posterior hypothalamus, which is the area of storage and release of GnRH-I. Monoamines are measured by HPLC with electrochemical detection. GnRH-I is measured by an EIA (enzyme immunoassay), using an antibody specific for GnRH-I. Aromatase will be measured by radiological enzymatic activity assay. Estradiol and androgens in fecal matter and thyroid hormone content of thyroid gland will be measured by RIA (radioimmunoassay), according to techniques used in our laboratory. RIAs will be validated for tree swallows and American kestrels. Additional tissue will be collected from hatchlings and stored for microarray analysis from any or all of the following: hypothalamus, gonad, bursa, thyroid, heart and liver. Lab notebooks and records from these assays will be dated and initialed.

SOPs:
"Histological Analysis Of Avian Tissue: Gonads and Bursa of Fabricius"
"EROD Assay For CYP450 Activity In Liver Samples"
"High Pressure Liquid Chromatography: Monoamine Analysis"
"GnRH-I ELISA"
"Protein Assay"
"Extraction of Steroid Hormones from Avian Feces"
"Radioimmunoassay of Androgens In Avian Serum or Fecal Extract"
"Radioimmunoassay of Estradiol In Avian Serum or Fecal Extract"
"Digestion Of Thyroid Gland And Radioimmunoassay of Thyroid Hormones".
"Analysis of Aromatase Activity in Hypothalamic Tissue"
"Tissue Storage, RNA Isolation and Microarray Analysis"

2.4.6 Statistical Analyses

Data will be analyzed by testing for 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 or effects. 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. A biostatistician has agreed to serve as a statistical consultant. These statistical tests may be revised, including not performed, by the Principal Investors (PI) based on data collection. Further, the PIs may conduct additional statistical tests not noted above.

The PIs plan to conduct the following comparisons. Null (HO) and alternative (HA) hypotheses are presented below. "PCB" refers to PCB 126 for work with tree swallows and PCB mixture for work with chickens and American kestrels.

2.4.6.1 Compare the embryo survival or hatchability of eggs injected with the PCB with eggs that are un-injected (control) or are injected with only the vehicle solution for the PCB (vehicle control).

- General Hypotheses
  HO: Hatchability of eggs injected with the PCB is equal to the hatchability of control eggs
  HA: Hatchability of eggs injected with the PCB 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.

2.4.6.2 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.

2.4.6.3 Compare organ weights of PCB exposed embryos and unexposed embryos.

- General Hypotheses
  HO: Organ weights in PCB exposed embryos are not different than controls
  HA: Organ weights in PCB exposed embryos are different compared to controls and are proportionally related to the dose of treatment

  For liver weight, HA: PCB exposure will enlarge liver.

- Statistical tests
  All data will be tested for normality and homogeneity of variances. If assumptions of normality and homogeneity of variances are met, organ weights will be compared across treatment groups by treatment and sex, and possible interactions examined, using 2-way ANOVA with appropriate post hoc comparisons. Alternately, 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.

2.6.4.4 Compare histology of gonad, bursa and heart of PCB exposed embryos to unexposed embryos.

- General Hypotheses
  HO: Gonad, bursa, and heart morphology in PCB exposed embryos are not different than controls
  HA: Gonad, bursa, and heart morphology PCB exposed embryos are different compared to controls and are proportionally related to the dose of treatment

- Statistical tests
  All data will be tested for normality and homogeneity of variances. If assumptions of normality and homogeneity of variances are met, histological indices of morphology will be compared across treatment groups by treatment and sex, and possible interactions examined, using 2-way ANOVA with appropriate post hoc comparisons. Alternately, 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.

2.4.6.5 Compare cytochrome P450 activity in liver microsomes from PCB exposed embryos to that of unexposed embryos.

- General Hypotheses
  HO: Cytochrome P450 activity in PCB exposed embryos is not different than controls
  HA: Cytochrome P450 activity in PCB exposed embryos is higher than controls and is proportionally related to the dose of treatment

- Statistical tests

  All data will be tested for normality and homogeneity of variances. If assumptions of normality and homogeneity of variances are met, cytochrome P450 activity will be compared across treatment groups by treatment and sex, and possible interactions examined, using 2-way ANOVA with appropriate post hoc comparisons. Alternately, 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.

2.4.6.6 Compare thyroid hormone (T3 and T4) content of thyroid glands from PCB exposed embryos to that of unexposed embryos.

- General Hypotheses
  HO: Thyroid hormone (T3 and T4) contents of thyroid glands in PCB exposed embryos are not different than controls
  HA: Thyroid hormone (T3 and T4) contents of thyroid glands in PCB exposed embryos are lower than controls and are proportionally related to the dose of treatment

- Statistical tests
  All data will be tested for normality and homogeneity of variances. If assumptions of normality and homogeneity of variances are met, thyroid hormone concentrations will be compared across treatment groups by treatment and sex, and possible interactions examined, using 2-way ANOVA with appropriate post hoc comparisons. Alternately, 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.

2.4.6.7 Compare estradiol and androgen content in fecal matter from PCB exposed embryos to that of unexposed embryos.

- General Hypotheses
  HO: Estradiol and androgen content in PCB exposed embryos are not different than controls
  HA: Estradiol and androgen content in PCB exposed embryos are different than controls and are proportionally related to the dose of treatment

- Statistical tests
  All data will be tested for normality and homogeneity of variances. If assumptions of normality and homogeneity of variances are met, steroid hormone concentrations will be compared across treatment groups by treatment and sex, and possible interactions examined, using 2-way ANOVA with appropriate post hoc comparisons. Alternately, 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.

2.4.6.8 Compare hypothalamic aromatase activity, catecholamine content and GnRH-I concentration in PCB exposed embryos to unexposed embryos.

- General Hypotheses
  HO: Hypothalamic aromatase activity, catecholamine content and GnRH-I concentration in PCB exposed embryos do not differ from controls
  HA: Hypothalamic aromatase activity, catecholamine content and GnRH-I concentration in PCB exposed embryos are different than controls and are proportionally related to the dose of treatment

- Statistical tests
  All data will be tested for normality and homogeneity of variances. If assumptions of normality and homogeneity of variances are met, hypothalamic aromatase activity, catecholamine content and GnRH-I concentration will be compared across treatment groups by treatment and sex, and possible interactions examined, using 2-way ANOVA with appropriate post hoc comparisons. Alternately, 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 PI based on data collected. Further, the PI may test other hypotheses and conduct additional statistical tests not noted above.

3. QUALITY ASSURANCE/QUALITY CONTROL

3.1 Data Quality Objectives, Indicators, and Assessment

3.1.1 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, observation and measurement of live birds, 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.

3.1.2 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 and 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 Coordinators 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 before the beginning of egg injection. Reliance on a detailed, explicit, and fully reviewed plan ensures that:

- Study objectives, methods, procedures, and details are documented before sampling.
- 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 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 and/or Co-Principal Investigator.

3.2 Data Generation and Acquisition

3.2.1 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.

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

Accuracy is 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 may be performed using a positive control sample or reference standard for most biological assays. Another measure of accuracy will be the closeness of duplicate determinations of the same sample in an assay.

Completeness is 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.

Comparability is 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.

3.2.2 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.

3.2.3 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 codes of AMKE and TRES will be used for American Kestrel and tree swallow samples respectively; CKN will be used for chicken samples. Each egg will be assigned a unique egg code as follows: Series of numbers 1-199 for Patuxent Research Refuge, 200-799 for Great Sacandaga Lake and 800-899 for Hudson River will be used for tree swallows. For American kestrels collected from Patuxent Research Colony, the unique egg code will reflect the nest number and clutch order number (e.g. 634-6 for the sixth egg laid in nest 634). For chicken a series of numbers 1000 and higher will be used. Samples from each egg/embryo will be identified by a sample ID encompassing the species, egg code and year,
e.g. TRES-1-2006. 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.

3.3 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 study will not begin until approval is received from the Quality Assurance Coordinator or their delegate. The Quality Assurance Coordinator will conduct an audit of the procedures and documentation of the study.

3.4 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.

3.5 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:

- Project name;
- Sample identification (unique for each sample);
- Sample matrix (e.g., egg contents, liver) which may be part of the sample ID;
- Name and signature of individual relinquishing custody;
- Name and signature of individual accepting custody;
- 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 airbill. 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.

4. PRINCIPAL INVESTIGATORS

Principal Investigator
The PI is a neuroendocrinologist with twenty five 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 avian toxicologist with experience in egg injection studies and immune and endocrine disruption studies in birds. The Co-PI will plan the logistics of all aspects of the study and participate in assays, data collection and data analysis.

Research Technicians with expertise in endpoints required as part of the study will conduct assays and analyze data as needed. The lead technician will devote 50% time to this work.

Other staff will assist with animal care, sampling and assays as needed.

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

5. LITERATURE CONSULTED

Barron, M. G., H. Galbraith, and D. Beltman. 1995. Comparative reproductive and developmental toxicology of PCBs to birds. Comp. Biochem. Physiol. , C: Comp. Pharmacol. Toxicol. 112C: 1-14.

Brunstrom, B., Halldin, K. 1998 EROD induction by environmental contaminants in avian embryo livers. Comparative Biochemistry and Physiology Part C 121: 213-219.

Brunstrom, B. and J. Lund. 1988. Differences between chick and turkey embryos in sensitivity to 3,3',4,4'-tetrachloro-biphenyl and in concentration/affinity of the hepatic receptor for 2,3,7,8-tetrachlorodibenzo-p-dioxin. Comp. Biochem. Physiol. , C 91: 507-512.

Brunström, B. and L. Reutergårdh. 1986. Differences in sensitivity of some avian species to the embryotoxicity of a PCB, 3,3',4,4'-tetrachlorobiphenyl, injected into the eggs. Environ. Pollut. (Series A) 42: 37-45.

DeWitt, J. C., E. B. Meyer, and D. S. Henshel. 2005. Environmental toxicity studies using chickens as surrogates for wildlife: effects of vehicle volume. Arch. Environ. Contam. Toxicol. 48: 260-269.

Fox, L. L. and K. A. Grasman. 1999. Effects of PCB 126 on primary immune organ development in chicken embryos. Journal of Toxicology and Environmental Health, Part A 58: 233-244.

Grasman, K. A. and L. L. Whitacre. 2001. Effects of PCB 126 on thymocyte surface marker expression and immune organ development in chicken embryos. Journal of Toxicology and Environmental Health, Part A 62: 191-206.

Henshel, D. S., B. Hehn, R. Wagey, M. Vo, and J. D. Steeves. 1997. The relative sensitivity of chicken embryos to yolk or air-cell injected 2,3,7,8-tetrachlorodibenzo-p-dioxin. Environ. Toxicol. Chem. 16: 725-732.

U.S. Environmental Protection Agency. 2001. Analyses of Laboratory and Field Studies of Reproductive Toxicity in Birds Exposed to Dioxin-Like Compounds for Use in Ecological Risk Assessment. EPA/600/R-03/114F, 1-39 plus appendices. Cincinnati, OH,

U.S. Environmental Protection Agency, Office of Research and Development, National Center for Environmental Assessment.

6. STANDARD OPERATING PROCEDURES

6.1 Recording and Handling Data for Avian Egg Injection Study

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

Procedure

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

6.2 Field Collection of Tree Swallow Eggs for Avian Egg Injection Study, Hudson River NRDA

INTRODUCTION

Avian egg injection is a well-established technique to assess the effects of contaminants on a developing avian embryo. To conduct an avian egg injection experiment, eggs from Patuxent Wildlife Research Refuge will be collected and brought into a laboratory where they are injected with the substance being tested. Proper handling of the eggs during collection and transit to the laboratory is essential to maintain viability in eggs that will subsequently be injected with contaminants and incubated. A subsample of eggs 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 foam padding)
- Labels
- Styrofoam or other type of cooler or ice chest
- Frozen cooler pack(s)
- 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. Eggs are generally laid at one day intervals. Monitor the laying of eggs, waiting till the fifth egg is laid. On the day the fifth egg is laid collect 2 eggs from that nest. Continue monitoring the nest. If another egg shows up in that nest the next day (so the total clutch would have been 6), a third egg can be collected (leaving three).
- For each egg collected, complete the appropriate information on the Avian Egg Collection Datasheet.
-
- For egg that are going to be incubated: Using a soft pencil, mark the Egg Code on the egg.
- Transport to lab in hard container with sufficient padding.
- Complete chain of custody transfer of samples from field collection crew to laboratory crew on Egg Collection Data Sheet.
- For eggs to be incubated: Follow Work Plan procedures.
- For eggs that are going to be analyzed for contaminants and not incubated: 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 for Removal of Avian Egg Contents for Contaminants Analysis, Hudson River NRDA.

Avian Egg Collection Data Sheet – Hudson River Avian Egg Injection Study Page ____ of ____

Collector ____ Data Recorder ____
Name Signature Name Signature

Egg Code[1]	Location[2]	Date Collected[3]	Time Collected[4]	Clutch Size	Eggs Warm Yes or No	Comments

[1] Egg Code: Numeric code beginning at 1; [2] Pond Name and Nest box number; [3] In MM/DD/YEAR format, such as 04/30/2006 for April 30, 2006; [4] In 24-hour format, such as 1300 for 1PM

Custody of samples listed above transferred from field collection crew to laboratory crew as follows:
Relinquished by: ____
Signature Print Name Company/Title Date Time
Received by: ____
Signature Print Name Company/Title Date Time

Data Sheet checked by: ________________________________ Date: _____________
Name/Initials

6.3 Removal of Avian Egg Contents for Contaminants Analysis
Hudson River NRDA

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

- ❑ Fill out the Avian Egg Processing Data Sheet; use one data sheet per egg.
- ❑ If debris is present, rinse egg in cool water while gently scrubbing with green scrubby or sponge. Do not soak the egg.
- ❑ Dry and weigh whole egg to the nearest .01 g
- ❑ Take three measurements each of egg length and maximum egg width with calipers. Compute average of three measurements for final width and length measurements.
- ❑ Transfer egg contents to chemically-clean jar using the following procedure:
  1. Use nitrile gloves for this part of the procedure. Avoid letting contents run over your hands into the sample jar.
  2. 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.
  3. Weigh the clean empty jar with lid on, and note this tare weight on data sheet.

4. Place jar in center of aluminum foil, and loosen the lid.
5. 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.
6. 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.
7. For swallows, hold the egg vertically with air cell end up. Using scissors cut the top of the eggshell off above the air cell if possible. Pour contents into the jar, or use the scalpel to gently scrape if that is necessary. Use forceps to remove any shell fragments from the jar. Avoid getting shell dust, or anything else besides the egg contents, in the jar.
8. 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. Wet weight conversion will be made based on the weight and egg measurements. 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. Using these procedures, the weight of each egg's contents will be measured, even for eggs whose contents are combined into a single 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.

- Place label on jar. Place clear tape over the label to keep it from getting wet.
- Prepare Chain of Custody records and maintain egg samples under chain of custody.
- Freeze samples. Ship under Chain of Custody 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.

Avian Egg Processing Data Sheet

Processor(s): Name ____________________________ Name ____________________________

Signature _________________________ Signature _________________________

Date Processed: _________________________________

Egg Code: _________

Sample ID: ____

Egg Length (three measurements, mm):_______ , _______ , ________ Average _______

Egg Width (three measurements, mm): _______ , _______ , ________ Average _______

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, ¼, ½, ¾ , full term

Data Sheet checked by: __________________________________ Date: _____________

Name/Initials

6.4 Egg Injection and Incubation Procedure for Tree Swallow (Tachycineta bicolor) Eggs

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

Robertson et al. (1992) in Birds of North America describe the eggs, their incubation and hatching of tree swallow chicks and is summarized herein:
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 more glossy 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 next 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.

Incubation Procedures

1) Upon receipt of the eggs, examine them noting any evidence of damage or embryonic development (by candling) and assign each egg a number. Note on the coding sheet the source, nest number, egg number for the clutch etc. Write the egg number on the egg in soft pencil on the pointed end of the egg.
2) Assign eggs to treatment groups with consideration of number of eggs available, number of eggs from the same clutch (consult the biostatistician).
3) Weigh eggs and note weight.
4) Place eggs in an incubator in an egg rack of suitable size on their sides. Incubation will be done at 99.5ºF and humidity adjusted as needed to ensure correct moisture loss (see no. 5). Eggs will be turned hourly by automatic rotation in the incubator for a total of 60º every two hours. In addition turn eggs 180º by hand or using a scoopula twice per day (before 10 am and after 4 pm). Mark the eggs with an X and an O on opposite side to confirm eggs are turned.
5) Check moisture loss by weighing a subset of eggs every other day and adjust the humidity appropriately to ensure correct moisture loss with egg mass loss averaging 14% over entire incubation period. For an average 1.9 g tree swallow egg over a 15 day incubation, moisture loss should average approximately 1% or 0.02 g per day.
   On days four, seven, and thirteen of incubation, candle the eggs and remove infertile and dead eggs. Retain infertile and dead eggs for contaminants analysis; follow SOP "Removal of Avian Egg Contents for Contaminants Analysis, Hudson River NRDA".
6) Two days before estimated hatch date, (~90% of incubation) open the eggs and sample tissues from the embryos.

Injection Procedures

1) Two and half days into incubation (expected equivalent age to a 3 day old quail embryo, i.e. 18% of incubation based on a 15 day incubation for a tree swallow egg), candle eggs, note center of air cell with a dot of sharpie and remove infertile or dead eggs. Retain any infertile and dead eggs for contaminant analysis if warranted.
2) Assign the eggs to treatment groups and 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 Dremel drill with a fine drill bit.
    c. Inject the vehicle or PCB 126 solution, 0.4 µL/g egg into the air cell, with a micro-pipettor and extended tip or Hamilton syringe.
    d. Seal the hole with paraffin.
    e. Allow the egg to sit pointed end down until the end of the 30 minute period.
4) Place eggs back into the incubator on their sides in an egg rack of suitable size. 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

Incubators: Natureform NMC2000 or GQF Sportsman 1502
Egg trays
Light for candling
Ethanol and tissue or alcohol wipes
22 gauge needles or 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

"Avian Egg Collection Data Sheet", "Incubator Record", "Egg Treatment and Incubation Log", "Log of checking eggs", "Moisture Loss Data Sheet", "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.

Incubator Record

Incubator	Date	Time	Temperature	% Humidity or Wet Bulb Temp	Eggs Turned (O/X)	List any adjustments	Initials

Reviewed by ______________________________ Date __________

Egg Treatment and Incubation Log **Chemical:**
Species: **Source:**

Treatment (ug/g)	Egg ID	Egg Mass (g)	Dosing Concentration ug/uL	uL injected	Date of Injection (ED2.5)	Date Death Detected	Stage at death	Initials

Make any additional notes on reverse. Reviewed by: ____________________

Log of checking of eggs

Species: ______________ **Egg Code Range:** ____________________

Date & Time Eggs Set: __________________________ **Date Injected:** ____________________

Date	Time	Investigator	Embryonic Day	# infertile	#dead	# remaining	Initials

Make any additional notes on reverse.

Reviewed by: ______________________________ Date: __________

Egg Moisture Loss

Species: **Source:**

Date	Time	Egg ID	Embryonic Day	Weight (g)	Comments	Initials

Reviewed by: ______________________

Date: ______________________

Deformity Score Sheet

Date	Species	Egg Code	Date Death Detected	Stage*	Cross Bill**	Short Upper Bill	Abnormal Eye Size	Neck/head Edema	Incomplete Skull	Clubbed Feet	Mal-position	Gastroschisis (post stage 45)	Other	Initials

Reviewed by: Date:

* If embryo is not old enough to detect a structure, or is too decomposed note "NS" for not scored under the deformity type.

** Note 'Y' (yes) or 'N' (no) to note presence or absence of the deformity.

6.5 *Egg Injection and Incubation Procedure for American Kestrel (*Falco sparverius*) Eggs*

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 (13ºC) 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 99.5ºF and 55-65% humidity (84ºF wet bulb). In addition to the hourly turning (60º) of the eggs done automatically by the incubator, turn the eggs 180º 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 Dr. Gary Heinz, Patuxent NWRC. Pisenti et al. (2001) described a 9% egg mass loss for embryos surviving to hatch and Dr. Heinz 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 (99.5ºF 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.

<u>*Injection Procedures*</u>

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 (consult the biostatistician). 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, 0.4 µL/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

"Avian Egg Collection Data Sheet", "Incubator Record", "Egg Treatment and Incubation Log", "Log of checking eggs", "Moisture Loss Data Sheet", "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.

6.6 Egg Injection Procedure for chicken (*Gallus domesticus*) Eggs

This protocol outlines procedures for incubating eggs and injecting chemicals into the eggs of domestic chickens. The purpose of these procedures is to mimic maternal deposition of chemicals into the egg for determination of toxicity to the embryo or chick.

1) Upon receipt from supplies, store the eggs at 15ºC until needed (but no more than 5 days).
2) Place eggs in an incubator in an egg rack of suitable size with the pointed end down. Incubate the eggs at 99.5ºF and 55-65% humidity (84ºF wet bulb) with hourly turning of the eggs.
3) On embryonic day four, candle the eggs and remove those that are infertile or which contain dead embryos.
4) Assign the eggs to treatment groups, mark each egg with unique code in pencil 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. Round the volume to the nearest 0.01 µL.
5) Make injections into the egg as follows, allowing the eggs to be outside the incubator for not more than 15 minutes:
    a. Wipe the blunt (air cell) end of the egg with 70% ethanol.
    b. Gently make a hole in the egg with a 20 gauge needle.
    c. Inject the vehicle or PCB mixture, 0.1 µL/g egg into the air cell, with a micro-pipettor and extended tip or Hamilton syringe.
    d. Seal the hole with paraffin.
6) Return eggs to the incubator.
7) On days five, eleven, and eighteen of incubation, candle the eggs and remove infertile and dead eggs.
8) On ED 18, transfer eggs to a 'hatcher' incubator, with each egg in an individual compartment.
9) Necropsy at approximately 24 hours after hatch.

Equipment Needed

Scales (510 - 0.001 g) Mettler Toledo PG503-S
Incubators: Natureform NMC2000
Egg trays
Light for candling
Ethanol and tissue or alcohol wipes
20 gauge needles
Rainin Pipettman with extended tips: one tip per egg
or Hamilton Syringe
Paraffin and tool to apply it to egg
Heating plate

Data Sheets: "Avian Egg Collection Data Sheet", "Incubator Record", "Egg Treatment and Incubation Log", "Log of checking eggs", "Moisture Loss Data Sheet", "Deformity Score Sheet".

6.7 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 before 24 hours after hatch. This protocol outlines appropriate dissection techniques and sample storage conditions for several tissues including:

Blood for serum
Brain for hypothalamic monoamines analysis
Liver for CYP450
Fecal matter from lower intestine for estradiol and androgen measurements
Thymus for mass and Bursa for mass and histology
Thyroid for thyroid hormone radioimmunoassay
Gonads for histology
Tissues for RNA isolation**

1) Bring ten to twenty hatchlings at a time to the necropsy room and keep warm in a table top incubator.
2) Record time necropsy is initiated and completed. Record all data on appropriate data sheet.
3) Weigh the hatchling.
4) 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.

** For tissues requiring microarray analysis, transfer a liver sample or hypothalamus/brain sample to a microcentrifuge tube and snap freeze in liquid nitrogen . Store at -80ºC.

5) Immediately remove the brain from the head, intact, and drop it directly into dry ice powder in an ice bucket. Make brain dissection as follows: cut along the sides of the skull on 3 sides including anterior; pull skull up; remove dura mater, break nerves and exteriorize the brain. After at least one minute on dry ice, fold the brain into a cold piece of aluminum foil and keep temporarily on dry ice.
6) By pinching around the cloaca, remove a fecal sample to a pre-tared 1.5 mL micro-centrifuge tube. Weigh and record the weight of the fecal sample. Store on dry ice.
7) Record the condition of the umbilicus and internal yolk sac, e.g. has it retracted properly into the abdomen? Is it adhered to the internal umbilicus? Tare a chemically clean jar on the scale and collect the yolk and transfer it to the jar, noting the weight.
8) Dissect the liver, remove the gall bladder and weigh the liver. Place the liver in a cryovial, or if it is too big for one vial, mince it and divide the tissue between multiple vials. Flash freeze the liver in liquid nitrogen for CYP450 analysis.
9) Dissect out the spleen, weigh it, then discard it.
10) Dissect out the heart, weigh it and preserve in appropriate fixative: Trim the heart of blood vessels in a standard manner from sample to sample, being careful not to remove any heart muscle. Weigh and then preserve the heart in 10% buffered formalin for 24-48hours. For storage longer than 48 hours, replace the formalin with 70% ethanol.
11) Remove both right and left thyroid at the same time. The thyroid is located at the caudal point of the thymus just anterior to the heart. Thyroid is within the thorax, ventral to and bound by fascia to the carotid artery. Weigh both thyroids separately but store together in a

1.5 mL micro-centrifuge tube. Weigh the thyroids on plastic, weighing paper will cause desiccation. Freeze thyroids on dry ice.

12) Remove the bursa, weigh it and place it in a 1.5 mL microcentrifuge tube in appropriate fixative.
13) Identify the gonads to determine gender. Males have two kidney shaped testicles. Females have one left ovary. Leave the gonads intact and remove them on a portion of the back of the carcass. Fix in 10% neutral buffered formalin or other appropriate fixative.
14) Place the remaining carcass in freezer bags by treatment and place in the freezor.

Long term storage:
Brains, serum, liver, thyroids, feces: -80ºC
Yolk: -20ºC
Fixed tissue: room temperature
Tissue for RNA analysis: -80ºC

Equipment Needed

Box
Heating plate
Scales sensitive to 0.00001 grams
(Mettler MT5)
Scales (510 - 0.001 g) Mettler Toledo PG503-S
Scissors for decapitation
Dissecting scissors and forceps
12x75 mm glass boroscillicate tubes
Dry Ice Powder
Aluminum foil
Cryovials
1.5 mL microcentrifuge tubes
Bouin's Fixative or Davidson's Fixative
10% buffered formalin
Liquid nitrogen
RNA Later if needed
Labor: ideally a minimum of 4 people participate to ensure rapid dissection and storage of tissues

Data Sheets

"Hatchling Sampling Data Sheet

Hatchling Sampling Data Sheet **Date** ________________ **Investigators:**

										Thyroid (mg)						
Egg Code	**H A**	**Time Start**	**Body Mass (g)**	**Blood & Brain***	**Yolk (g)**	**Feces (mg)**	**Liver (mg)**	**Heart (mg)**	**Spleen (mg)**	**Left**	**Right**	**Bursa (mg)**	**Gen -der**	**Gonads ***	**Time Finish**	**Initials**

HA = hatchling Appearance: note deformities

Reviewed by: ________________ Date: ________________

* Check off if collected, weigh gonads before embedding for histological analysis

6.8 Histological Analysis Of Avian Embryo Tissue: Gonads and Bursa of Fabricius

This protocol describes the process of dehydrating, embedding, sectioning and staining tissue for histological study under light microscopy.

Procedure

1. Fix tissue with 10% buffered formalin

2. Dehydration:

a.	70% EtOH	1 hour	
b.	85% EtOH	1 hour	
c.	95% EtOH	40 minutes	
d.	95% EtOH	40 minutes	
e.	100% EtOH	40 minutes	
f.	100% EtOH	1 hour	
g.	AMERICLEAR	40 minutes	
h.	AMERICLEAR	1 hour	
i.	Paraffin	40 minutes	@ 56°C
j.	Paraffin	1 hour	@ 56°C
k.	Paraffin	1 hour	@ 56°C

3. Embed tissue in paraffin with desired orientation (longitudinal orientation for bursas; **this is very important**). This step must happen quickly in order for the paraffin to solidify in one block. See note below for gonad orientation (##).

4. Section tissue embedded in paraffin into 5-10 μm sections. Make three slides. Place 3-5 sections on one slide. Use plus-treated slides, as the tissue will stay on the slides while they are taken through the washing and staining process.

5. Place slides in an oven or on a hotplate at 60°C for 30-60 minutes. This helps with keeping the sections bonded to the slide.

6. Stain the tissue by washing the slides as follows:

a.	Xylenes	2 minutes
b.	Xylenes	2 minutes
c.	100% EtOH	1 minute
d.	100% EtOH	1 minute
e.	95% EtOH	1 minute
f.	95% EtOH	1 minute
g.	Tap water (non-running)	10 minute
h.	Mayer's hematoxylin	15 minutes
i.	Lukewarm running tap water	20 minutes
j.	Eosin	2 minutes

k.	Non-running tap water	1 minute
l.	95% EtOH	2 minutes
m.	95% EtOH	2 minutes
n.	100% EtOH	2 minutes
o.	100% EtOH	2 minutes
p.	100% EtOH	2 minutes
q.	Xylenes	2 minutes
r.	Xylenes	2 minutes
s.	Xylenes	2 minutes

7. Set slides out to dry.

8. Mount cover slip with mounting medium after the slides are dry. Try not to use too much mounting medium.

Notes for gonads:

Cut tissue in 3-5 µM sections. Place multiple sections on each of at least 5 slides for every embedded block of tissue. For testes cut transverse. For ovaries, cut to acquire both end and middle regions, i.e., several sections of each of three regions per ovary. For ease cut the organ before dehydration and embedding.

Alternative staining: For immature gonads stain with Toluidine Blue. For mature ovaries stain with Toluidine Blue. For mature testes stain with Berg's stain to distinguish spermatozoa.

Endpoints:

Bursa: 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.

a. Male gonad:
    i. Differentiate cortex and medulla
    ii. Area of seminiferous tubules
    iii. Number of spermatozoa
    iv. Abnormalities in Sertoli or Leydig cells and outer cortex
    v. Presence of lipid filled vacuoles indicating steroidogenic activity
b. Female gonad:
    i. Number of primordial follicles and oocytes
    ii. Differentiate cortex and medulla
    iii. Relative area of cortex and medulla
    iv. Abnormalities

Record a digital image of each section used.

Equipment Needed:
Tissue Tek(VIP1000) for tissue dehydration and embedding
Microtome for tissue sectioning
Warm water bath for mounting tissue onto slides
Hot plate for warming slides prior to washing

Additional Supplies:
Paraplast
10-20 baths for washing fluids
Plus-treated slides
Cover slips
Mounting medium
Hematoxylin
Eosin
Ethanol
Xylenes

Literature Consulted

Reaves, M.E. 2002. Effects of organochlorines on reproductive structures of wild fish-eating birds and domestic chickens. PhD Dissertation, Wright State University, Dayton, OH.

6.9 Ethoxyresorufin-o-dealkylase (EROD) Assay For CYP450 Activity In Liver Samples

This protocol outlines a method for preparing microsomes from hatchling avian liver tissue by differential centrifugation and measuring enzyme activity of cytochrome P450-1A (a monooxygenase or mixed function oxidase) in those microsomes. The expression/activity of CYP450 is elevated by exposure to xenobiotics. Halogenated aromatic hydrocarbons (HAHs), especially planar dioxins, furans and polychlorinated biphenyls increase CYP450 expression via interaction with the aryl hydrocarbon receptor.

Expression of CYP450 is a biomarker of exposure to HAHs. CYP450 is a phase I metabolic enzyme that has dealklyase activity. The enzyme activity is measured by assaying EROD (ethoxyresorufin-o-dealkylase) activity. 7-ethoxyresorufin serves as a substrate for this enzyme which yields resorufin as a fluorescent product. NADPH is the cofactor which donates electrons. The reaction is $NADPH_2$ + substrate + $O_2 \rightarrow$ substrate-O + H_2O + NADP. Enzyme activity is expressed as nmol or pmol of resorufin per mg protein per minute. This protocol is adapted from Melancon (1997) and Brunstrom and Halldin (1998).

Reagents

Homogenization buffer: Na/K Phosphate pH 7.4

Stock A 0.2M KH_2PO_4 = 27.22 g/L Potassium Phosphate-Monobasic, using distilled water

Stock B 0.2 M Na_2PO_4 = 28.40 g/L Sodium Phosphate-Dibasic, using distilled water

The homogenizing buffer, at pH 7.4, is prepared by mixing approximately 1 part stock A to 4 parts stock B until a pH 7.4 is reached. (Stock A will decrease pH while stock B will increase it)

Microsomal resuspension buffer: 0.05M Na/K Phosphate containing 10^{-3}M Disodium Ethylenediamine Tetraacetate (EDTA), pH 7.6

Stock C = 6.80g KH_2PO_4 + 0.372g EDTA/L using distilled water

Stock D = 7.10g Na_2PO_4 + 0.372g EDTA/L using distilled water

The resuspension buffer is prepared by starting with Stock C and raising pH to 7.6 using Stock D.

Tris Buffer

0.066 M Tris-HCl, pH 7.4

Start with 7.99 g of Trizma base and lower pH to 7.4 or start with 10.4 g of Trizma HCl and raise pH with NaOH to 7.4.

For avian sample:

Stock Solutions::

1.00 mM solution of 7-ethoxyresorufin(Sigma E3763 "Resorufin ethyl ether") in methanol (HPLC-grade)

2.0mM solution of Resorufin sodium salt in methanol (HPLC grade)

Working Solutions:

2.5 µM 7-ethoxyresorufin in Tris-HCl
1 x 10^{-6} M resorufin in Tris-HCl
0.25 mM solution of NADPH in Tris-HCl

Procedure

Keep all samples on cool on wet ice between each step

7) Weigh excised liver (without gall bladder). Place the liver in a cryovial and flash freeze in liquid nitrogen if storage is necessary before microsomal preparation.
8) Thaw liver sample on crushed ice.
9) Using Melancon's approach for small liver samples, homogenize the liver in **4X** of ice cold **homogenizing** buffer per **weight** of tissue. Keep the tube in a beaker of crushed ice while homogenizing. Homogenizer: motor driven stainless steel and teflon pestle in a glass homogenizing vessel is suggested. Use **no less** than **1 mL** of buffer! Divide the homogenate into two. Retain one half of the homogenate for potential additional endpoints and continue the procedure with the second half of the homogenate.
10) Centrifuge homogenate for 20 min at 11,000g in the **pre-cooled**, 4ºC, Eppendorf 5402 centrifug (~11500 rpm).
11) Collect **supernatant** from step 4 and centrifuge it at 100,000 xg for 60 min at 4ºC to obtain microsomes. Use the Sorvall MC120 centrifuge, rotor RP80-T at 46,000 rpm. If extra volume in the tubes is needed, it is permissible to add more homogenizing buffer.
12) Resuspend microsomes in **resuspension** buffer. If necessary, aliquout samples into 2 or more tubes to allow one tube to be kept in the ultracold without all microsomes undergoing freeze thaw cycle. Store the microsomes in aliquots at -80ºC and upon thawing vortex vigorously before proceeding. Multiple freeze/thaw cycles can affect activity. Refer to literature for guidance on microsome concentration for each species, or if unavailable, literature for a species with similar size liver to the species you are analyzing.

The quantity of microsomes used should be that which gives a linear response over the time of the assay, within the range of the standard curve. If enzyme activity is highly induced, one may need to re-run the assay with a smaller number of microsomes. In one microtiter plate run 7 triplicate samples, four resorufin standard curves of 10^{-8}, 10^{-9}, 10^{-12}, and 10^{-15} dilutions and 4 wells of non-induced reference microsomes, e.g. from Japanese quail. A total volume of 260 uL per well is optimal.
***As of August 2005, samples (S1, S2, etc.) were made in 1x(100%), 1:2(50%) and 1:4(25%) dilutions.

1:2 = 100ul of tris + 100ul of microsomes = 200ul, 50ul of this mixture per well and 50ul left for protein

1:4 = 150ul of tris + 50ul of microsomes = 200ul, 50ul of this mixture per well and 50ul left for protein

Example Plate Layout:

	1	2	3	4	5	6	7	8	9	10	11	12
A	250 ul 10^{-8}	250 ul 10^{-9}	250 ul 10^{-12}	250 ul 10^{-15}	S1 1x	S1 1x	S1 1x	S3 1:4	S3 1:4	S3 1:4	S6 1:2	S6 1:4
B	**100ul 10^{-8}** 100ul tris	**100ul 10^{-9}** 100ul tris	**100ul 10^{-12}** 100ul tris	**100ul 10^{-15}** 100ul tris	S1 1:2	S1 1:2	S1 1:2	S4 1x	S4 1x	S4 1x	S6 1:2	S6 1:4
C	100ul tris 100ul B1	100ul tris 100ul B2	100ul tris 100ul B3	100ul tris 100ul B4	S1 1:4	S1 1:4	S1 1:4	S4 1:2	S4 1:2	S4 1:2	S6 1:2	S6 1:4
D	100ul tris 100ul C1	100ul tris 100ul C2	100ul tris 100ul C3	100ul tris 100ul C4	S2 1x	S2 1x	S2 1x	S4 1:4	S4 1:4	S4 1:4	S7 1x	S7 1:4
E	100ul tris 100ul D1	100ul tris 100ul D2	100ul tris 100ul D3	100ul tris 100ul D4	S2 1:2	S2 1:2	S2 1:2	S5 1x	S5 1x	S5 1x	S7 1x	S7 1:4
F	100ul tris 100ul E1	100ul tris 100ul E2	100ul tris 100ul E3	100ul tris 100ul E4	S2 1:4	S2 1:4	S2 1:4	S5 1:2	S5 1:2	S5 1:2	S7 1x	S7 1:4
G	100ul tris 100ul F1	100ul tris 100ul F2	100ul tris 100ul F3	100ul tris 100ul F4	S3 1x	S3 1x	S3 1x	S5 1:4	S5 1:4	S5 1:4	S7 1:2	S7 1:2
H	100ul tris 100ul G1	100ul tris 100ul G2	100ul tris 100ul G3	100ul tris 100ul G4	S3 1:2	S3 1:2	S3 1:2	S6 1x	S6 1x	S6 1x	S7 1:2	250ul Tris

13) Using a round bottom, 96 well plate, suitable for fluorescence assays: To each well add 200 uL of Tris buffer containing ethoxyresorufin and 50 uL of Tris buffer containing microsomes.
14) Preincubate in the dark at 37ºC for 10 mins.
15) Add 10 uL of Tris buffer containing NADPH (0.25 mM) to each well and place the plate in the fluorescent reader. Take an initial reading then seven additional readings at 90 sec intervals (75 sec for each plate reading), excitation 544 nm, emission 590 nm. Calculate the results from the initial reading. The additional multiple readings serve only to determine that there is a stable increase in product over time. This serves as a quality control to ensure nothing unusual occurred.
16) Determine protein concentrations at the dilution used for the assay.
17) Calculate results using standard curve and protein concentration to nmol (or pmol) product per mg protein per minute.

Equipment Needed

Homogenizer

Centrifuge (11,000 xg)

Ultra centrifuge (100,000 xg)

Fluorescence microplate reader, excitation 544 nm, emission 590 nm

Literature Consulted

Melancon, M. J., **"Development of Cytochromes P450 in Avian Species as a Biomarker for Environmental Contaminant Exposure and Effect: Procedures and Baseline Values",** Environmental Toxicology and Risk Assessment: Biomarkers and Risk Assessment (5th. Volume), ASTM STP 1306, David A. Bengston and Diane S. Henshel, Eds., American Society for Testing and Materials, Philadelphia, 1997.

Brunstrom, B, Halldin, K. 1998 EROD induction by environmental contaminants in avian embryo livers. Comparative Biochemistry and Physiology Part C 121: 213-219.

Personal Communication with Mark Melancon provided some detail.

6.10 High Pressure Liquid Chromatography (HPLC):Monoamine Analysis

Background:

HPLC analysis allows determination of monoamines in brain homogenates. This SOP covers machine set-up, standard preparation and typical tissue analysis used in our lab. The Bioanalytical Systems (BAS, West Lafayette, IN; also provides analysis software package) manual provides further information regarding machine set-up. Heat and light degrade catecholamines, so acidified samples are stored in the dark at –80^{o}C. There is also freeze/thaw degradation, so standards and samples are prepared at assay concentrations the same day they are to be analyzed.

Brain sample monoamines are expressed per mg protein. Protein measurement protocol is listed under Protein Measurement.

These samples may be further analyzed for GnRH-I.

Materials:
Use all HPLC grade chemicals and deionized water (diH_2O) .
We use a Bioanalytical Systems (BAS) HPLC with the following BAS parts and subunits:
Electrochemical detector unit (2 of these) CC-5
Temperature controller LC 22C
Amperometric Detector LC 4C
Solvent Delivery System PM 80
Data Acquisition System DA 5
Injection loop: 10 ul using a Hamilton 25 ul syringe (Fisher # 80275, 1702N, 22s/ 2"/3)
BAS software upgraded 12/03 to Epsilon LC, ver. 2.34B (epsilon-web.net)
Column: BAS # MF 6213, Phase ll, ODS, 3um, 100x3.2 mm
Working electrode: BAS # F1000 glassy carbon
Reference electrode RE6 (pack of 3): BAS # MF2078
Some parts are available through BAS while others (e.g., stainless tubing precut lengths) are available from Upchurch. BAS can recommend other suppliers.

Procedure:

Three steps are required to analyze samples with the HPLC:
I- Preparation of the standard, mobile phase and samples.
II- Setting the machine.
III- Data analysis: using the BAS software

I. Standard Preparation:
In order to develop a standard curve for each chemical, we inject (analyze) at least 2 concentrations for each compound. A concentration of 2ng/10 ul is diluted serially (1:2) to 1ng and 0.5 ng as follow:

Stock A- to be kept frozen –80°C

Compound name	abbr.	molecular weight of compound	molecular weight of parent compound	equivalent weight of parent compound	amount of compound weighed (gm)
L-Arterenol bitartrate	NE	319.3	169.2	25mg	0.0472
5-Hydroxytryptamine creatine sulfate complex	5HT	387.4	176.2	25mg	0 .0549
5-Hydroxyindole-3-acetic acid	5HIAA	191.2	191.2	25mg	0.0250
3-Hydroxytryamine	DA	189.6	153.2	25mg	0.0310

Weigh out 25.0 mg or equivalent weight from the previous table (last column) in separate volumetric flasks (5 mL.).

Dissolve in 5 ml. HCL 0.1 M.
To prepare 250 ml. of HCl 0.1M, add 2.05 ml. Conc. acid to 250 diH_2O.

Stock B (to be kept frozen in small brown glass vials)
Take 10 ul from each chemical of stock A and add to 25 ml of HCl 0.1M in separate 25 ml volumetric flasks

Stock C (prepared at the time it is to be used- keep on wet ice and in the dark)
Take 100 ul of each of the 8 standards from stock B and add 200 ul HCl 0.1 M, so the total volume is 1000 uL. This stock now is ready for injection. If an individual standard compound is to be injected, use 100 uL of that B stock standard and 900 ul HCl. If there will be a 4 compound standard (eg NE, 5HIAA, DA, and 5HT) use 100 ul of each of those B stocks and 600 ul HCl.

To inject, vortex prepared standard and draw up into a syringe (this will overfill the 10 ul loop). Follow the procedure for the BAS Epsilon software to inject/begin the data acquisition.

NOTE:
stock A- 25 mg is dissolved in 5 ml, so 10 ul contains 0.010 x 5/25=.050mg= 50 ug
stock B- 50 ug is dissolved in 25 ml, so 100 ul contains 0.100 x 50/25= .200 ug= 200 ng
stock C- 200 ng is dissolved in 1000 ul, so 10 ul (injection volume) contains 10 x 200/1000=2 ng/10 ul.

The maximum capacity or volume of the stainless steel loop going from the injector to HPLC is 10 ul. The loop is overfilled to be sure that it fills to its 10 ul capacity.

Mobile Phase: to prepare 1 Liter
Chemicals:
EDTA (0.67 mM): 0.250 g (MW 372.2)

Monochloroacetic Acid (MCAA, 0.13 M) : 12.3 g (MW 94.4)

Sodium Hydroxide (NaOH 0.1N): 4.5 g (MW 40)

Procedure
This acidic mobile phase is stable under refrigeration and can be prepared (4 L / time). Note that harmful chemicals are used in the preparation of this mobile phase.
Dispose of waste chemicals using approved procedures.
Measured chemicals are added with enough diH_2O to bring the volume
to 1L.
Adjust Ph to 3.1 using NaOH to raise pH or MCAA to lower it.
Add: Octyl Sodium Sulfate (SOS 0.86mM) : 0.200 g (MW 232.3)
Store in the refrigerator until use.
When ready to use the mobile phase:
Filter solution through a 0.2 um filter using a water vacuum system.
Allow the solution to degas 20-45 min.(stir under vacuum)

Prior to beginning a cycle of analysis and then periodically during that series, one may need to follow the BAS suggested procedure for cleaning the column using 100% acetonitrile followed by 40:60 acetonitrile:diH_2O. Inspect the BAS manual troubleshooting guide for indications of the latter condition.
Degas the required volume of the mobile phase (0.5L- 1.0L).
Add acetonitrtrile (HPLC-Grade; 2%) and Tetrahydrofuran,THF (HPLC-Grade; 0.1%). For each liter we need 20 mL acetonitrile and 1 mL THF. Measure this mobile phase + additions in a volumetric flask for consistency's sake.
Begin the process of establishing a quiet baseline and standard runs with good peak separation. This may take a few days.

<u>Tissues sample preparation:</u>
Add 500 ul of 0.1M HCl to tissue samples in 12 x 75 borosilicate tubes.
Using a small bore probe and an electric tissue grinder (PowerGen125, Fisher Scientific), homogenize to completely and evenly grind tissue. Avian brain punches will be homogenized in 5 sec or less (sample hypothalamus and median eminence with a 2mm biopsy scoop, Roboz Co. #6384). If a sample is larger and needs longer homogenizing, do not overheat; homogenize no more than 10 seconds and then return the sample to the ice bucket. Keep samples in refrigerator or on ice/ in dark except during the homogenization. Punched, non-homogenized samples may be stored at $-80^{o}C$ in 0.1 M HCl.
Rinse homogenizer in distilled water after after each sample, and blot dry. Change the rinse water with each sample.
Centrifuge homogenized samples at 4C for 20 min at 2000 RPM (or long enough to get a firm pellet) in the Sorvall RT6000-D centrifuge.

Pour the supernatant into labeled spin-filters, Costar Spin-x #8161. Centrifuge the spin-filters at 4^0C for 40 min at 9000 rpm in an Eppendorf model 5402 centrifuge.
Keep the spin-filtered sample on ice in the dark together with its standard set until the entire sample group (for that day) has been processed (injected). After processing, the remainders of the standard and sample groups are stored in the dark at -80^0C.

Comment
If samples are to be analyzed for GnRH-I, a <u>minimum</u> of 100 ul of supernatant is required for each assay replicate.

II. Setting the Machine:
The HPLC machine should display the following settings (turning off the power to the unit will return the settings to default values):
Potential level : +
Manual offset : LEFT toggle:OFF & RIGHT toggle:100
Temperature : 30^{o}C keep the detector cell cabinet shut during operation to better stabilize the temperature of the column
Output : as close to 0 as possible; toggle the autozero lever to zero when needed to keep the reading near 0
AppE potential : 0.800
E gen : -0.200 V
I gen : .000 uA
Flow rate : 1.0 mL/ min this is during active operation. Reduce flow rate to .3 ml/min during non-test/ off use periods
Local/ remote : local
Detector Range : W1, the top detector in our arrangement, 5 nA: W2, the bottom detector in our arrangement, 2 nA. Remember that 5 nA is the LESS sensitive, and 2 nA is the MORE sensitive
Typical flow pressure for our mobile phase is 3000-3400 psi. The pressure will be lower for 100% acetonitrile and 40:60 acetonitrile:water solutions. The machine is set to shut down at pressures greater than 4000 psi. The electrochemical detector is left ON at all times except during servicing at which time it is in STANDBY. During off/non test periods (e.g., overnight) the flow rate is reduced and the temperature controller is turned off.
Adjusting the range depends on the concentration of the chemical on the tissue. The lower the value the higher the sensitivity.

Reference:
C.S. Duchala, M.A. Ottinger, and E. Russek. 1984 The Developmental Distribution of Monoamines in the Brain of Male Japanese Quail (*Coturnix coturnix japonica*). Poultry Science 63:1052-1060.

6.11 GnRH-I ELISA

This assay measures GnRH-I in tissue extracts or serum.

Procedure

1. Coat plates, 50 uL/ well, goat anti rabbit IgG (5ug/ml in coating buffer) for 1 hour @37^0 C or overnight @ 4^0C
2. Wash plate 3 times with wash buffer, 250 uL/ well, then blot plate
3. Pipet PEG (2%) blocker, 200 uL/ well, then incubate for 1 hour @ 37^0 C
4. Wash plate 3 times with wash buffer, 250 uL/ well, then blot plate
5. Pipet GnRH (1:200 of purified Ab in standard diluent), 50 uL/ well, then incubate for 1.5 hour @ 37^0 C
6. Wash plate 3 times with wash buffer, 250 uL/ well, then blot plate
7. Pipet standards or samples (prepared in standard diluent, SEE **Notes**,) 100 uL/ well, then incubate for 1.5 hour @ 37^0 C
8. DO NOT WASH PLATE BEFORE THE NEXT STEP
9. Pipet GnRH conjugate (1:2500 in standard diluent), 50 uL/ well, then incubate for 1.25 hour @ 37^0 C
10. Wash plate 5 times with wash buffer, 250 uL/ well, allowing the last wash to remain on the plate, room temperature, 10 minutes, then blot plate
11. Pipet SAP (1:2000 in enzyme diluent), 50 uL/ well, then incubate for 0.5 hour @ 37^0 C
12. Wash plate 5 times with wash buffer, 250 uL/ well; pipet 200 uL/ well PBS, allowing the buffer to remain on the plate, room temperature, 10 minutes, then blot plate
13. Pipet PNPP substrate (1 mg/ ml in substrate buffer), 100 uL/ well, then incubate for at least 1 hour @ 37^0 C while periodically checking color development
14. Read the plate @ 405 nm and plot using a cubic-spline vs. log-linear method.

Standards: pipet in duplicate with column placement for best space efficiency. Use 7 serial 1:10 dilutions of Avian LhRH with 1 ug/ml as the highest concentration and an 8^{th} well for a standard diluent blank.
The standards will have these concentrations:

1. 1 ug/ ml
2. .1 ug/ ml
3. .01 ug/ ml
4. .001 ug/ ml
5. .0001 ug/ ml
6. .00001 ug/ ml
7. .000001 ug/ ml

Plate sealers may be reused as long as they are completely sealing the rim of the plate to prevent dehydration of the wells.
Plates may be coated in advance and stored; plates sealed and in a plastic bag, @ -200 C. follow the method through step 3. Do not remove the PEG before freezing.
PNPP is light sensitive. Prepare it at the beginning of step 11 to allow it time to dissolve. If there is a pronounced yellow color, the PNPP is unusable.

Follow good lab techniques in handling and pipetting ELISA plates. Touching the underside of the plate or splashing reagents out of a well will distort readings. Examine the %CV for any duplicates; only less than 10% is acceptable. See the HPLC method for preparing tissue extracts. Serum is used without any treatment. There must be 100 uL of any standard or sample. Any volume less than that for a sample can be corrected using standard diluent and the dilution of the sample noted for later mathematical correction.

Coating Buffer (pH 9.6)

1. Weigh 15.9 g Na_2CO_3.
2. Weigh 29.3 g $NaHCO_3$.
3. Add all into 1000 mL Distilled water.
4. Adjust pH to 9.6.
5. Store in refrigerator.

Standard Diluent- Stock Solution (1% BSA, 1% NaN_3 solution in 10X PBS)

1. Weigh 0.5 g BSA (bovine serum albumin).
2. Weigh 0.5 g NaN_3.
3. Add both to 50 mL 10X PB
4. Mix well.

*** Put 5 mL of solution in 50 mL plastic tubes and store at 0 degrees C (or put 1.5 mL in 15 mL tubes)***

Standard Diluent – Working Solution

1. Weigh 0.844 g $NaHCO_3$.
2. Weigh 1.255 g Na_2CO_3.
3. Weigh 0.203 g $MgCl_2$.
4. Add all to 1000 mL Distilled water and mix well.

Wash Buffer

1. Measure out 8 mL of Tween-20.
2. Weigh 4 g of NaN_3.
3. Measure out 400 mL of PBS (10X).

Add all to 3600 mL of Distilled Water and mix well.

Equipment Needed

Platewasher
Pipettors, repeater pipetors, and multi-tip pipettors
pH meter
96 well plates with disposable sealers
warming oven
refrigerator
-70^0 C freezer
centrifuge

Homogenizer

Literature Consulted

Q. Li, L. Tamarkin, P. Levantine, M.A. Ottinger. 1994 Estradiol and Androgen Modulate Chicken Luteinizing Hormone-Releasing Hormone-I Release in Vitro. Biology of Reproduction 51: 896-903.

6.12 Protein Assay

This protocol describes a procedure for measuring protein content using a commercially available kit, it is used with the GnRH-I assay and HPLC analysis of hypothalamic monoamines to generate protein values. BCA Protein Assay Reagent Kit (Pierce 23225X)

Procedure:

Making Standards:

Always run a test curve with a few samples to see if they fall in the middle range of the curve. If the samples do not fit the curve, dilute the samples appropriately.

1. Standard #1 is the bovine serum albumin provided in the kit (2mg/mL)
2. Standard #2 is 0.5mL of standard #1 mixed with 0.5mL 1X phosphate buffered saline (PBS).
3. Standards #3-7 are serial dilutions of standard #2, made with 0.5mL of standard and 0.5mL 1X PBS.
4. Standard #8 is 1X PBS (a negative control).

Preparing Working Solution of Assay Reagent:

1. Mix 50 parts solution A with 1 part solution B.
2. If using the test tube method, mix 50mL of solution A with 1 ml solution B for one plate.
3. If using the microtiter plate method mix 25mL solution A with 0.5mL solution B for one plate.

Assay Using Microtiter Plates:

1. Measure samples in duplicates at a minimum.
2. Add 10 µL of standard and samples into wells.
3. Quickly add 200 µL of working assay reagent into each well.
4. Mix by gentle agitation and incubate 30 minutes at 37°C
5. Remove plate from incubation and allow to equilibrate at room temperature for 15 minutes.
6. Read at 570nm on Bio-Rad plate reader.

6.13 Extraction Of Steroid Hormones From Avian Feces

For samples from HATCHLINGS: Weigh feces WHEN THEY ARE COLLECTED - you will extract the entire sample. If feces were not weighed at collection they must be weighed before extraction which can result in sample loss. The extraction should be done in glass.

Add 1 mL of distilled water and 9 mL of 100% ethanol (ETOH) to each tube. If running an extraction efficiency the samples can be spiked with radiolabeled hormone (i.e. probably 20 uL of hot estradiol and 20 uL of hot testosterone[1]). The labeled/unlabeled hormone should be added first along with the water and then vortexed for one minute. After vortexing the ethanol should be added and vortexed again for one minute. Remember everything is radioactive from now on so proceed accordingly.

Cap tubes with purple/green lids and parafilm on top of the caps.

Secure tubes horizontally to a shaker/rocker (room 3144) for end-to-end mixing **OVERNIGHT** (12 hr minimum).

The next day centrifuge samples at ~3000 RPM for 25minutes at 4^{o}C using the table top Sorvall centrifuge in the RIA room.

Pour off supernatant into a clean 16 x 125 test tube. **SAVE THE SUPERNATANT** and discard old sample tube (*into radiation waste if necessary).

Place tubes with supernatant into water bath heated to 60 ^{o}C. (Any hotter and the supernatant might pop/boil resulting in lost sample and possible radioactive contamination). Place the water bath under the multi-tube manifold apparatus in the hood (room 3144) and gently blow off the ethanol using nitrogen gas. The combination of heat from the water bath and nitrogen gas will cause the ethanol to evaporate off much quicker.

Once the tubes are **completely dry**, resuspend the samples in 3 mL of 100% ETOH. Sonicate and vortex for one minute each.

This next step is designed for analysis of small samples, i.e., hatchlings, and is necessary for measuring estradiol and androgens from the same extract with minimal dilution of the hormone because we use two different diluents for the two assays.

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[1] Before you add tracer to the extraction you need to check expiration date for the estradiol and cpms for the testosterone - see androgen RIA SOP for the appropriate CPM range for the testosterone and check it on the scintillation counter and run the efficiency from the same batches of tracer. Write down everything you do clearly.

Remove 1.5 mL (i.e. one half) of the ethanol/hormone extract to a new tube. Pipet accurately since ethanol is volatile and tends to leak out of the pipette tip if not transferred quickly. Label one extract tube 'E' for estradiol and one extract tube 'A' for androgens.

Return samples to water bath/nitrogen gas setup and evaporate off ETOH for the final time. The hormone has now been extracted from the fecal sample and should be resuspended in RIA standard diluent depending on RIA needs:

Resuspend the 'E' tube extract in MP Biomedicals E_2 RIA kit - zero pg/mL standard. Since the estradiol assay requires 50 uL of extract per tube, should be done in duplicate and you need to have enough extract to measure hormone recovery, resuspend the dried 'E' extract in 150 uL of zero standard - that should yield 2x 50 uL for assay, 1x 20 uL for recovery and extra that will be stuck in the tube and not removable.

Resuspend the 'A' tube extract in androgen assay standard diluent. Remember there are two recipes for this diluent - one from the old fecal extract protocol and one from the RIA protocol. It probably doesn't matter which one you use, just be consistent although I would say use the recipe from the RIA SOP. Since the androgen assay requires 100 uL of extract per tube, should be done in duplicate and you need to have enough extract to measure hormone recovery, resuspend the dried 'A' extract in 250 uL of zero standard - that should yield 2x 100 uL for assay, 1x 20 uL for recovery and extra that will be stuck in the tube and not removable.

Sonicate and vortex the reconstituted extracts and allow equilibration for 30 minutes, i.e., let stand at room temperature.

Securely cap the extracts and freeze at -80ºC until analysis. They are radioactive - label and store them appropriately!!

Analyze extracts for androgens and estradiol using SOPs: "Radioimmunoassay of Androgens In Avian Serum or Fecal Extract" and "Radioimmunoassay of Estradiol In Avian Serum or Fecal Extract"

This will ensure that the sample hormone will not be overdiluted and still in the range of the standard curve for the RIA. IF after all this, the hormone is not detectable on the standard curve, you will probably have to reduce the amount of diluent and only run the samples in singlets, not duplicates.

For extraction efficiencies (these are done at the time of RIA):
Estradiol: For each sample place 20 uL of extract into a tube for counts for recovery. Prepare a 'total count' tube containing 20 uL of the hot estradiol, i.e. exactly what you spiked the fecal sample with. Count on the gamma counter. Cpms of extract *2 then divided by cpms of total count and the result multiplied by 100 will give the extraction efficiency as a percent. The sample cpms are multiplied by two because we divided the sample into two after extraction.

Androgens: For each sample, place 20 uL of extract into a scintillation vial. Add 5 mL of scintillation cocktail. Prepare 'total count' vials with 20 uL of hot androgen that you originally spiked the sample with and add 5 mL scintialltion cocktail. Measure cpms on the scintillation counter. As for estradiol:

$$\frac{\text{CPM extract * 2}}{\text{CPM total counts}}$$ and multiply result by 100 to express % extraction efficiency

6.14 Radioimmunoassay of Androgens In Avian Serum or Fecal Extract

The purpose of this protocol is to measure androgens in serum from blood of birds. The method and reagents described here have been validated for hatchling and adult Japanese quail and adult bobwhite quail. To use this method with any other species or age requires validation. The radioimmunoassay is a competitive binding assay that includes use of a tritiated testosterone and requires training in radiation safety for any personnel working on the assay. The method was validated for quail by serum extraction of steroids and parallelism experiments.

Procedure

Steroid extraction with spiking for recovery

1) Calculate the amount of serum required for the assay. This is dependent upon results of the validation which should be done whenever a series of assays is started, whenever a procedure is adapted (different sex, age or species) or when a different kit is tried. Serum may need to be concentrated or diluted depending on androgen concentrations.
2) For serum volume less than 1 mL, double extract with 2 mL ethyl ether:
    a. Add serum to a 16x100 mm boroscillicate glass tube labelled ‘A’ and with the sample number.
    b. Spike sample with 10 µL ^{3}H testosterone (‘hot’) to estimate extraction efficiency
    c. Under the fume hood add 2 mL of ether and vortex vigorously.
    d. Wait for the serum and ether layers to separate (~1 min).
    e. Freeze the sample either in a ethanol and dry ice bath in a beaker or covered with foil in a -80º freezer if working with many samples. The serum will freeze, the ether fraction will not.
    f. Pour off the ether fraction into tube ‘B’.
    g. Allow the serum fraction in tube A to thaw and repeat steps c to f.
    h. Dry down the sample by evaporation of the ether leaving the steroid residue on the glass tube either by leaving the tubes in a fume hood overnight or by forcing nitrogen gas over the sample in a fume hood for rapid drying.
3) Assay requires the following volumes (samples should be assayed in duplicate unless samples are from hatchlings and volume is limiting):

    Japanese Quail:

    Hatchling (male) serum assayed in singlets (most samples have to be pooled): 120 µL, assayed in duplicates, 220 µL. 100 µL per assay tube, 10 µL for recovery, 10 µL lost on tube. Serum reconstituted 1:1.

    Adult serum assayed in duplicate: 250 µL, reconstituted 1:1 for males and females yielding 250 µL: 100 µL per assay tube and 20 µL for recovery = 220 µL.

    Bobwhite Quail:

    Adult serum assayed in duplicate: 150 µL reconstituted 1:2 to yield 300 µL serum for assay: 100 µL per assay tube and 20 µL for recovery = 220 µL.

Validation/Parallelism

1) Acquire a sample of pooled serum. Volume is dependent upon number of serial dilutions or repeated measurements to be done. An example is as follows:

   To validate a 2X concentrate and 1:1 through 1:16 serial dilution in duplicate requires 600 µL of serum. Double extract the 600 µL and reconstitute in 300 µL of standard diluent. This yields a 2x concentrated sample. Remove 150 µL of this 2X concentration to a new tube, dilute it 1:1 with zero standard and label the tube "X" for the 1:1 reconstitution. From the "X" tube remove 150 µL to a tube labelled "1:2" and add 150 µL standard diluent. From that "1:2" dilution remove 150 µL to another tube, labelled "1:4" and add 150 µL standard diluent to yield a 1:4 dilution. Continue as such to create a 1:8 and 1:16 dilution. From these serial dilution tubes take 50 µL of reconstituted sample for each assay tube (assay in duplicate). Follow the methods for the radioimmunoassay below. Compare the resulting data to the standard curve. The serially diluted samples' curve should parallel that of the standard and final concentrations should be close to one half of the higher dilution for each sample. The non-linear portion of the standard curve is usually the upper and lower 5%. Unknowns in these high and low areas of the curve are more likely to have some error. Therefore, samples are run at a dilution predicted to be in the working range of the assay. Precision is determined by repeated (5-8 samples) assay of a sample. The CV should be no more than 10%. Sample data in Appendix A.

Radioimmunoassay

- Run a standard before you begin to check that the assay and all reagents are acting normally.

1. Label 12x75mm boroscillicate glass tubes in duplicate with Total, NSB (non-specific binding), Bo and S1-S10 and then number tubes of unknowns in duplicate.
2. Prepare standards:

Take 10 µL of testosterone standard solution C and dilute it in 990 µL standard diluent yielding 1 mL of 10 ng/mL, this is solution D and is not used in the assay.

Prepare 10 standards by serial dilution solution D to be used in the assay and designated S1 to S10.
S10 = 250 µL of solution D plus 250 µL of standard diluent and is 5 ng/mL.
S9 = 250 µL of S10 plus 250 µL standard diluent and is 2.5 ng/mL.
S8 = 250 µL of S9 plus 250 µL standard diluent and is 1.25 ng/mL.
S7 = 250 µL of S8 plus 250 µL standard diluent and is 0.625 ng/mL.
S6 = 250 µL of S7 plus 250 µL standard diluent and is 0.3125 ng/mL.
S5 = 250 µL of S6 plus 250 µL standard diluent and is 0.156 ng/mL.
S4 = 250 µL of S5 plus 250 µL standard diluent and is 0.08 ng/mL.
S3 = 250 µL of S4 plus 250 µL standard diluent and is 0.04 ng/mL.

S2 = 250 µL of S3 plus 250 µL standard diluent and is 0.02 ng/mL.
S1 = 250 µL of S2 plus 250 µL standard diluent and is 0.01 ng/mL.

3. Prepare anti-androgen antibody: 100 µL antibody to 9.9 mL standard diluent.
4. Add 100 µL standard diluent to Total, NSB and Bo tubes.
5. Add 100 µL of standards to each respective tube.
6. Add 100 µL of unknowns and control sera to each respective tube.
7. Add 100 µL of antibody solution to all tubes EXCEPT total and NSB.
8. Add 150 µL of 3H-testosterone to all tubes.
9. Cover, vortex and incubate at room temperature for 1.5-2 hours.
10. Place tubes in ice bath for 5 minutes to stop the reaction.
11. Add 400 µL per tube of dextran coated charcoal EXCEPT total tubes, add 400 µL phosphate buffer to total tubes.
12. Vortex and incubate on ice for 15 minutes.
13. Centrifuge 3000 rpm on Sorvall table top centrifuge for 15 minutes at 2-8ºC.
14. Remove 0.5 mL supernatant, carefully without disturbing the charcoal, and add to a scintillation vial.
15. Add 5 mL of scintillation cocktail and vortex vigorously.
16. Count on scintillation counter for four minutes per sample.

Guidelines:
NSB should be less than 10% of the total count.
Bo should be 20-25% of the total count.
CV of duplicate samples should be less than 5% ideally.

Waste Disposal

A and B tubes from the extraction, assay tubes must be disposed of in containers provided by radiation safety for ^3H waste (cardboard containers). Scintillation tube waste must be placed in the appropriate drum. Amount of radiation must be calculated and listed on waste containers.

All tips used with radioactive samples must be disposed of in a plastic bag and the bag disposed of in the dry waste container.

Conduct wipe tests of lab and equipment on a monthly basis to check for radioactive contamination.

Calculations

Using Graphpad Prism, convert counts per minute to androgen concentration as follows:
1. Create new project
2. Format data for duplicate 'Y' values.
3. Type standard curve concentrations into X values column
4. Type standard curve counts per minute (cpm) and unknown cpms into A-Y values column
5. Type NSB cpms into B-Y values column

6. “Analyze” data - remove baseline and column math
   Baseline values = B, D, F
   Calculate = difference: value-baseline
   Replicates = calculate the mean of the replicates
7. “Analyze” data - transforms
   X = log(X)
   Y = Y/K (where K=Bo (baseline subtracted)/100)
8. “Analyze” data - nonlinear regression (curve fit)
   Choose an equation = sigmoidal dose response (variable slope)
   Also calculate = unknowns from standard curve
9. Pull down: interpolate X values
10. “Analyze” data - transforms X = 10^X
11. “Graph” data using log(X) vs. % binding

Equipment Needed

Ethyl ether
Boroscillicate glass tubes, 16x100 and 12x75 mm, tube racks
Pipettors
Ice
Vortex
Radioactive waste disposal containers
Scintillation Counter

Reagents

0.1 M Phosphate Buffer, pH 7.6

K_2HPO_4	13.93g
NaH_2PO_4	3.12g
NaCl	8.76g
NaN_3	1.0g
DDW	to 1000 mL

Adjust pH to 7.6 and store at 4ºC

Standard Diluent, pH 7.4

0.1 M phosphate buffer	100 mL
DDW	900 mL
NaCl	7.6g

Bovine Serum Albumin, fraction V, Sigma A-7888, suitable for insulin RIA 10g

NaN_3	1.0g

Adjust to pH 7.4 and store at 4ºC

Dextran Coated Charcoal

Activated charcoal 1.5g

Dextran 0.15g
0.1 M phosphate buffer 300 mL
Mix and store at 4ºC (keep away from the light)

Testosterone Standard

1. Weigh 10 mg powdered testosterone and dissolve in 10 mL EtOH to make a 1000 µg/mL solution and label this 'A'.
2. In a new tube dilute 100 µL of A with 900 µL EtOH to make solution B (100 µg/mL).
3. Prepare solution C with 10 µL of B into 990 µL of standard diluent (1000 ng/mL).
4. Make 20 µL aliquots of solution C and store at -80ºC.

Testosterone from Amersham Biosciences, UK.
1,2,6,7-^{3}H-testosterone, 250 µCi, 250 µL, catalog # TRK402, store at -20ºC.
"Tracer" 3-5 µL of tritiated testosterone in 100 mL standard diluent and stir well.
Count 20 µL with scintillation cocktail. Want cpm between 1000 and 2000.

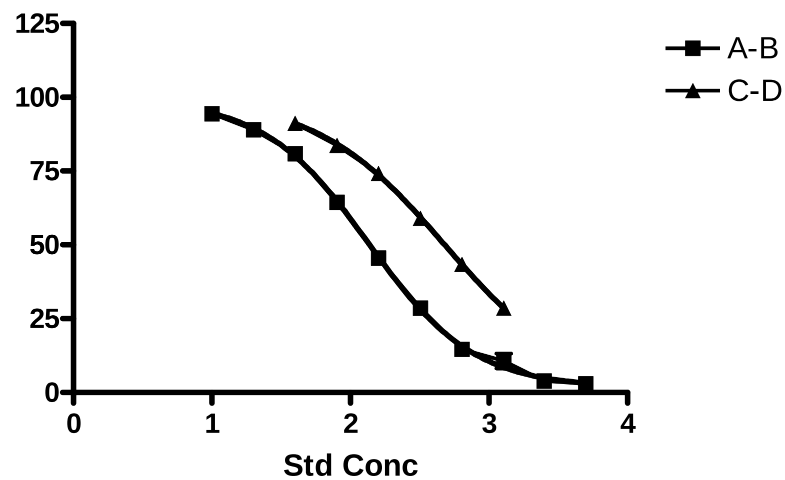
Anti-androgen from Esoterix (previously Endocrine Sciences, Inc) Calabasas Hills, CA
Store at -80ºC, rehydrate per directions on vial (1.2 mL water) and prepare 110 µL aliquots, refreeze at -80ºC. Working solution (prepare shortly before use) = 100 µL of frozen stock in 9.9 mL of standard diluent (1:100 dilution).

Appendices

A. Sample validation/parallelism data:

Transform of Baseline-corrected of Data 1:Transformed data

Std Conc	A-B
16.507	91.234
31.888	83.675
53.783	74.180
99.652	59.014
174.657	43.427
314.677	28.549



On graph:
A-B is standard
C-D is serial dilution of unknown
On table:

A-B is percent binding (Y axis)
Std Conc are unknowns from greatest dilution to 2X concentrate (X axis shown log transformed).

Literature Consulted

Bales, KL, Abdelnabi, M, Cushing, BS, Ottinger, MA, Carter CS. 2004 Effects of neonatal oxytocin manipulations on male reproductive potential in prairie voles. Physiology and Behavior 81: 519-526.

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Ottinger, MA, Pitts, S, Abdelnabi, MA. 2001 Steroid hormones during embryonic development in Japanese Quail: Plasma, gonadal and adrenal levels. Poultry Science 80:795-799.

Ottinger, MA and Brinkley, HJ. 1979. Testosterone and sex related physical characteristics during the maturation of the male Japanese quail. Biology of Reproduction 20:905-909.

6.15 Radioimmunoassay of Estradiol In Avian Serum or Fecal Extract

The purpose of this protocol is to measure estradiol in serum from blood of birds. The method and reagents described here have been validated for hatchling and adult Japanese quail and adult bobwhite quail. To use this method with any other species or age requires validation. The radioimmunoassay is a competitive binding assay that includes use of a iodinated estradiol and requires training in radiation safety for any personnel working on the assay. Currently we use a double-antibody kit from MP Biomedicals designed for human serum. The kit was validated for quail by serum extraction of steroids and parallelism and precision experiments. Steroids are first extracted from the serum sample by ether partition which removes interfering aqueous compounds.

Procedure

Steroid extraction with spiking for recovery

1) Calculate the amount of serum required for the assay. This is dependent upon results of the validation which should be done whenever a series of assays is started, whenever a procedure is adapted (different sex, age or species) or when a different source of reagents is tried. Serum may need to be concentrated or diluted depending on estradiol concentrations.
2) For serum volume less than 1 mL, double extract with 2 mL ethyl ether:
    a. Add serum to a 16x100 mm boroscillicate glass tube labelled 'A' and with the sample number.
    b. Spike sample with 10 µL ^{125}I estradiol ('hot') to estimate extraction recovery (efficiency) and incubate for at least 30 minutes at room temperature to equilibrate.
    c. Under the fume hood add 2 mL of ethyl ether and vortex vigorously.
    d. Wait for the serum and ether layers to separate (~1 min).
    e. Freeze the sample either in a ethanol and dry ice bath in a beaker or covered with foil in a -80º freezer if working with many samples. The serum (bottom, aqueous layer) will freeze, the ether fraction will not (top layer including steroid hormones)
    f. Pour off the ether fraction into corresponding tube 'B'.
    g. Allow the serum fraction in tube A to thaw and repeat steps c to f.
    h. Dry down the sample in tube B by evaporation of the ether leaving the steroid residue on the glass tube either by leaving the tubes in a fume hood overnight or by forcing nitrogen gas over the sample in a fume hood for rapid drying.
3) Recent assays have required the following volumes (samples should be assayed in duplicate unless samples are from hatchlings and volume is limiting):

    Japanese Quail:

Hatchling (female) serum assayed in singlets (some samples have to be pooled): 80 µL, assayed in duplicates, 130 µL. 50 µL per assay tube, 20 µL for recovery, 10 µL lost on tube. If assayed in singlets. Serum reconstituted 1:1.

80µL of serum is extracted as described above then reconstituted with the same amount of buffer: 50µL for the assay and 20 µL for calculating the recovery.

Adult serum assayed in duplicate: 100 µL plasma extracted then reconstituted (1:4) with buffer for females yielding 400 µL and 1:3 for males yielding 300 µL: 50 µL per assay tube and 50 µL for recovery = 150 µL.

Bobwhite Quail:

Adult serum assayed same as adult Japanese quail serum.

Validation/Parallelism

1. Acquire a sample of pooled serum from each new species. Volume is dependent upon number of serial dilutions or repeated measurements to be done. An example follows: To validate a 2X concentrate and 1:1 through 1:16 serial dilution in duplicate requires 600 µL of serum. Double extract the 600 µL and reconstitute in 300 µL of standard diluent solution. This yields a 2x concentrated sample. Remove 150 µL of this 2X concentration to a new tube, dilute it 1:1 with zero standard and label the tube "X" for the 1:1 reconstitution. From the "X" tube remove 150 µL to a tube labelled "1:2" and add 150 µL zero standard. From that "1:2" dilution remove 150 µL to another tube, labelled "1:4" and add 150 µL zero standard to yield a 1:4 dilution. Continue as such to create a 1:8 and 1:16 dilution. From these serial dilution tubes take 50 µL of reconstituted sample for each assay tube (assay in duplicate). Follow the methods for the radioimmunoassay below. Compare the resulting data to the standard curve. The serially diluted samples' curve should parallel that of the standard and final concentrations should be close to one half of the higher dilution for each sample. The non-linear portion of the standard curve is usually the upper and lower 5%. Unknowns in these high and low areas of the curve are more likely to have some error. Therefore, samples are run at a dilution predicted to be in the working range of the assay. Precision is determined by repeated (5-8 samples) assay of a sample. The CV should be no more than 10%. Sample data in Appendix A.

Radioimmunoassay

1. Follow the directions given in the MP Biomedicals protocol. A description follows:
2. Bring all reagents to room temperature. Reconstitute extracted samples with zero standard, vortex and allow to stand at least 30 minutes.
3. Label 12x 75 mm tubes 1-16 for standards and 17+ for unknowns. 1-2 is non-specific binding (NSB), 3-4 is zero with 15-16 being 3000 pg/mL.

4. Add 500 µL of the steroid diluent into tubes 1-2.
5. Add 50 µL of zero standard into tubes 1-4 and 50µL from each standard to its respective tube. (Known amount in duplicate 5-16). Add 50 µL of each unknown and control sera to each appropriate tube.
6. Vortex each sample and add 50 µL of each into its appropriate tubes.
7. Add 500 µL of ^{125}I estradiol (blue) to all tubes.
8. Add 500 µL of anti-estradiol antibody (yellow) to all tubes EXCEPT 1-2.
9. Vortex, cover tubes with foil, label as radioactive, and incubate for 90 minutes in a 37ºC waterbath.
10. Add 500 µL of precipitant solution (red) to all tubes and vortex.
11. Centrifuge all samples at 2300 rpm (~1000 xg) for 25 minutes* at 2-8˚C on the Sorvall table top centrifuge. (*This is a slight increase in time from the MP Biomedicals protocol and reduces slippage of pellets when decanting).
12. Decant supernatants into a radioactive waste container and blot each tube on absorbent bench paper. If pellets start to slip, centrifuge the samples again for 5 minutes.
13. Count the precipitate on a gamma counter calibrated for ^{125}I for 1 minute. Also count two or more tubes of 10 uL tracer each for Total Counts for recovery calculations.

Waste Disposal

A and B tubes from the extraction, assay tubes must be disposed of in containers provided by radiation safety for ^{125}I waste (blue plastic barrels). Liquid ^{125}I waste must be poured into plastic carbuoy supplied by radiation safety. Amount of radiation must be calculated and listed on waste containers. All tips used with radioactive samples must be disposed of in a plastic bag and the bag disposed of in the dry waste container. Conduct wipe tests of lab and equipment on a monthly basis to check for radioactive contamination.

Calculations

Using Graphpad Prism, convert counts per minute to estradiol concentration as follows:

1. Create new project
2. Format data for duplicate 'Y' values.
3. Type standard curve concentrations into X values column
4. Type standard curve and unknowns cpms into A-Y values column
5. Type NSB cpms into B-Y values column
6. "Analyze" data - remove baseline and column math
   Baseline values = B, D, F
   Calculate = difference: value-baseline
   Replicates = calculate the mean of the replicates
7. "Analyze" data - transforms
   X = log(X)
   Y = Y/K (where K=Bo (baseline subtracted)/100)
8. "Analyze" data - nonlinear regression (curve fit)

Choose an equation = sigmoidal dose response (variable slope)
Also calculate = unknowns from standard curve

9. Pull down: interpolate X values
10. "Analyze" data - transforms X = 10^X
11. "Graph" data using log(X) vs. % binding

Equipment Needed

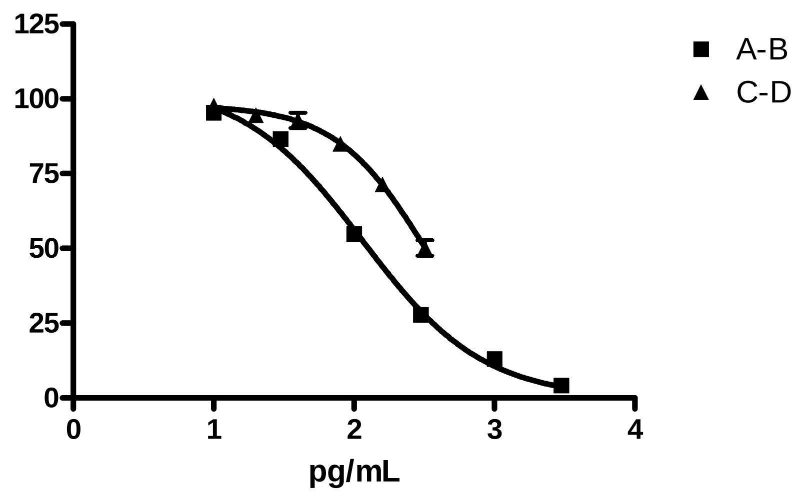
17β estradiol double antibody kit
07-138-102 MP Biomedicals
Ethyl ether
Boroscillicate glass tubes, 16x100 and 12x75 mm, tube racks
Pipettors
Extra 0 pg/mL standard ("zero standard")
 # 07138171 for 50 mL MP Biomedicals
Radioactive waste disposal containers
Gamma Counter

Appendices

A. Sample validation/parallelism data:

Transform of Baseline-corrected of Data 1:Transformed data

pg/mL	A-B
	97.795
13.335	94.503
15.636	92.852
27.813	84.967
54.925	71.388
126.210	50.157



On graph:
A-B is standard
C-D is serial dilution of unknown
On table:

A-B is percent binding (Y axis)
pg/mL are unknowns from greatest dilution to 2X concentrate (X axis shown log transformed).

Literature Consulted

This method and MP Biomedicals reagents have recently been validated for Japanese and bobwhite quail. Prior work was similar but with different sources for reagents.

Ottinger, MA, Pitts, S, Abdelnabi, MA. 2001 Steroid hormones during embryonic development in Japanese Quail: Plasma, gonadal and adrenal levels. Poultry Science 80:795-799.

Watson, JT, Abdelnabi, M, Wersinger, X, Ottinger, MA, Adkins-Regan, E. 1990 Circulating Estradiol and activation of male and female copulatory behavior in Japanese quail (*Coturnix japonica*). General Comparative Endocrinology 77:229-238.

6.16 Digestion Of Thyroid Gland And Radioimmunoassay of Thyroid Hormones

The thyroid gland is site of thyroid hormone synthesis and storage. While measuring circulating thyroid hormone in the blood is a potentially useful endpoint, measurement of thyroid hormone stores in the thyroid gland is a more sensitive indicator of thyroid function (McNabb et al. 2004). The thyroid gland must maintain a store of hormone for times of growth or stress. This protocol outlines methods for digesting thyroid glands to extract hormone and radioimmunoassay for quantification of T_3 (triiodothyronine) and T_4 (thyroxine) content.

Procedure

Digestion of thyroid gland

This procedure was supplied by Anne McNabb, Virginia Polytechnic University, Blacksburg VA in August 2004.

Reagents:

Digestion Mix (100 mL)

1.21g TRIS
153.65mg L-Glutathione reduced, Sigma G4251
42.55mg PTU – light sensitive (6-n-propyl-2-thiouracil Cat# P-3755 from Sigma)
1ml Triton X
100ml dd H2O

1. Weigh all dry components in the following order: a)TRIS, b)Glutathione, c)PTU
2. Combine 100 mL ddH2O and dry components in an Erlenmeyer beaker and stir on stir-plate at low speed.
3. As the solution is stirring, add the Triton X. (To ease the Triton in and out of the pipette tip, cut about 1cm off the pipette tip before putting it on the pipette.) Cover the beaker with aluminum foil as it stirs.
4. Pour the solution into a amber bottle (it is light sensitive) and store at 2-8ºC.

Pronase Solution

Pronase: Calbiochem Cat#53702

1. The average weight of thyroid glands (TG) to be digested is approximately 5mg.
2. Calculate the amount of Pronase needed. E.g. For 68 thyroid glands and 25 mg Pronase per gland, 68x25 = 1700 mg (or 1.7 g) Pronase for entire group. Use a volume of 350 µL of digestion mixture per gland, 0.35 mL x 68 glands = 23.8 mL. Mix the 1.7 g of

Pronase into 23.8 mL of digestion mix by swirling it around until all the Pronase has gone into solution (do not use a stirring rod; Pronase tends to stick to anything that touches it). Make your actual calculations for a few more glands than you have to ensure you have enough solution since some is lost in the container or when pippetting.

3. Pour a small amount of the mixed Pronase/digestion mixture into the weigh boat that was used to weigh the Pronase and swirl (to remove any Pronase that is stuck to the sides of the boat).

100% EtOH (ice cold)

1. Pipette 350 µL of pronase solution in each 1.5 mL tube that holds the gland tissue. Vortex, and incubate in a 37ºC water bath for 24 hours.
2. Place about 100 mL of 100% EtOH into the freezer for about 20 minutes. Remove tubes from the water bath and add 1 ml of the ice cold 100% EtOH (for a total of 1.35ml).
3. Vortex and store tubes at -20ºC for 24 hours.
4. Remove tubes from the freezer and centrifuge at 14,000 RPM and -4˚C for 8 minutes.
5. Decant supernatant into a clean, labeled, 1.5ml microcentrifuge tube and discard the tube with the pellet. Store the extract at -20ºC until analysis.

Radioimmunoassay

Measure T3 and T4 using DPC coat-a-count kits for total canine T3 and T4. Follow manufacturers directions. Include an interassay control if running samples over more than one assay. Prepare standards for appropriate hormone ranges from scratch in 75% ethanol as needed.

Equipment Needed

Stir plate Water bath 37ºC Gamma Counter
DPC coat-a-count kits for total canine T3 and T4 (Catalog #s TKC3 and TKC4)

Literature Consulted

McNabb, FMA, Cheng, MF. 1985. Thyroid development in altricial ring doves, *Streptopelia risoria.* General and Comparative Endocrinology 58: 243-251.

McNabb, FMA, Larsen, CT, Pooler, PS. 2004 Ammonium perchlorate effects on thyroid function and growth in bobwhite quail chicks. Environmental Toxicology and Chemistry 23(4):997-1003.

6.17 Analysis of Aromatase Activity in Hypothalamic Tissue

Background:

Aromatase is responsible for the biosynthesis of estradiol. This assay measures amounts of aromatase. Testosterone is converted to estradiol in the brain by aromatase.

Materials:

- Charcoal Pellets
- K-phosphate buffer
- NADPH/buffer solution
- Ice
- 16 x 100 test tubes
- microcentrifuge tubes
- androstenedione
- scintillation vials

Procedures:

***Directions for 25 samples**
*Allow 2 days for assay completion

<u>Day 1</u>

Prepare:

a.) Charcoal Pellets
b.) K-phosphate buffer
c.) Prepare NADPH/buffer solution

A.) Charcoal Pellet Preparation

0.5 ml of a 5% aqueous charcoal solution is placed into a 1.5 ml Eppendorf snap top centrifuge tube. Centrifuge (3000 RPM in Sorval for 10 min) and draw off supernatant which is discarded. Tubes must air dry before use, so let sit with caps open overnight – 2 days. Store in a cool dry place with the lids closed.

B.) Prepare K-phospate buffer - into deionized water add:

1.) K-phosphate monobasic (0.1M)
x(g)/136.09g/mol = 0.1M (mol/L)
x = 13.69 g (for 1 L)
x = 6.8045 g (for 0.5 L)
x= 2.738 g (for 200 ml)

2.) K-phosphate dibasic, anhydrous (0.1M)

x/174.18g/mol = 0.1M
x = 17.418 g (for 1 L)
x = 8.709 g (for 0.5 L)
x= 3.483 g (for 200ml)

3.) Sucrose (0.25M)
x/342.30 = 0.25
x = 85.575g (for 1 L)
x = 42.7875g (for 0.5 L)
x= 17.115 g (for 200ml)

Bring solution to pH 7.2 (pH 7.4 for poikilotherms) and store in refrigerator until use

C.) NADPH -***keep on ice***

1. Dissolve 0.01775g NADPH in 1mL K-phosphate buffer.
2. Store at -80^{o}C with samples in microcentrifuge tube.

Day 2

Preparation:

1. Turn on water bath to 37^{o} C and double check temperature (for quail; for other poikilotherms, water bath must be at the temperature animals were acclimated to).
2. Set shake to 2.5
3. Label (16x100) test tubes: 2 for each sample, 2 blanks
4. Obtain ice.

Experiment:

1. Defrost samples but keep on ice in microcentrifuge tubes.
2. Add K-phosphate buffer and homogenize for 30 seconds (100 µl for adult dissections or 50ul for hatchling dissections).
3. Dissolve 72 µL androstenedione in 1mL propylene glycol.
   Do this under hood or radioactive labeled countertop.
4. To each test tube that will hold sample add:
   - 233 µL K-phosphate buffer
   - 30 µL dissolved NADPH
   - 12 µL dissolved androstenedione
5. To each test tube that will hold blanks add:
   258 µL K-phosphate buffer
   30 µL dissolved NADPH
   12 µL dissolved androstenedione
6. Add 25 µL sample homogenate to each appropriately labeled sample test tube.
7. Place all tubes in gently shaking water bath for 3 hours.

Check temperature of bath regularly to insure it is the correct temperature.

8. Remove tubes from bath, place under hood in room 3144 and add 4mL methylene chloride to stop the reaction.
9. Add 1.7 mL deionized water to each tube and vortex GENTLY.
10. Centrifuge in bucket centrifuge for 5 minutes @ 1000 rpm.
    Ensure buckets are balanced exactly by using balance located next to centrifuge.
11. Remove 1mL aqueous portion (top layer) and add to charcoal pellets in microcentrifuge tubes.
12. Let incubate for 20 minutes at 4^{o}C in radioactive fridge vortex every 5 minutes.
13. Centrifuge for 5 minutes @ 14,000g or 6,750 rpm (using centrifuge in room 3120).
14. Add 200 µL supernatant to scintillation vial, .
    300 µL MilleQ water to scintillation vial.
    5 mL scintillation cocktail to scintillation vial.
15. Vortex until white precipitate is swirled.
16. Place vials in scintillation counter.

Notes: order information

Sigma: *B* NADPH tetra Na salt N1630, buy in lots of 250 mg or more
Perkin Elmer/ NEN : Androst-4-ene 3,17- dione [1 *B* – ^{3}H(N)] NET926250UC, buy as 250 uCi or more
Round bottom snap top tubes
Campus Chemistry store for methylene chloride

SAMPLE Calculation: **Androst-ene dione** calculation- for 200 samples, 12 ul x 200= 2,400 ul or 2.4 ml… so need 216 ul ^{3}H dissolved in 3 ml propylene glycol. **NADPH** calculation- .01775 g in 1 ml K-phosphate & 30 ul /sample x 200 = 6 ml or 0.1065gx0.01775g NADPH so would need to order 2 x 250 mg

6.19 Tissue Storage, RNA Isolation And Microarray Analysis

1. Collect tissue and make cuts using a sterile razor blade. Place tissue sample in a clean microcentrifuge tube and snap freeze in liquid nitrogen. Store at -80ºC.
2. Isolate RNA using Qiagen RNeasy kits. Use appropriate kit depending upon type and size of tissue as described in RNeasy handbook.
3. Submit RNA samples for microarray analysis.

- Tissues will be collected and prepared as in previous experiments and microarrays have been validated for several avian species. Because these microarrays are constructed with normalized cDNAs, they offer higher cross species utility than those produces with oligos, especially with species having a high homology to galliforms. Our previous study has used the following procedure.

1. Hypothalamic tissue samples from were frozen in liquid nitrogen and stored at -80ºC. Tissue samples ranged from 115 to 238 mg in weight.
2. RNA was isolated with RNeasy MAXI kit per manufacturers instructions.
3. Between 75 and 190 µg of RNA was obtained from each tissue sample.
4. 260/280 ratio for isolated RNA was between 1.5 and 1.6.
5. RNA was run on a gel to check for degradation; degradation was minimal.
6. 25 µg RNA from each sample was submitted for microarray analysis on slides developed for chickens.
7. The RNA successfully hybridized with the chicken oligonucleotides on the chicken microarray.

Detailed descriptions of the microarrays, including the characterization and analytical approaches can be found in:

Laura E. Ellestad, Wilfrid Carre, Michael Muchow, Sultan A. Jenkins, Xiaofei Wang, Larry A. Cogburn, Tom E. Porter. Gene expression profiling during cellular differentiat***ion in the embryonic pituitary*** gland using cDNA microarrays. ***In*** **"**Functional Avian Endocrinology", Allistair Dawson and Peter Sharp***, Editors***.

Wilfrid Carre, Xiaofei Wang, Tom E. Porter, Yves Nys, Jianshan Tang, Erin Bernberg, Robin Morgan, Joan Burnside, Samuel E. Aggrey, Jean Simon and Larry A. Cogburn. 2006 Chicken Genomics Resource: Sequencing and Annotation of 35,407 ESTs from Single and Multiple Tissue cDNA Libraries and CAP3 Assembly of a Chicken Gene Index. Physiological Genomics, Articles in Press. (March 21, 2006).

6.20 Field Collection of Tree Swallow Eggs from Greater Sacandaga Lake, New York for Avian Egg Injection Study, Hudson River NRDA

INTRODUCTION

Avian egg injection is a well-established technique to assess the effects of contaminants on a developing avian embryo. To conduct an avian egg injection experiment, eggs are collected from the field and brought into a laboratory where they are injected with the substance being tested. Proper handling of the eggs during collection and transit to the laboratory is essential to maintain viability in eggs that will subsequently be injected with contaminants and incubated. A subsample of eggs 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. Eggs are generally laid at one day intervals. Monitor the laying of eggs daily. Tree swallows generally lay eggs at one day intervals with a maximum clutch size of about 5-7 eggs. When the number of eggs in the nest has not increased since the day before the clutch should be considered complete and should be collected promptly. Collect all eggs in the clutch.
- For each egg collected, complete the appropriate information on the Avian Egg Collection Datasheet.
- Place eggs in individually numbered compartments (one for each egg or eggs from each clutch). A list of the nest box 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 New York State Department of Environmental Conservation (NYSDEC) laboratory in a hard container avoiding temperature extremes and jostling.

- At the NYSDEC laboratory, eggs which are going to be incubated should be separated from eggs which are going to undergo contaminants analysis.
- For eggs that are going to be analyzed for contaminants and not incubated: Randomly select 10 nests from among those from which eggs were collected and from each of those 10 nests, randomly select 2 eggs from each nest. These 2 eggs will each form a composite sample for chemical analysis. 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. Note that the eggs were taken for contaminant analysis on the Egg Collection Data Sheet. Freeze samples at NYSDEC laboratory. Ship under Chain of Custody overnight on dry ice to the sample archive or analytical laboratory, as directed by the Trustees.
- For eggs that are going to be incubated: Transport promptly to the laboratory. Injection of tree swallow eggs at day ED2.5-3 will be done in the laboratory; as eggs will have already undergone potentially one day of incubation in the nest in the field, prompt transport under appropriate conditions is essential. Use of a "Koolatron" to maintain a proper temperature of eggs during transport is recommended. Maintain a temperature of about 90 to 100 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 NYSDEC field collection crew to the laboratory crew on Egg Collection Data Sheet.

Avian Egg Collection Data Sheet – Hudson River Avian Egg Injection Study – Greater Sacandaga Lake, New York

Collector: ______________________________ Data Recorder: ______________________________
Print Name Signature Print Name Signature

Species: Tree Swallow

Compartment #	Egg Code[1]	Location[2]	Date Collected[3]	Time Collected[4]	Clutch Size	Eggs Warm Yes/No	Prior Day Clutch Size	Prior Day Time	Comments	Fresh Weight (g)[5]

[1] Egg Code: Numeric code between 200 and 799; [2] Site and nest number, such as Gr Sac Box 2 where Gr Sac = Greater Sacandaga Lake Box Number 2; [3] In MM/DD/YEAR format, such as 04/30/2006 for April 30, 2006; [4] In 24-hour format, such as 1300 for 1PM; [5] Record at the laboratory

Custody of samples listed above transferred from field collection crew to laboratory crew as follows:

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

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

Data Sheet checked by: ______________________ Date: __________

6.21 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. For the duration of the study, monitor nests every two to three days. Greater time intervals may occur because of bad weather (e.g. rain or thunderstorms) or because the hatching dates on all remaining target nests are known. Tree swallows generally lay eggs at one day intervals with a maximum clutch size of about 5-7 eggs. Eggs will not be collected from 4-egg clutches. 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 laboratory and the other 2 sibling eggs will be subject to contents collection for contaminants analysis. In order to facilitate transport of eggs to the laboratory, eggs for contaminant analysis can be collected independently of those to be transported to the laboratory. Eggs should be collected from a total of 20 nests (60 eggs total). If available, more eggs from more nests may be collected.
- 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 nest box 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 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 the laboratory crew on Egg Collection Data Sheet.

Avian Egg Collection Data Sheet – Hudson River Avian Egg Injection Study – Upper Hudson River

Page _1 of _1_

Collector: ______________________________ Data Recorder: ______________________________

Print Name Signature Print Name Signature

Species: Tree Swallow

Compartment #	Egg Code[1]	Location[2]	Date Collected[3]	Time Collected[4]	Clutch Size	Eggs Warm Yes/No	Embryonic day of incubation	Comments	Fresh Weight (g)[5]

[1] Egg Code: Numeric code between 800 and 899; [2] Site followed by box number, such as UHR313 where UHR = Upper Hudson River, Box Number 313; [3] In MM/DD/YEAR format, such as 04/30/2006 for April 30, 2006; [4] In 24-hour format, such as 1300 for 1PM; [5] Weighed at the laboratory

Custody of samples listed above transferred from field collection crew to laboratory crew as follows:

Relinquished by: ______________________________

Signature Print Name Company/Title Date Time

Received by: ______________________________

Signature Print Name Company/Title Date Time

Relinquished by: ______________________________

Signature Print Name Company/Title Date Time

Received by: ______________________________

Signature Print Name Company/Title Date Time

Data Sheet checked by: ______________________________ Date: ______________

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Appendix B

Design and Preparation of a Custom 58-Congener PCB Mixture Dosing Solution For Avian Egg Injection Studies

HUDSON RIVER

Study Plan for Avian Egg Injection Study

DESIGN AND PREPARATION OF A CUSTOM 58-CONGENER PCB MIXTURE DOSING SOLUTION FOR AVIAN EGG INJECTION STUDIES

HUDSON RIVER NATURAL RESOURCE DAMAGE ASSESSMENT

HUDSON RIVER NATURAL RESOURCE TRUSTEES

STATE OF NEW YORK

U.S. DEPARTMENT OF COMMERCE

U.S. DEPARTMENT OF THE INTERIOR

FINAL

PUBLIC RELEASE VERSION*

DECEMBER 20, 2006

Available from:
U.S. Department of Commerce
National Oceanic and Atmospheric Administration
Hudson River NRDA, Lead Administrative Trustee
Assessment and Restoration Division, N/ORR3
1305 East-West Highway, Rm 10219
Silver Spring, MD 20910-3281

Names of certain individuals and affiliations have been removed to protect confidentiality.

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 Hudson River NRDA, the Trustees are conducting an avian egg injection study. As described in the Trustees' Avian Egg Injection Study Plan, one of the contaminants of concern being tested in the study is a PCB mixture. This report provides the U.S. Geological Survey (USGS) report on the preparation of the PCB mixture used in the Trustees' avian egg injection studies. The USGS report also describes the rationale for the selection of PCB congeners used in the mixture and the preparation of dosing solutions used in the avian egg injection studies.

Table of Contents

Appendices

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.

In 2002, the Hudson River Natural Resource Trustees released an Assessment Plan for the Hudson River (Hudson River Natural Resource Trustees, 2002). That Assessment Plan documented that natural resources of the Hudson River had been, and continued to be, exposed to contamination by PCBs, and provided information regarding three major steps in the assessment: pathway and injury determination, injury quantification, and damage determination and restoration. That Assessment Plan further noted that the Trustees were considering conducting injury determination and quantification for a number of Hudson River resources, including birds, and that the Trustees' currently proposed approach to injury determination and quantification entailed several specific investigations focused on birds, including a preliminary avian evaluation, a breeding bird survey, a bird egg study, an evaluation of avian exposure from feeding on floodplain organisms, and bald eagle monitoring.

In 2004, the Trustees released the "Study Plan for Year 2004 Avian Investigations for the Hudson River - Final, Public Release Version" (Avian Injury Study Plan), dated June 15, 2004 (Hudson River Natural Resource Trustees, 2004). That Avian Injury Study Plan described the activities that constituted the Trustees' planned approach to conducting investigations of injury to avian species as part of the Hudson River NRDA, including an avian egg injection study.

In 2006, the Trustees released the "Study Plan for Avian Egg Injection Study" (Avian Egg Injection Study Plan), dated May 12, 2006 (Hudson River Natural Resource Trustees, 2006, 2007).

2.0 INTRODUCTION

The Avian Egg Injection Study Plan noted that the objective of the investigation is to evaluate the toxicity and adverse effects of embryonic exposure of multiple avian species to dose ranges of PCB 126 or a PCB mixture. That PCB mixture, made up of individual PCB congeners, fits a profile similar to the mixture of PCBs occurring in the eggs of birds nesting in the Upper Hudson River. Appendix A of this report provides the U.S. Geological Survey (USGS) report on the preparation of the PCB mixture used in the Trustees' avian egg injection studies. The USGS report also describes the rationale for the selection of PCB congeners used in the mixture and the preparation of dosing solutions used in the avian egg injection studies.

3.0 LITERATURE CITED

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Appendix A

Design and Preparation of a Custom 58-Congener PCB Mixture Dosing Solution for Avian Egg Injection Studies (U.S. Geological Survey report)

Design and preparation of a custom 58-congener PCB mixture dosing solutions for Japanese quail egg injection studies.

Biochemistry & Physiology Branch Final Laboratory Report FY 2006

12 July 2006

Prepared for: Kathryn Jahn
US Fish and Wildlife Service
New York Field Office, Region 5
3817 Luker Rd.
Courtland, New York 13045

Prepared by: U.S. Geological Survey
Columbia Environmental Research Center
4200 New Haven Road
Columbia, Missouri 65201

Table of Contents

List of Tables

Attachments

Attachment 1. AccuStandard custom PCB 58-congener mixture certificate of analysis.

Attachment 2. AccuStandard isooctane procedural blank certificate of analysis.

Introduction

The Hudson River Natural Resource Trustees are conducting studies to evaluate whether avian reproduction and/or development is injured as a result of exposure to PCBs from the Hudson River. As part of the NRDA, Japanese quail egg injection studies have been conducted in order to establish the effective dosage and reliable measurement end points that will then be applied to tests in other avian species. This report describes the rationale for selection of the PCB congeners selected for testing in avian species and the preparation of the mixture used in the avian egg injection studies with Japanese quail.

The mixture of PCBs, and possibly dioxins and furans, present in biota from the Hudson River are largely the result of releases of Aroclors into the river (see NRDA websites for details: http://www.fws.gov/contaminants/restorationplans/hudsonriver/hudsonriver.cfm or http://www.dec.state.ny.us/website/dfwmr/habitat/nrd/). Aroclors are the commercial names for heat transfer fluids used in electrical components such as capacitors and transformers. Aroclors were chiefly comprised of polychlorinated biphenyls, but also contained polychlorinated dibenzofurans (PCDFs) as a by-product of manufacture and use (Erickson et al. 1989). Based on our current knowledge of toxicity in avian species, PCBs have a subset of congeners which can bind the Ah-receptor and also cause dioxin-like toxicity. PCBs with chlorine atoms at one (mono-) or no (non-) ortho positions of the biphenyl rings and chlorine atoms on the lateral positions (meta and para) of the biphenyl rings fall into this dioxin-like category of toxicity. These non-ortho-chloro PCBs and mono-ortho-chloro PCBs are referred to as “dioxin-like PCBs.” Other PCB congeners have been shown to work through different modes of action and can cause neurotoxicity, malignant transformations of cells and tissues, as well as other untoward effects in exposed species. However, knowledge of the exact mechanisms by which the non-dioxin-like PCB congeners cause these effects is limited.

The amounts of PCDFs in Aroclors although small on a mass basis, are significant on the basis of toxicological potency. Thus, any mixture that is prepared to evaluate the toxicological potency of chemicals associated with the use and release of Aroclors into the Hudson River, must consider the toxic potency of PCDFs as well as PCBs. The relative toxicological importance of PCDFs in fish and wildlife species in the Hudson River is a direct function of the exposure experienced by the particular species. Fundamental principles of toxicokinetics (uptake, distribution, metabolism, and elimination) in the organism are important variables for the assessment of PCDFs, as well as PCBs and other chemicals which bioaccumulate and contribute to dioxin-like toxic equivalents (TEQs). The environmental fate of the PCDFs and PCBs present in Aroclors is dependent, in part, on physical chemical properties of the individual congeners (persistence, volatility, sorption, etc). Thus, the chemical exposure experienced by a species in the Hudson River ecosystem is a function of its trophic level and placement in the food web.

Rationale and Design of PCB Congener Mixture

The rationale for selection of chemicals to include in a mixture representing avian species found in the Hudson River basin was based on the toxicological concerns for these chemicals (ie. exposure and toxicity). Polychlorinated dibenzodioxins (PCDDs) and PCDFs are both referred to as “dioxin-like” because they exert their toxicity through the same cytosolic receptor (Ah-receptor) and cause the same suite of toxicological symptoms in avian species. Certainly, no model of toxicity comparable to that of dioxin-like toxicity (van den Berg et al. 1998) is available for the non-dioxin-like PCB congeners. Therefore, toxicological evaluation and assessment of PCBs is logically divided into two categories, dioxin-like PCBs and the non-dioxin-like PCBs. The lack of a detailed model of toxicity for the non-dioxin-like PCB congeners forces us to evaluate these congeners on the basis of mass or simple concentrations. The dioxin-like PCBs, on the other hand, can be evaluated based on concentration and their individual potency (toxic equivalency factor, TEF). Thus, evaluation and selection of PCB congeners to include in a mixture for toxicological testing in Japanese quail was based on concentrations of the non-dioxin-like PCB congeners and the toxic potencies (concentrations X TEF) for each of the dioxin-like PCB congeners. The evaluation of PCDD and PCDF congeners was based on their potency to cause dioxin-like effects and the relative amounts present in avian wildlife from the Hudson River.

Chemical Data on Belted Kingfisher, Spotted Sandpiper, and Tree Swallows from the Hudson River

The exposure data on belted kingfisher, spotted sandpiper, and tree swallows collected from the Hudson River in 2004 (Hudson River Natural Resource Trustees 2005) was the main source of information for the selection of chemicals that comprised the mixture for toxicity testing. A summary of the chemical exposure data used for the selection of congeners that were added to the synthetic mixture are provided for this report. The data summary for the selection process is provided in two manners: 1) toxic potencies or TEQs for the dioxin-like PCB, PCDD, and PCDF congeners (Table 1); and 2) mean concentrations and their ranks for the non-dioxin-like PCBs (Table 2).

The TEQs present in the avian species collected from the Hudson River in 2004 were remarkable with respect to the concentrations of PCB-related TEQs as compared to PCDF- and PCDD-related TEQs (Table 1). In all three of these species the PCB-related TEQs were 99% or greater of the total TEQs observed in each species. This is remarkable in the sense that even though we know that Aroclors released into the Hudson River were first and foremost PCBs, they also contained PCDFs as manufacturing by-products. Some of these PCDFs would be subject to metabolism (eg. 2,3,7,8-TCDF) and might not be observed at significant concentrations for this reason. The overall conclusion that can be drawn from these data is that any dioxin-like toxicity observed or expected in developing embryos of birds from the Hudson River study area appears to be almost exclusively related to PCBs. Consequently, our toxicity testing in Japanese quail is focused on PCB congeners that are present. Testing of the contributions of PCDFs and PCDDs to the developmental toxicity of the dioxin-like chemicals in these avian species would necessarily have to take a much smaller research or assessment priority.

Evaluation of the non-dioxin-like PCB congeners present in these avian species from the Hudson River basin was based on the concentrations of the chemicals present, the rank order of their concentrations within the eggs of a species, and the cumulative percent of total PCBs attributable to individual congeners (Table 2). The PCB congener concentrations (ng/g, ranked from greatest to least) are followed by a column of the relative percentage of each congener as compared to the total PCB concentration for the species, and the last column under each species is a cumulative percentage as compared to the total PCB content in that species.

The composition of the PCB congeners with the greatest concentrations in each species was fairly consistent. As one evaluates the cumulative percentage of the congeners, starting from the congener with the greatest concentration, there are a large number of congeners (or congener pairs) which are the same in each of the three species. If one looks at the first 36 congeners for each species, 29 of 36 congeners are the same when comparing the congeners found in greatest concentrations in the tree swallow to that of the belted kingfisher, while 31 of the first 36 congeners are the same between the spotted sandpiper and the belted kingfisher. The cumulative percentages of individual PCB congener contributions to total PCBs up until the 36th ranked congener (or congener set) are approximately 95%, 90% and 88% for spotted sandpiper, belted kingfisher, and tree swallow, respectively. That is to say, the first 36 PCB congeners (or congener sets) account for approximately 88% of the total PCB content in tree swallows; 90% of the total PCB content in belted kingfisher; and 95% of the total PCB content in spotted sandpiper.

Design of a PCB mixture similar to Hudson River spotted sandpipers

The congener composition developed for the laboratory toxicity testing was based on the spotted sandpiper eggs collected from the Hudson River in 2004. The rationale for the selection was based on: 1) the greater concentrations of total PCBs and TEQs in the eggs of the spotted sandpiper; 2) the large degree of similarity between the congener patterns in tree swallows, spotted sandpipers and belted kingfisher eggs from the Hudson River samples; and 3) the high concentrations of PCBs observed in spotted sandpiper eggs in 2004, suggesting that this species might be a candidate for an egg injection study. The first 36 PCB congeners (or congener sets) in the spotted sandpiper were selected from the list (Table 2). This represented approximately 95% of the total PCB content measured in spotted sandpiper eggs on a mass basis. Additionally, the dioxin-like PCBs were added to the mixture based upon their importance from a toxicological standpoint. Thus, the dioxin-like PCB congeners that were not among in the first 36 congeners or congener sets were added to the PCB mixture in direct proportion to their concentrations found in spotted sandpiper eggs (Table 2), even when their concentrations were small and the ranks of the dioxin-like PCBs were below the first 36 congeners. Accordingly, another 9 congeners were included in the synthetic PCB mixture on this basis. The exact concentrations of the dioxin-like and non-dioxin-like PCB congeners in the mixture reflect the concentrations found in the spotted sandpiper egg samples.

The final selection of congeners to be included into the PCB mixture was based on the composition of PCBs in the spotted sandpiper eggs taken in the initial 11 samples collected from the Hudson River in 2004, the presence of these congeners in Aroclor mixtures, and the relative amounts of some congeners in Aroclors. The field identification and species of these 11 samples were as follows: 1) BK04-105-E, Belted Kingfisher; 2) BK04-150-E, Belted Kingfisher; 3) BK04-171-E, Belted Kingfisher; 4) BK04-216-E, Belted Kingfisher; 5) SS04-22-E1, Spotted Sandpiper; 6) SS04-140-A-E, Spotted Sandpiper; 7) SS04-237-A-E1, Spotted Sandpiper; 8) TS04-JV-40-EA, Tree Swallow; 9) TS04-JV-42-EA, Tree Swallow; 10) TS04-REM-83-EA, Tree Swallow; and 11) TS04-RI-128-EA, Tree Swallow. The analysis of PCB congeners included co-elution of some congeners (Table 2). The first 36 ranked congeners or congener sets included 18 pairs of congeners that co-eluted and 5 congener sets that potentially included three co-eluting congeners (Table 3). Construction of the congener mix for toxicity testing required us to evaluate the possible composition of each individual congener within a set of co-eluting congeners. The composition of individual congeners within a set was estimated from the composition of these congeners within a 1:1:1:1 Aroclor 1242:1248:1254:1260 mixture. Of the 18 pairs of co-eluting congeners, 13 of the pairs contained a congener that was present in only very small amounts (<1%) relative to the amount of the other co-eluting congener. The PCB congeners with relative proportions of <0.01 (ie. <1%) as compared to the other co-eluting congener were omitted from the PCB mixture (Table 3). Thus, PCB congeners 80, 61, 106, 73, 76, 127, 120, 160, 86, 93, 182, 111, and 107 were not added to the PCB mixture, due to negligible concentrations assumed to be present. The individual congeners of the 5 remaining co-eluting pairs of congeners were added into the PCB mixture at concentrations proportional to their relative amounts in the Aroclor 1:1:1:1 mixture (Table 3).

The same selection criteria and rules for proportions were used for the 5 sets of co-eluting congeners that appeared in the top 36 ranked congeners or congener sets from the original data set on spotted sandpiper eggs (Table 2). Congeners 90 and 116 were omitted from the mixture, due to assumed negligible amounts. The remainder of the individual congeners in the 5 sets of triplet peaks were added into the PCB mixture at the amounts given in Table 3. The composition of the PCB mixture resulted in 58 individual PCB congeners; the 49 congeners selected based on their importance to the total mass of PCBs and the 9 congeners selected based on their dioxin-like toxicological properties (Table 3). The 58-congener PCB mixture, with proportions equivalent to that of the congener composition of spotted sandpiper eggs from the Hudson River, was special ordered from AccuStandard, Inc. (New Haven, CT; Table 4).

The objective of this work was to prepare the mixture of PCB congeners and develop a serial dilution set of dosing solutions to be used to study PCB-related toxicity in avian embryos and chicks. The remainder of this report describes the preparation of six concentrations (doses) of the custom 58-congener PCB mixture emulsified in 0.75% (v/v) corn oil:1,2-propanediol and an emulsified 0.75% corn oil:1,2-propanediol blank under sterile conditions. These solutions were prepared for use as egg injection dosing solutions in a Japanese quail egg dosing study conducted at the University of Maryland. The solutions may potentially be used in subsequent egg injection studies on other species.

Preparation of custom 58-congener PCB mixture dosing solutions

PCB mixture order and receipt

The custom 58-congener PCB standard dissolved in 250-mL of isooctane and corresponding 250-mL of blank isooctane were purchased from AccuStandard, Inc. (New Haven, CT; Invoice # 270876). The custom PCB congener solution and isooctane blank were received in separate brown glass bottles with Teflon-lined screw caps. AccuStandard personnel used their exact measured weights of each congener and GC/MS –based congener purities to generate a list of "prepared concentrations" and "certified analyte concentrations" provided in the Certificate of Analysis for the custom PCB congener standard solution (Attachment 1). A similar certificate of Analysis was provided for the isooctane blank (Attachment 2).

Upon receipt at CERC, the custom PCB congener package was opened by CERC staff and the bottles were inspected for concurrence with attached documentation. Both bottles were received in good condition and documentation was verified. To verify these solution concentrations triplicate 100-µL sub-samples of each were taken with a 100-µL Hamilton syringe and placed in cleaned amber 1.5-mL autosample vials with yellow Teflon-faced septa screw caps. The autosample vials were weighed before and after sample addition using both a Mettler AE260 and a Mettler-Toledo A6245 so that mass of the transferred volume could be determined. The six samples were given to the CERC Organic Chemistry section for analysis. The 58-congener PCB mixture and the isooctane blank were secured and stored in a locked box placed in the CERC Biochemistry Section hazardous compound storage area.

Glassware preparation

Transfer tubes - Two 30-mL glass culture tubes with Teflon-faced rubber lined screw caps (Fisher # 149 3010G, 20 x 125 mm) were used for transfer and sub-sampling of the original 58-congener PCB mixture from AccuStandard. We prepared these transfer tubes by solvent rinsing with three consecutive

Preparation of PCB mixture stock emulsion and procedural blank emulsion

The dosing solutions of the PCB mixture were prepared as emulsions of 0.75% (v/v) corn oil in 1,2-propanediol (propylene glycol) to attain concentrations great enough for toxicity testing. A PCB stock emulsion (in vial labeled 3-2) and procedural blank emulsion (in vial labeled 3-1) were prepared from the sub-samples described above. The targeted concentration of the PCB stock emulsion dosing solution was 246 µg PCB/ µL in a final volume of 1.6-mL. A procedural blank emulsion was also prepared. These emulsions were prepared as follows. A small amount of sterile corn oil (Sigma, Cat. # C8267, batch 103K0107, density 0.9 g/mL) was added to each of the 3-mL vials; 12.2-µL to vial 3-1 (blank) and 12.7-µL to vial 3-2 (PCB mixture), calculated based density. Each vial was gently mixed by slow vortexing. This was done to allow the corn oil to act as a co-solvent for the PCB congeners and the rest of the isooctane was allowed to evaporate. Any residual isooctane was allowed to evaporate under gentle nitrogen purge with the vials maintained at approximately 25 ^{o}C. The vials were periodically mixed by slow vortexing. The evaporation was continued until vials reached a constant weight and the masses of the contents (PCB congeners) in each of the two vials were determined by difference.

Filter sterilized 1,2-propanediol (Fisher # P355-1, density 1.036 g/mL) was added to both vials to bring them to the previously marked volume of 1.6-mL. Calculated volumes based on the masses of the 1,2-propanediol added confirmed the volume added to each vial. The contents of each 3-mL vial were vigorously vortex-mixed until they became thoroughly emulsified. The resulting emulsion of the 58-congener PCB standard dosing stock solution (vial 3-2) was at a target concentration of 246 µg PCB/uL, while the procedural blank emulsion (vial 3-1) was the negative control for the toxicity testing blank dosing solution.

Serial dilution of the PCB mixture stock emulsion

The various dosing solutions were prepared by serial dilution of the stock PCB mixture emulsion. A sterile emulsion of corn oil and 1,2-propanediol (propylene glycol) was first prepared to make all of the subsequent dilutions. The sterile stock emulsion of 0.75% (v/v) corn oil:1,2-propanediol was prepared by combining 101.2-µL of sterile corn oil with 13.4710-mL of sterile 1,2-propanediol in a sterile 15-mL glass culture tube with a Teflon-faced rubber-lined screw cap (volumes confirmed by weight). This was vigorously vortex-mixed to produce a stable emulsion. The six 3-mL vials previously prepared, weighed and marked at 0.5 and 1.0-mL were arranged so that they were consecutively numbered, 3-8 to 3-12. A 500-µL aliquant of the 58-congener PCB standard dosing solution stock emulsion (vial 3-2) was transferred to the empty vial 3-7 under sterile conditions. In the same manner, a 500-µL aliquant of the procedural blank dosing solution emulsion (vial 3-1) was transferred to vial 3-4. Under sterile conditions, a 500-µL aliquant of the freshly vortex-mixed sterile stock emulsion of 0.75% (v/v) corn oil:1,2-propanediol from the 15-mL culture tube was transferred to each of the vials 3-8, 3-9, 3-10, 3-11, and 3-12. The 58-congener PCB mixture emulsion dosing solution stock (vial 3-2) was freshly vortex-mixed and using a calibrated Rainin P-1000, 500-µL was transferred to vial 3-8. This resulted in a 2-fold dilution and vial 3-8 was vigorously vortex-mixed. In the same manner, 500-µL was transferred from vial 3-8 to vial 3-9 resulting in a 4-fold dilution of the original PCB emulsion. This process was repeated to produce the entire dosing solution dilution series (Table 5).

Sub-sampling of dosing solution emulsions for chemical analysis

A 20-µL sample of each dosing solution (Table 5) was taken and placed in previously prepared amber 1.5-mL autosample vial with Teflon-faced septa screw caps. The autosample vials were weighed before and after sample addition using a Mettler AE260 so that mass of the transferred volume could be determined. The seven samples were given to the CERC Environmental Chemistry Branch for analysis. The results of these analyses will be reported in a separate report from the Environmental Chemistry Branch, CERC.

Shipment of the dosing solution emulsions

Each vial listed in Table 5 was individually bagged in a 4 x 4 “Zip-loc” type plastic bag, placed in a 3 mL vial shipping box and all were stored at -80 ^{o}C in preparation for shipment. All other vials or bottles containing solutions prepared in this procedure were returned and secured to a locked box. The 3 mL vial shipping box containing the dosing solutions was packed in a Styrofoam shipping box with approximately 10 lbs. dry ice and shipped via FedEx with the appropriate documentation to the laboratory conducting the avian egg injection studies with Japanese quail.

Nominal concentrations of PCBs and TEQs in the dosing solutions and eggs

The certified concentrations provided by AccuStandard (Attachment 1) were used to calculate the nominal concentrations of individual PCB congeners in each of the emulsified egg dosing solutions (Table 6). Briefly, for each congener we calculated the mass in 50.0-mL of the original 250-mL 58-congener PCB standard stock solution from AccuStandard (Table 4). This mass was converted to concentration units (µg/µL) using the final volume of the PCB stock dosing solution emulsion (1.60-mL in vial 3-2). Subsequently, dosing solution concentrations for the individual congeners were calculated based on dilution factors (Table 5). Finally, individual PCB congener egg dose concentrations were calculated by multiplying the dosing solution concentration of each congener times the injection volume (4 µL/egg) and then dividing by the average mass of a Japanese quail egg (10 g/egg). Expected doses of each of the 58 PCB congeners were calculated in this fashion, for each of the six dosing solutions (Table 6).

For example: The AccuStandard certified concentration for PCB congener 28 (2, 4, 4’-trichlorobiphenyl) of 817.6 µg/mL, was multiplied by 50.0 ml, resulting in 40,880 µg of PCB congener 28. Next, we divided by 1.60 mL and then by 1000 µL/mL to give 25.55 µg/µL; the concentration of PCB congener 28 in the emulsified stock dosing solution (98 µg total PCB/g egg, vial 3-7). The PCB congener 28 concentration/g egg (expected nominal dose) was calculated by multiplying 25.55 µg/µL by 4 µL, dividing by 10 g/egg and finally multiplying by 1000 ng/µg to obtain 10,220 ng/g (Table 6). The names for each of the dosing solution emulsions (column headers) were given as the nominal total PCB dose expected to be delivered to an individual Japanese quail egg from that solution (Table 6). Thus, the six dosing solution emulsions were referred to as 98, 49, 25, 12, 6, and 3 µg PCB/g egg doses (column headers in Table 6).

The dioxin toxic equivalents (TEQs) expected to be delivered to a quail egg at each dose were also estimated (Table 7). The dose from the 12 PCB congeners with dioxin-like potency (from Table 6) was multiplied times the potency of the congener (toxic equivalency factor, TEF) as determined for avian wildlife (van den Berg et al. 1998). The sum of the individual congener TEQs was estimated for each of the dosing solutions. The six dosing solution emulsions, referred to as 98, 49, 25, 12, 6, and 3 µg PCB/g egg (Table 6), had total TEQs of approximately12,600 pg/g, 6,300 pg/g, 3,100 pg/g, 1,600 pg/g, 790 pg/g, and 390 pg/g, respectively (Table 7).

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Tables

Table 1. Concentrations (pg/g) of dioxin-like toxic equivalents (TEQs) derived from PCBs, PCDDs, and PCDFs measured in spotted sandpiper, belted kingfisher, and tree swallow eggs collected from the Hudson River basin in 2004*.

	Tree swallow TEQ (pg/g)	Belted Kingfisher TEQ (pg/g)	Spotted Sandpiper TEQ (pg/g)
PCB-81	600	600	1700
PCB-77	2000	650	1500
PCB-123	0	0	0
PCB-114	1	2	6
PCB-126	200	900	500
PCB-167	0	0	0
PCB-156	4	5	16
PCB-157	1	1	3
PCB-169	0	0	0
PCB-189	0	0	0
PCB 118/106	4	5	16
PCB 105/127	17	23	76
PCB Sub-total	2828	2187	3818
2,3,7,8-TCDD	1	1	1
1,2,3,7,8-PECDD	3	2	1
1,2,3,4,7,8-HXCDD	0	0	0
1,2,3,6,7,8-HXCDD	0	0	0
1,2,3,7,8,9-HXCDD	0	0	0
1,2,3,4,6,7,8-HPCDD	0	0	0
OCDD	0	0	0
PCDD Sub-total	4	3	2
2,3,7,8-TCDF	1	1	1
1,2,3,7,8-PECDF	0	0	0
2,3,4,7,8-PECDF	5	2	2
1,2,3,4,7,8-HXCDF	0	0	0
1,2,3,6,7,8-HXCDF	0	0	0
2,3,4,6,7,8-HXCDF	0	0	0
1,2,3,7,8,9-HXCDF	0	0	0
1,2,3,4,6,7,8-HPCDF	0	0	0
1,2,3,4,7,8,9-HPCDF	0	0	0
OCDF	0	0	0
PCDF Sub-total	6	3	3
Total TEQs	2837	2194	3823

TEQs based on concentrations of polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs) measured in eggs collected in 2004 and avian TEF values (van den Berg et al. 1998). Concentrations of co-eluting congeners PCB-118/PCB-106 and PCB-105/PCB-127, were assumed to be all PCB-118 and PCB-105, respectively, for TEQ estimation purposes. Concentrations for the PCB congeners are based on GC-LR/MS analysis and concentrations of PCDDs and PCDFs are based on GC-HR/MS analysis of the initial 11 avian egg samples taken in 2004 (see text for sample identification numbers).

Table 2. Concentrations (ng/g egg), relative amount (% of total), and cummulative percentage of rank ordered polychlorinated biphenyl (PCB) congeners measured in the eggs of birds collected along the Hudson River in 2004.

	Tree swallow averages (ng/g)	Rel. %	Cumm.%		Belted Kingfisher averages (ng/g)	Rel. %	Cumm.%		Spotted Sandpiper averages (ng/g)	Rel. %	Cumm.%
TOTAL PCB	8203	100		TOTAL PCB	9198	100		TOTAL PCB	25579	100	
PCB 52/73	506	6	6.2	PCB 138/163/164	642	7	7.0	PCB 28	2548	10	10.0
PCB 28	459	6	11.8	PCB 153	580	6	13.3	PCB 66/80	2290	9	18.9
PCB 66/80	426	5	17.0	PCB 52/73	566	6	19.4	PCB 74/61	1679	7	25.5
PCB 70/76	409	5	21.9	PCB 118/106	548	6	25.4	PCB 118/106	1604	6	31.7
PCB 31	382	5	26.6	PCB 66/80	523	6	31.1	PCB 47/48/75	1538	6	37.8
PCB 49/43	370	5	31.1	PCB 47/48/75	492	5	36.4	PCB 138/163/164	1410	6	43.3
PCB 118/106	368	4	35.6	PCB 74/61	428	5	41.1	PCB 90/101/89	1289	5	48.3
PCB 138/163/164	347	4	39.8	PCB 90/101/89	410	4	45.5	PCB 52/73	1250	5	53.2
PCB 90/101/89	318	4	43.7	PCB 99	395	4	49.8	PCB 49/43	1099	4	57.5
PCB 41/71/64/68	316	4	47.5	PCB 49/43	386	4	54.0	PCB 153	1016	4	61.5
PCB 74/61	305	4	51.3	PCB 110	304	3	57.3	PCB 99	998	4	65.4
PCB 47/48/75	283	3	54.7	PCB 28	282	3	60.4	PCB 70/76	913	4	68.9
PCB 153	266	3	58.0	PCB 105/127	227	2	62.9	PCB 105/127	764	3	71.9
PCB 110	259	3	61.1	PCB 149/139	218	2	65.2	PCB 31	743	3	74.8
PCB 44	231	3	63.9	PCB 41/71/64/68	181	2	67.2	PCB 56/60	720	3	77.6
PCB 56/60	214	3	66.5	PCB 187/182	164	2	69.0	PCB 41/71/64/68	645	3	80.2
PCB 99	178	2	68.7	PCB 180	160	2	70.7	PCB 110	453	2	81.9
PCB 105/127	172	2	70.8	PCB 56/60	144	2	72.3	PCB 85/120	437	2	83.6
PCB 95/93	146	2	72.6	PCB 31	139	2	73.8	PCB 87/115/116	330	1	84.9
PCB 87/115/116	138	2	74.3	PCB 70/76	136	1	75.3	PCB 128	250	1	85.9
PCB 42/59	137	2	76.0	PCB 85/120	128	1	76.7	PCB 149/139	217	1	86.8
PCB 149/139	125	2	77.5	PCB 95/93	123	1	78.0	PCB 92	213	1	87.6
PCB 37	119	1	78.9	PCB 87/115/116	120	1	79.3	PCB 180	180	1	88.3
PCB 97/86	99	1	80.1	PCB 92	115	1	80.6	PCB 158/160	178	1	89.0
PCB 85/120	83	1	81.1	PCB 146	108	1	81.7	PCB 146	165	1	89.6
PCB 180	70	1	82.0	PCB 170/190	99	1	82.8	PCB 97/86	159	1	90.3
PCB 128	61	1	82.7	PCB 128	87	1	83.8	PCB 156	155	1	90.9
PCB 26	52	1	83.4	PCB 111/117	86	1	84.7	PCB 95/93	155	1	91.5
PCB 92	50	1	84.0	PCB 63	74	1	85.5	PCB 187/182	141	1	92.0
PCB 132/168	48	1	84.6	PCB 91	62	1	86.2	PCB 170/190	132	1	92.5
PCB 16/32	47	1	85.1	PCB 158/160	54	1	86.8	PCB 111/117	130	1	93.0
PCB 170/190	47	1	85.7	PCB 156	53	1	87.3	PCB 141	118	0.5	93.5
PCB 22	46	1	86.3	PCB 199	52	1	87.9	PCB 130	84	0.3	93.8
PCB 158/160	44	1	86.8	PCB 151	51	1	88.4	PCB 107/109	81	0.3	94.1
PCB 91	44	1	87.3	PCB 44	49	1	89.0	PCB 137	78	0.3	94.5
PCB 146	42	1	87.9	PCB 107/109	47	1	89.5	PCB 42/59	71	0.3	94.7
PCB 156	42	1	88.4	PCB 196/203	46	0.5	90.0	PCB 144/135	65	0.3	95.0
PCB 77	40	0.5	88.8	PCB 132/168	42	0.5	90.4	PCB 91	65	0.3	95.2
PCB 33/20/21	38	0.5	89.3	PCB 72	41	0.4	90.9	PCB 132/168	60	0.2	95.5
PCB 17	38	0.5	89.8	PCB 97/86	38	0.4	91.3	PCB 63	60	0.2	95.7
PCB 18	36	0.4	90.2	PCB 183	38	0.4	91.7	PCB 114	57	0.2	95.9
PCB 141	35	0.4	90.6	PCB 177	37	0.4	92.1	PCB 119	50	0.2	96.1
PCB 63	34	0.4	91.0	PCB 141	36	0.4	92.5	PCB 167	49	0.2	96.3
PCB 15	33	0.4	91.4	PCB 130	31	0.3	92.8	PCB 183	44	0.2	96.5
PCB 187/182	33	0.4	91.8	PCB 144/135	30	0.3	93.2	PCB 177	40	0.2	96.6
PCB 67	31	0.4	92.2	PCB 194	30	0.3	93.5	PCB 123	39	0.2	96.8
PCB 53	29	0.4	92.6	PCB 178	29	0.3	93.8	PCB 174/181	39	0.2	96.9
PCB 107/109	28	0.3	92.9	PCB 42/59	28	0.3	94.1	PCB 82	38	0.1	97.1
PCB 82	27	0.3	93.3	PCB 137	28	0.3	94.4	PCB 151	37	0.1	97.2
PCB 151	25	0.3	93.6	PCB 174/181	27	0.3	94.7	PCB 157	33	0.1	97.4
PCB 111/117	24	0.3	93.9	PCB 119	26	0.3	95.0	PCB 147	32	0.1	97.5
PCB 25	23	0.3	94.1	PCB 133	25	0.3	95.3	PCB 72	31	0.1	97.6
PCB 144/135	23	0.3	94.4	PCB 167	22	0.2	95.5	PCB 77	30	0.1	97.7
PCB 84	22	0.3	94.7	PCB 114	22	0.2	95.8	PCB 22	30	0.1	97.8
PCB 40	20	0.2	94.9	PCB 26	22	0.2	96.0	PCB 129	29	0.1	98.0
PCB 130	20	0.2	95.2	PCB 147	21	0.2	96.2	PCB 199	26	0.1	98.1
PCB 137	20	0.2	95.4	PCB 123	18	0.2	96.4	PCB 196/203	25	0.1	98.2
PCB 167	16	0.2	95.6	PCB 206	17	0.2	96.6	PCB 171	23	0.1	98.2
PCB 183	16	0.2	95.8	PCB 154	17	0.2	96.8	PCB 133	21	0.1	98.3
PCB 174/181	14	0.2	96.0	PCB 193	15	0.2	97.0	PCB 33/20/21	20	0.1	98.4

	Tree swallow averages (ng/g)	Rel. %	Cumm.%		Belted Kingfisher averages (ng/g)	Rel. %	Cumm.%		Spotted Sandpiper averages (ng/g)	Rel. %	Cumm.%
PCB 136	13	0.2	96.1	PCB 171	14	0.2	97.1	PCB 124	20	0.1	98.5
PCB 114	13	0.2	96.3	PCB 100	14	0.1	97.3	PCB 178	19	0.1	98.6
PCB 196/203	12	0.1	96.4	PCB 172/192	14	0.1	97.4	PCB 16/32	18	0.1	98.6
PCB 124	12	0.1	96.6	PCB 77	13	0.1	97.6	PCB 194	18	0.1	98.7
PCB 129	12	0.1	96.7	PCB 202	13	0.1	97.7	PCB 81	17	0.1	98.8
PCB 177	12	0.1	96.9	PCB 157	12	0.1	97.8	PCB 100	15	0.1	98.8
PCB 51	12	0.1	97.0	PCB 136	11	0.1	98.0	PCB 103	14	0.1	98.9
PCB 72	11	0.1	97.2	PCB 195	10	0.1	98.1	PCB 172/192	14	0.1	98.9
PCB 98/102	11	0.1	97.3	PCB 126	9	0.1	98.2	PCB 154	14	0.1	99.0
PCB 24/27	11	0.1	97.4	PCB 208	8	0.1	98.2	PCB 53	14	0.1	99.1
PCB 199	11	0.1	97.6	PCB 98/102	8	0.1	98.3	PCB 26	14	0.1	99.1
PCB 123	10	0.1	97.7	PCB 103	7	0.1	98.4	PCB 193	12	0.05	99.2
PCB 157	9	0.1	97.8	PCB 16/32	7	0.1	98.5	PCB 8/5	11	0.04	99.2
PCB 119	9	0.1	97.9	PCB 84	6	0.1	98.5	PCB 44	11	0.04	99.2
PCB 194	9	0.1	98.0	PCB 159	6	0.1	98.6	PCB 67	11	0.04	99.3
PCB 83/108	8	0.1	98.1	PCB 81	6	0.1	98.7	PCB 37	11	0.04	99.3
PCB 171	7	0.1	98.2	PCB 113	5	0.1	98.7	PCB 25	10	0.04	99.4
PCB 147	7	0.1	98.3	PCB 129	5	0.1	98.8	PCB 166	8	0.03	99.4
PCB 46	7	0.1	98.4	PCB 82	5	0.1	98.8	PCB 98/102	7	0.03	99.4
PCB 81	6	0.1	98.4	PCB 53	4	0.05	98.9	PCB 206	7	0.03	99.5
PCB 45	6	0.1	98.5	PCB 67	4	0.05	98.9	PCB 24/27	7	0.03	99.5
PCB 178	6	0.1	98.6	PCB 209	4	0.04	99.0	PCB 162	7	0.03	99.5
PCB 19	6	0.1	98.6	PCB 162	4	0.04	99.0	PCB 202	7	0.03	99.5
PCB 55	5	0.1	98.7	PCB 201	4	0.04	99.1	PCB 113	6	0.02	99.6
PCB 38	5	0.1	98.8	PCB 165	4	0.04	99.1	PCB 136	6	0.02	99.6
PCB 133	5	0.1	98.8	PCB 166	4	0.04	99.1	PCB 195	6	0.02	99.6
PCB 172/192	5	0.1	98.9	PCB 25	4	0.04	99.2	PCB 69	5	0.02	99.6
PCB 8/5	5	0.1	99.0	PCB 69	3	0.04	99.2	PCB 159	5	0.02	99.6
PCB 206	5	0.1	99.0	PCB 124	3	0.04	99.3	PCB 126	5	0.02	99.7
PCB 4/10	5	0.1	99.1	PCB 179	3	0.04	99.3	PCB 140	5	0.02	99.7
PCB 193	4	0.05	99.1	PCB 189	3	0.03	99.3	PCB 185	5	0.02	99.7
PCB 57	4	0.04	99.2	PCB 176	3	0.03	99.4	PCB 189	4	0.02	99.7
PCB 154	3	0.04	99.2	PCB 112	3	0.03	99.4	PCB 17	4	0.02	99.7
PCB 103	3	0.04	99.3	PCB 185	3	0.03	99.4	PCB 191	4	0.01	99.8
PCB 202	3	0.03	99.3	PCB 24/27	3	0.03	99.5	PCB 83/108	4	0.01	99.8
PCB 134/143	3	0.03	99.3	PCB 140	3	0.03	99.5	PCB 84	3	0.01	99.8
PCB 179	3	0.03	99.4	PCB 191	3	0.03	99.5	PCB 131/142	3	0.01	99.8
PCB 131/142	3	0.03	99.4	PCB 57	3	0.03	99.5	PCB 208	3	0.01	99.8
PCB 195	3	0.03	99.4	PCB 125	2	0.03	99.6	PCB 175	3	0.01	99.8
PCB 122	2	0.03	99.5	PCB 4/10	2	0.03	99.6	PCB 40	3	0.01	99.8
PCB 100	2	0.03	99.5	PCB 148	2	0.02	99.6	PCB 179	3	0.01	99.8
PCB 166	2	0.03	99.5	PCB 83/108	2	0.02	99.6	PCB 55	3	0.01	99.8
PCB 162	2	0.03	99.5	PCB 18	2	0.02	99.7	PCB 18	2	0.01	99.9
PCB 208	2	0.03	99.6	PCB 175	2	0.02	99.7	PCB 4/10	2	0.01	99.9
PCB 126	2	0.03	99.6	PCB 207	2	0.02	99.7	PCB 122	2	0.01	99.9
PCB 96	2	0.02	99.6	PCB 88/121	2	0.02	99.7	PCB 165	2	0.01	99.9
PCB 189	2	0.02	99.6	PCB 198	2	0.02	99.7	PCB 201	2	0.01	99.9
PCB 69	2	0.02	99.7	PCB 19	2	0.02	99.8	PCB 94	2	0.01	99.9
PCB 29	2	0.02	99.7	PCB 51	2	0.02	99.8	PCB 176	2	0.01	99.9
PCB 113	2	0.02	99.7	PCB 40	2	0.02	99.8	PCB 15	2	0.01	99.9
PCB 185	1	0.02	99.7	PCB 22	2	0.02	99.8	PCB 148	2	0.01	99.9
PCB 191	1	0.02	99.7	PCB 200	1	0.02	99.8	PCB 125	2	0.01	99.9
PCB 176	1	0.02	99.8	PCB 94	1	0.01	99.8	PCB 57	2	0.01	99.9
PCB 159	1	0.02	99.8	PCB 205	1	0.01	99.9	PCB 88/121	1	0.01	99.9
PCB 209	1	0.02	99.8	PCB 17	1	0.01	99.9	PCB 134/143	1	0.01	99.9
PCB 125	1	0.01	99.8	PCB 197	1	0.01	99.9	PCB 209	1	0.004	99.9
PCB 94	1	0.01	99.8	PCB 37	1	0.01	99.9	PCB 198	1	0.004	99.9
PCB 175	1	0.01	99.8	PCB 150	1	0.01	99.9	PCB 51	1	0.004	99.95
PCB 58	1	0.01	99.8	PCB 131/142	1	0.01	99.9	PCB 205	1	0.003	99.96
PCB 140	1	0.01	99.9	PCB 45	1	0.01	99.9	PCB 200	1	0.003	99.96
PCB 88/121	1	0.01	99.9	PCB 33/20/21	1	0.01	99.9	PCB 45	1	0.003	99.96
PCB 201	1	0.01	99.9	PCB 58	1	0.01	99.9	PCB 19	1	0.003	99.97
PCB 50	1	0.01	99.9	PCB 122	1	0.01	99.9	PCB 207	1	0.003	99.97
PCB 6	1	0.01	99.9	PCB 34/23	1	0.01	99.95	PCB 173	1	0.003	99.97
PCB 7/9	1	0.01	99.9	PCB 96	1	0.01	99.96	PCB 197	1	0.002	99.97
PCB 112	1	0.01	99.9	PCB 188	0.4	0.005	99.96	PCB 29	1	0.002	99.98

	Tree swallow averages (ng/g)	Rel. %	Cumm.%		Belted Kingfisher averages (ng/g)	Rel. %	Cumm.%		Spotted Sandpiper averages (ng/g)	Rel. %	Cumm.%
PCB 54	1	0.01	99.9	PCB 134/143	0.4	0.004	99.97	PCB 150	0.5	0.002	99.98
PCB 12/13	1	0.01	99.9	PCB 54	0.4	0.004	99.97	PCB 6	0.4	0.002	99.98
PCB 207	1	0.01	99.9	PCB 8/5	0.3	0.004	99.98	PCB 96	0.4	0.002	99.98
PCB 198	1	0.01	99.9	PCB 155	0.3	0.003	99.98	PCB 58	0.4	0.002	99.98
PCB 173	1	0.01	99.9	PCB 50	0.2	0.003	99.98	PCB 155	0.4	0.001	99.98
PCB 34/23	0.5	0.01	99.9	PCB 55	0.2	0.002	99.98	PCB 184	0.4	0.001	99.99
PCB 35	0.4	0.01	99.95	PCB 173	0.2	0.002	99.99	PCB 65/62	0.3	0.001	99.99
PCB 165	0.4	0.01	99.96	PCB 46	0.2	0.002	99.99	PCB 112	0.3	0.001	99.99
PCB 205	0.4	0.01	99.97	PCB 152	0.2	0.002	99.99	PCB 161	0.3	0.001	99.99
PCB 148	0.4	0.005	99.97	PCB 15	0.2	0.002	99.99	PCB 54	0.3	0.001	99.99
PCB 200	0.3	0.004	99.97	PCB 78	0.1	0.002	99.99	PCB 7/9	0.3	0.001	99.99
PCB 39	0.3	0.004	99.98	PCB 39	0.1	0.002	99.99	PCB 38	0.2	0.001	99.99
PCB 197	0.2	0.003	99.98	PCB 161	0.1	0.001	99.996	PCB 46	0.2	0.001	99.99
PCB 150	0.2	0.002	99.98	PCB 184	0.1	0.001	99.998	PCB 188	0.2	0.001	99.99
PCB 30	0.2	0.002	99.98	PCB 65/62	0.1	0.001	99.999	PCB 34/23	0.2	0.001	99.99
PCB 65/62	0.1	0.002	99.99	PCB 104	0.1	0.001	100	PCB 50	0.2	0.001	99.99
PCB 152	0.1	0.001	99.99	PCB 7/9	0.1	0.001	100	PCB 39	0.1	0.001	99.99
PCB 78	0.1	0.001	99.99	PCB 6	0.1	0.001	100	PCB 12/13	0.1	0.0005	99.995
PCB 184	0.1	0.001	99.99	PCB 35	0.04	0.0005	100	PCB 78	0.1	0.0005	99.996
PCB 161	0.1	0.001	99.99	PCB 169	0.04	0.0004	100	PCB 152	0.1	0.0004	99.996
PCB 145	0.1	0.001	99.99	PCB 38	0.03	0.0004	100	PCB 79	0.1	0.0004	99.997
PCB 155	0.1	0.001	99.99	PCB 29	0.03	0.0003	100	PCB 35	0.1	0.0003	99.997
PCB 104	0.1	0.001	99.99	PCB 145	0.03	0.0003	100	PCB 36	0.1	0.0002	99.997
PCB 11	0.1	0.001	99.995	PCB 1	0	0	100	PCB 30	0.1	0.0002	99.998
PCB 79	0.1	0.001	99.996	PCB 2	0	0	100	PCB 145	0.05	0.0002	99.998
PCB 188	0.03	0.0004	99.997	PCB 3	0	0	100	PCB 186	0.05	0.0002	99.998
PCB 36	0.03	0.0004	100	PCB 14	0	0	100	PCB 169	0.05	0.0002	99.998
PCB 1	0	0	100	PCB 11	0	0	100	PCB 14	0.04	0.0002	99.998
PCB 2	0	0	100	PCB 12/13	0	0	100	PCB 204	0.04	0.0001	100
PCB 3	0	0	100	PCB 30	0	0	100	PCB 1	0	0	100
PCB 14	0	0	100	PCB 36	0	0	100	PCB 2	0	0	100
PCB 169	0	0	100	PCB 79	0	0	100	PCB 3	0	0	100
PCB 186	0	0	100	PCB 186	0	0	100	PCB 11	0	0	100
PCB 204	0	0	100	PCB 204	0	0	100	PCB 104	0	0	100

The concentrations presented in this table are means of the initial 11 avian egg samples taken from the Hudson River by the trustees in 2004, with analysis by GC-LR/MS.

Table 3. Calculations of the relative amounts for individual congeners added to the PCB mixture representing spotted sandpiper eggs from the Hudson River.

	Ratio of the components in an Aroclor mixture*	Major PCB component of combined peak	Congener concentration (ng/g)	Congener contribution (%)	Cummulative contribution (%)
TOTAL PCB			25579	100	
PCB 28			2548	10	10
PCB 66/80		66	2290	9	19
PCB 74/61		74	1679	7	25
PCB 118/106		118	1604	6	32
PCB 47/48/75	(1:0.65:0.05)		1538		
47	1	47	905	4	35
48	0.65	48	588	2	38
75	0.05	75	45	0.18	38
PCB 138/163/164	(1:0.23:0.1)		1410		
138	1	138	1060	4	42
163	0.23	163	244	1	43
164	0.1	164	106	0.41	43
PCB 90/101/89	(0:1:0.028)		1289		
90	0	90	NS		
101	1	101	1253	5	48
89	0.028	89	35	0.14	48
PCB 52/73		52	1250	5	53
PCB 49/43	(1:0.12)		1099		
49	1	49	981	4	57
43	0.12	43	118	0.46	57
PCB 153			1016	4	61
PCB 99			998	4	65
PCB 70/76		70	913	4	69
PCB 105/127		105	764	3	72
PCB 31			743	3	75
PCB 56/60	(1:1)		720		
56	1	56	360	1	76
60	1	60	360	1	78
PCB 41/71/64/68	(0.28:0.22:1:0)		645		
41	0.28	41	120	0.47	78
71	0.22	71	95	0.37	78
64	1	64	430	2	80
PCB 110			453	2	82
PCB 85/120		85	437	2	84
PCB 87/115/116	(1:0.1:0)		330		
87	1	87	300	1	85
115	0.1	115	30	0.12	85
116	0	116	NS		
PCB 128			250	1	86
PCB 149/139	(1:0.02)		217		
149	1	149	213	1	87
139	0.02	139	4	0.02	87

		Ratio of the components in an Aroclor mixture*	Major PCB component of combined peak	Congener concentration (ng/g)	Congener contribution (%)	Cummulative contribution (%)
PCB 92				213	1	88
PCB 180				180	1	88
PCB 158/160			158	178	1	89
PCB 146				165	1	90
PCB 97/86			97	159	1	90
PCB 156				155	1	91
PCB 95/93			95	155	1	91
PCB 187/182			187	141	1	92
PCB 170/190		(1:0.22)	170	132		
	170	1	190	108	0.42	92
	190	0.22		24	0.09	93
PCB 111/117			117	130	1	93
PCB 141				118	0.46	94
PCB 130				84	0.33	94
PCB 107/109			109	81	0.32	94
PCB 137				78	0.30	94
PCB 42/59		(1:0.3)		71		
	42	1	42	54	0.21	95
	59	0.3	59	16	0.06	95
PCB 114				57	0.22	95
PCB 167				49	0.19	95
PCB 123				39	0.15	95
PCB 157				33	0.13	95
PCB 77				30	0.12	96
PCB 81				17	0.07	96
PCB 126				5	0.02	96
PCB 189				4	0.02	96
PCB 169				0.05	0.0002	96

* The ratios of co-eluting congeners measured in an Aroclor 1:1:1:1 mixture of Aroclor 1242:1248:1254:1260. Congeners with relative concentrations <1% of the total for the co-eluting peaks were excluded. Congener concentrations for the co-eluting PCBs were based on the concentration of the entire co-eluting set of congeners in sandpiper eggs multiplied times the relative amount (%) of each individual congener in the set.

Table 4. Final selected PCB congeners, total mass and percentage of total PCB in the stock 50mL aliquant of the original PCB mixture solution.

PCB Congener	PCB congener mass (ug) in the 50 mL aliquant of the original stock solution received from AccuStandard.	Relative amount (%)
PCB 28	40880	10.46%
PCB 66	36660	9.38%
PCB 74	26840	6.87%
PCB 118	25530	6.53%
PCB 47	14420	3.69%
PCB 48	9405	2.41%
PCB 75	802	0.21%
PCB 138	17000	4.35%
PCB 163	3980	1.02%
PCB 164	1607	0.41%
PCB 101	19980	5.11%
PCB 89	600	0.15%
PCB 52	20100	5.14%
PCB 49	15820	4.05%
PCB 43	1816	0.46%
PCB 153	16215	4.15%
PCB 99	15935	4.08%
PCB 70	14515	3.72%
PCB 105	12280	3.14%
PCB 31	11800	3.02%
PCB 56	5780	1.48%
PCB 60	5760	1.47%
PCB 41	1998	0.51%
PCB 71	1614	0.41%
PCB 64	6780	1.74%
PCB 110	7220	1.85%
PCB 85	6940	1.78%
PCB 87	4818	1.23%
PCB 115	398.4	0.10%
PCB 128	4026	1.03%
PCB 149	3380	0.87%
PCB 139	68	0.02%
PCB 92	3413.5	0.87%
PCB 180	2802	0.72%
PCB 158	2822	0.72%
PCB 146	2583.5	0.66%

PCB Congener	PCB congener mass (ug) in the 50 mL aliquant of the original stock solution received from AccuStandard.	Relative amount (%)
PCB 97	2584	0.66%
PCB 156	2412	0.62%
PCB 95	2398	0.61%
PCB 187	2196.5	0.56%
PCB 170	1814	0.46%
PCB 190	400.8	0.10%
PCB 117	1992	0.51%
PCB 141	1788	0.46%
PCB 130	1390	0.36%
PCB 109	1206	0.31%
PCB 137	1198.5	0.31%
PCB 42	795	0.20%
PCB 59	197.2	0.05%
PCB 114	1008	0.26%
PCB 167	796	0.20%
PCB 123	599	0.15%
PCB 157	594	0.15%
PCB 77	400	0.10%
PCB 81	200.4	0.05%
PCB 126	84.35	0.02%
PCB 189	70.2	0.02%
PCB 169	0.72	0.0002%
Total	390713	100.0%

Table 5. Target and nominal concentrations of total PCB in each dosing solution.

Dose ID	Vial & Cap #	Solution Description	Target Concentration (µg/µL)[1]	Nominal Concentration (µg/µL)[2]
7	3-7	Stock 58-congener PCB mixture	246	244
6	3-8	2-fold dilution	123	122
5	3-9	4-fold dilution	62	61
4	3-10	8-fold dilution	31	30.5
3	3-11	16-fold dilution	15	15
2	3-12	32-fold dilution	8	7.5
1	3-4	Isooctane blank	0	0

[1] Target concentrations for the dosing solutions of the custom 58-congener PCB mixture.

[2] Nominal total PCB concentrations are based on the sum of the certified analyte concentrations from AccuStandard (Attachment 1), a volume of 50 mL of the 250-mL of original custom 58-congener PCB mixture, a stock PCB emulsion volume of 1.6 mL, and the appropriate serial dilution for each dose.

Table 6. Nominal individual PCB congener doses (ng/g egg) expected in the eggs of Japanese quail injected with each of the dosing solutions.

	Nominal egg dose (ng/g egg)					
PCB congener	98 µg PCB/g dose (vial 3-7)	49 µg PCB/g dose (vial 3-8)	24 µg PCB/g dose (vial 3-9)	12 µg PCB/g dose (vial 3-10)	6 µg PCB/g dose (vial 3-11)	3 µg PCB/g dose (vial 3-12)
PCB 28	10220	5110	2555	1278	639	319
PCB 66	9165	4583	2291	1146	573	286
PCB 74	6710	3355	1678	839	419	210
PCB 118	6383	3191	1596	798	399	199
PCB 47	3605	1803	901	451	225	113
PCB 48	2351	1176	588	294	147	73
PCB 75	201	100	50	25	13	6
PCB 138	4250	2125	1063	531	266	133
PCB 163	995	498	249	124	62	31
PCB 164	402	201	100	50	25	13
PCB 101	4995	2498	1249	624	312	156
PCB 89	150	75	38	19	9	5
PCB 52	5025	2513	1256	628	314	157
PCB 49	3955	1978	989	494	247	124
PCB 43	454	227	114	57	28	14
PCB 153	4054	2027	1013	507	253	127
PCB 99	3984	1992	996	498	249	124
PCB 70	3629	1814	907	454	227	113
PCB 105	3070	1535	768	384	192	96
PCB 31	2950	1475	738	369	184	92
PCB 56	1445	723	361	181	90	45
PCB 60	1440	720	360	180	90	45
PCB 41	500	250	125	62	31	16
PCB 71	404	202	101	50	25	13
PCB 64	1695	848	424	212	106	53
PCB 110	1805	903	451	226	113	56
PCB 85	1735	868	434	217	108	54
PCB 87	1205	602	301	151	75	38
PCB 115	100	50	25	12	6	3
PCB 128	1007	503	252	126	63	31
PCB 149	845	423	211	106	53	26
PCB 139	17	9	4	2	1	1
PCB 92	853	427	213	107	53	27
PCB 180	701	350	175	88	44	22
PCB 158	706	353	176	88	44	22
PCB 146	646	323	161	81	40	20
PCB 97	646	323	162	81	40	20
PCB 156	603	302	151	75	38	19
PCB 95	600	300	150	75	37	19
PCB 187	549	275	137	69	34	17
PCB 170	454	227	113	57	28	14
PCB 190	100	50	25	13	6	3
PCB 117	498	249	125	62	31	16
PCB 141	447	224	112	56	28	14

PCB congener	98 µg PCB/g dose (vial 3-7)	49 µg PCB/g dose (vial 3-8)	24 µg PCB/g dose (vial 3-9)	12 µg PCB/g dose (vial 3-10)	6 µg PCB/g dose (vial 3-11)	3 µg PCB/g dose (vial 3-12)
PCB 130	348	174	87	43	22	11
PCB 109	302	151	75	38	19	9
PCB 137	300	150	75	37	19	9
PCB 42	199	99	50	25	12	6
PCB 59	49	25	12	6	3	2
PCB 114	252	126	63	32	16	8
PCB 167	199	100	50	25	12	6
PCB 123	150	75	37	19	9	5
PCB 157	149	74	37	19	9	5
PCB 77	100	50	25	13	6	3
PCB 81	50	25	13	6	3	2
PCB 126	21	11	5	3	1	1
PCB 189	18	9	4	2	1	1
PCB 169	0.18	0.09	0.05	0.02	0.01	0.01
Total PCB	97678	48839	24420	12210	6105	3052

Estimation based on the following:
Highest Dose = 98 µg/g egg
Injection volume = 0.4 µL/g egg
Egg weight = 10 g

Table 7. Calculated doses and contributions to TEQs (pg/g egg) of individual dioxin-like PCB congeners for each dose solution of the 58-congener PCB mixture prepared for the Japanese quail egg injection studies.

			TEQ (pg/g)					
PCB congener	WHO TEF-value	Rel. TEQ (% total)	98 µg PCB/g egg dose	49 µg PCB/g egg dose	24 µg PCB/g egg dose	12 µg PCB/g egg dose	6 µg PCB/g egg dose	3 µg PCB/g egg dose
PCB 118	0.00001	0.5	64	32	16	8	4	2
PCB 105	0.0001	2.4	307	154	77	38	19	10
PCB 156	0.0001	0.5	60	30	15	8	4	2
PCB 114	0.0001	0.2	25	13	6	3	2	1
PCB 167	0.00001	0.0	2	1	0.50	0.25	0.12	0.06
PCB 123	0.00001	0.0	1	1	0.37	0.19	0.09	0.05
PCB 157	0.0001	0.1	15	7	4	2	1	0.46
PCB 77	0.05	39.7	5000	2500	1250	625	313	156
PCB 81	0.1	39.8	5010	2505	1253	626	313	157
PCB 126	0.1	16.7	2109	1054	527	264	132	66
PCB 189	0.00001	0.0	0.18	0.09	0.04	0.02	0.01	0.01
PCB 169	0.001	0.0	0.18	0.09	0.05	0.02	0.01	0.01
Total TEQ		100.0	12594	6297	3148	1574	787	394

Estimated based on a 4 µL/egg injection volume and 10 g/egg assumed egg weight. Relative TEQ (%) was calculated based on the contribution of each congener to the total TEQ.

Attachments

AccuStandard Inc.
125 Market Street
New Haven, CT 06513
USA

Ph: 203-786-5290
Fax: 203-786-5287
E-mail: usa@accustandard.com
www.accustandard.com

ISO 9001

CERTIFICATE OF ANALYSIS

CATALOG NO: S-13907-250ML
DESCRIPTION: Custom PCB Congener Standard
LOT: B5110052
SOLVENT: Isooctane

EXPIRATION: Nov 9,2015

See reverse for additional certification information.

This product is guaranteed accurate to + 0.5% of the Certified Analyte concen[illegible] Expiration Date on the Label.

Component	CAS #	Purity % (GC/MS)	Prepared Concentration[1] (µg/mL)		Certified Analyte Concentration[2] (µg/mL)
2,4,4'-Trichlorobiphenyl	7012-37-5	100	817.6	±32.70	817.6
2,3',4,4'-Tetrachlorobiphenyl	32598-10-0	100	733.2	±29.33	733.2
2,4,4',5-Tetrachlorobiphenyl	32690-93-0	100	536.8	±21.47	536.8
2,3',4,4',5-Pentachlorobiphenyl 118	31508-00-6	99.5	513.2	±20.53	510.6
2,2',4,4'-Tetrachlorobiphenyl	2437-79-8	100	288.4	±11.54	288.4
2,2',4,5-Tetrachlorobiphenyl 48	70362-47-9	99.9	188.3	±7.53	188.1
2,4,4',6-Tetrachlorobiphenyl	32598-12-2	99.5	16.12	±0.64	16.04
2,2',3,4,4',5'-Hexachlorobiphenyl 138	35065-28-2	100	340.0	±13.60	340.0
2,3,3',4',5,6-Hexachlorobiphenyl 163	74472-44-9	99.0	80.40	±3.22	79.60
2,3,3',4',5',6-Hexachlorobiphenyl 164	74472-45-0	99.7	32.24	±1.29	32.14
2,2',4,5,5'-Pentachlorobiphenyl 101	37680-73-2	99.4	402.0	±16.08	399.6
2,2',3,4,6'-Pentachlorobiphenyl 89	73555-57-2	99.9	12.01	±0.48	12.00
2,2',5,5'-Tetrachlorobiphenyl 52	35693-99-3	100	402.0	±16.08	402.0
2,2',4,5'-Tetrachlorobiphenyl 49	41464-40-8	100	316.4	±12.66	316.4
2,2',3,5-Tetrachlorobiphenyl 43	70362-46-8	99.9	36.36	1.45	36.32
2,2',4,4',5,5'-Hexachlorobiphenyl 153	35065-27-1	99.6	325.6	±13.02	324.3
2,2',4,4',5-Pentachlorobiphenyl 99	38380-01-7	99.6	320.0	±12.80	318.7
2,3',4',5-Tetrachlorobiphenyl 70	32598-11-1	99.0	293.2	±11.73	290.3
2,3,3',4,4'-Pentachlorobiphenyl 105	32598-14-4	100	245.6	±9.82	245.6
2,4',5-Trichlorobiphenyl 31	16606-02-3	100	236.0	±9.44	236.0
2,3,3',4'-Tetrachlorobiphenyl 56	41464-43-1	99.6	116.1	±4.64	115.6
2,3,4,4'-Tetrachlorobiphenyl 60	33025-41-1	99.0	116.4	±4.66	115.2
2,2',3,4-Tetrachlorobiphenyl 41	52663-59-9	W.3	40.24	±1.61	39.96
2,3',4',6-Tetrachlorobiphenyl 71	41464-46-4	100	32.28	±1.29	32.28
2,3,4',6-Tetrachlorobiphenyl 64	52663-58-8	99.0	137.0	a5.43	135.6
2,3,3',4',6-Pentachlorobiphenyl 110	38380-03-9	99.7	144 8	±5.79	144.4
2,2',3,4,4'-Pentachlorobiphenyl 85	65510-45-4	99.0	140.2	±5.61	138.8
2,2',3,4,5'-Pentachlorobiphenyl 87	38380-02-8	W.5	96.84	±3.87	96.36
2,3,4,4',6-Pentachlorobiphenyl	74472-38-1	99.5	8.008	±0.32	7.968
2,2',3,3',4,4'-Hexachlorobiphenyl 128	38380-07-3	99.7	80.76	±3.23	80.52
2,2',3,4',5',6-Hexachlorobiphenyl 149	38380-04-0	99.0	68.28	±2.73	67.60
2,2',3,4,4',6-Hexachlorobiphenyl 139	56030-56-9	99.4	1.368	±0.05	1.360
2,2',3,5,5'-Pentachlorobiphenyl 92	52663-61-3	99.7	68.48	±2.74	68.27
2,2',3,4,4',5,5'-Heptachlorobiphenyl 180	35065-29-3	100	56.04	±2.24	56.04
2,3,3',4,4',6-Hexachlorobiphenyl 158	74472-42-7	100	56.44	±2.26	56.44
2,2',3,4',5,5'-Hexachlorobiphenyl 146	51908-16-8	98.9	52.24	±2.09	51.67
2,2',3',4,5-Pentachlorobiphenyl 97	41464-51-1	99.0	52.20	±2.09	51.68

1. All weights are traceable through NIST, Test No 822/270236-04
2. Certified Analyte Concentration = Purity x Prepared Concentration. The Uncertainty calculated for this product is the Combined Uncertainty $u_c(y)$. It represents an estimated standard deviation equal to the positive square root of the total variance of the uncertainty of components. The Expanded Uncertainty is U which is $U_c(y)$ *K where K is the coverage factor at the 95% confidence level (K=2). Values reported above are Expanded Combined Uncertainty
3. A product with a suffix (-1A, -2B, etc.) on its lot# has had its expiration date extended and is identical to the same lot# without the suffix

Certified by: R. Cooper

This product was manufactured to meet the quality system requirements of ISO 9001

QR-ORGINOH002

CERTIFICATION REPORT

1. Intended Use: The product covered by this Certificate is designed for Calibration or for use in Quality Control procedures for the specified chemical compounds listed on the reverse side. This product can be used for Identification and/or Quantification. This product can also be used as a Reference Material to validate analytical procedures, subject to the conditions under Section 8.

2. Raw Materials: Reference Standards are prepared from the highest quality starting materials with defined purities. All analytes and solvents are obtained from pre-qualified vendors and then analyzed or evaluated prior to use according to ISO9001 requirements.

3. Manufacturing: AccuStandard, Inc, manufactures its products under an ISO 9001 certified quality system. Balances used in the manufacturing process are calibrated regularly. All weights are traceable through the National Institute of Standards and Technology (NIST), Test No. 8221254480.

4. **Homogeneity Assessment**: Homogeneity of the finished product is assessed by analyzing sample batches or by other methods consistent with the intended use of the product and by procedures that comply with the ISO 9001 Quality System.

5. **Stability Assessment**: AccuStandard, Inc. guarantees the stability of this solution through the expiration date stated on the label, when handled and stored according to the conditions stated on the label. To ensure a uniform solution, mix the contents of the sealed container thoroughly prior to use. Care should be taken not to contaminate the contents of the original container.

6. **Analytical Quality Control**: Products are tested by validated analytical methods covered under the company's ISO 9001 Quality System.

7. **Uncertainty Statistics and Confidence Limits**: The maximum Uncertainty stated on the face of this certificate has been calculated in accordance with the EURACHEM/CITAC Guide – Quantifying Uncertainty in Analytical Measurement - Second Edition, The Uncertainty given is the Expanded Combined Uncertainty and represents an estimated Standard Deviation equal to the positive square root of the total variance of the uncertainty of components. The Expanded Uncertainty is U which is $U_c(y)$ *K, where K is the coverage factor at the 95% confidence level (K=2). The Expanded Uncertainty is based on the combination of uncertainties associated with each individual operation involved in the preparation of the product.

8. **Legal Notice and Limit of Liability**: This product is for research use only. No warranty for any particular application is expressed or implied. Due to their hazardous nature, they should be handled by trained personnel. The company's liability will be limited to replacement of product or refund of purchase price. Notice of claims must be made within thirty (30) days from date of delivery.

AccuStandard Inc.
125 Market Street
New Haven, CT 06513
USA

Ph: 203-786-5290
Fax: 203-786-5287
E-mail: usa@accustandard.com
www.accustandard.com

CERTIFICATE OF ANALYSIS

CATALOG NO: S-13907-250ML

EXPIRATION: Nov 9, 2015

DESCRIPTION: Custom PCB Congener Standard

LOT: B5110052 **See reverse for additional certification information.**

SOLVENT: Isooctane

This product is guaranteed accurate to + 0.5% of the Certified Analyte concentration through the Expiration Date on the Label.

Component	CAS #	Purity % (GC/MS)	Prepared Concentration[1] (μg/mL)		Certified Analyte Concentration[2] (μg/mL)
2,3,3',4,4',5-Hexachlorobiphenyl 156	38380-08-4	100	48.24	±1.93	48.24
2,2',3,5',6-Pentachlorobiphenyl 95	38379-99-6	99.1	48.40	±1.94	47.96
2,2',3,4',5,5',6-Heptachlorobiphenyl	52663-68-0	99 4	44.20	±1.77	43.93
2,2',3,3',4,4',5-Heptachlorobiphenyl 170	35065-30-6	100	36.28	±1.45	36.28
2,3,3',4,4',5,6-Heptachlorobiphenyl 190	41411-64-7	1 0	8.016	±0.32	8.016
2,3,4',5,6-Pentachlorobiphenyl [illegible]	68194-11-6	99.0	40.24	±1.61	39.84
2,2',3,4,5,5'-Hexachlorobiphenyl 141	52712-04-6	99.0	36.12	±1.44	35.76
2,2',3,3',4,5'-Hexachlorobiphenyl 130	52663-66-8	99.3	28.00	±1.12	27.80
2,3,3',4,6-Pentachlorobiphenyl	74472-35-8	100	24.12	±0.96	24.12
2,2',3,4,4',5-Hexachlorobiphenyl 137	35694-06-5	99.7	24.04	±0.96	23.97
2,2',3,4'-Tetrachlorobiphenyl 42	36559-22-5	99.4	16.00	±0.64	15.90
2,3,3',6-Tetrachlorobiphenyl 59	74472-33-6	98.5	4.004	±0.16	3.944
2,3,4,4',5-Pentachlorobiphenyl 114	74472-37-0	100	20.16	±0.81	20.16
2,3',4,4',5,5'-Hexachlorobiphenyl 167	52663-72-6	99.0	16.08	±0 64	15.92
2',3,4,4',5-Pentachlorobiphenyl 123	65510-44-3	[illegible]	12.00	±0.48	11 98
2,3,3',4,4',5'-Hexachlorobiphenyl 157	69782-90-7	[illegible]	12.00	±0.48	11.88
3,3',4,4'-Tetrachlorobiphenyl 77	32598-13-3	100	8.000	±0.32	8.000
3,4,4',5-Tetrachlorobiphenyl 81	70362-50-4	100	4.008	±0.16	4.008
3,3',4,4',5-Pentachlorobiphenyl 1	57465-28-8	100	1.687	±0.07	1.687
2,3,3',4,4',5,5'-Heptachlorobiphenyl 189	39635-31-9	100	1.404	±0.06	1.404
3,3',4,4',5,5'-Hexachlorobiphenyl 169	32774-16-6	100	0.0144	0.00	.0144

58 Components

1. All weights are traceable through NIST, Test No 822/270236-04
2. Certified Analyte Concentration = Purity x Prepared Concentration. The Uncertainty calculated for this product is the Combined Uncertainty $u_c(y)$ It represents an estimated standard deviation equal to the positive square root of the total variance of the uncertainty of components. The Expanded Uncertainty is U which is $U_c(y)$ *K where K is the coverage factor at the 95% confidence level (K=2). Values reported above are Expanded Combined Uncertainty
3. A product with a suffix (-1A 2B, etc.) on its lot# has had its expiration date extended and is identical to the same lot# without the suffix.

Certified by: R. Cooper

This product was manufactured to meet the quality system requirements of ISO 9001

CERTIFICATION REPORT

1. **Intended Use**: The product covered by this Certificate is designed for Calibration or for use in Quality Control procedures for the specified chemical compounds listed on the reverse side. This product can be used for Identification and/or Quantification. This product can also be used as a Reference Material to validate analytical procedures, subject to the conditions under Section 8.

2. **Raw Materials**: Reference standards are prepared from the highest quality starting materials with defined purities. All analytes and solvents are obtained from pre-qualified vendors and then analyzed or evaluated prior to use according to IS09001 requirements.

3. **Manufacturing**: AccuStandard, Inc. manufactures its products under an ISO 9001 certified quality system. Balances used in the manufacturing process are calibrated regularly. All weights are traceable through the National Institute of Standards and Technology (NIST), Test No. 822/254480.

4. **Homogeneity Assessment**: Homogeneity of the finished product is assessed by analyzing sample batches or by other methods consistent with the intended use of the product and by procedures that comply with the ISO 9001 Quality System.

5. **Stability Assessment**: AccuStandard, Inc. guarantees the stability of this solution through the expiration date stated on the label, when handled and stored according to the conditions stated on the label. To ensure a uniform solution, mix the contents of the sealed container thoroughly prior to use. Care should be Wen not to contaminate the contents of the original container.

6. **Analytical Quality Control**: Products are tested by validated analytical methods covered under the company's ISO 9001 Quality System.

7. **Uncertainty Statistics and Confidence Limits**: The maximum Uncertainty stated on the face of this certificate has been calculated in accordance with the EURACHEM/CITAC Guide – Quantifying Uncertainty in Analytical Measurement - Second Edition. The Uncertainty given is the Expanded Combined Uncertainty and represents an estimated Standard Deviation equal to the positive square root of the total variance of the uncertainty of components. The Expanded Uncertainty is U which is $U_c(y)$ *K, where K is the coverage factor at fhe 95% confidence level (K=2). The Expanded Uncertainty is based on the combination of uncertainties associated with each individual operation involved in the preparation of the product.

8. **Legal Notice and Limit of Liability**: This product is for research use only. No warranty for any particular application is expressed or implied. Due to their hazardous nature, they should be handled by trained personnel. The company's liability will be limited to replacement of product or refund of purchase price. Notice of claims must be made within thirty (30) days from date of delivery.

AccuStandard®, Inc.
Chemical Reference Standards • The Standard for Excellence

WARRANTIES:

Manufacture]: (AccuStandard®, Inc.) warrants that its products shall conform to the description of such products as provided in its catalog or on the specific products' label. This warranty is exclusive, and AccuStandard, Inc. makes no other Warranty, express or implied, including any implied warranty of merchantability or fitness for any particular purpose.

PRODUCT STABILITY:

AccuStandard's products are monitored regularly to ensure they meet Catalog Specifications (on-going stability studies). The integrity of these products is dependent upon proper handling and storage by the end-user.

AccuStandard recommends the following storage conditions:

Volatiles	-10 to –20 °C
Semi-Volatiles	4 °C

Exceptions: Highly concentrated soludons (e.g. Z-014J) should be stored at room temperature.

Note: Allow ampules to equilibrate to 20 °C prior to opening.

LIABILITY:

AccuStandard, Inc. products are for research use only. Due to their hazardous nature, they should be handled by trained personnel. AccuStandard's liability will be limited to replacement of products or refund of purchase price. Failure to give notice of claim within thirty (30) days from *date* of delivery will constitute a waiver by buyer of any and all claims.

125 Market Street
Mew Haven CT 06513 • USA
Website: www.accustandard.com

Tel (800) 442-5290 • (203) 786-5290
Fax (877) 786-5287 • (203) 786-5287
Email: usa@accustandard.com

AccuStandard Inc.
125 Market Street
New Haven, CT 06513
USA

CERTIFICATE OF ANALYSIS

Ph: 203-786-5290
Fax: 203-786-5287
E-mail: usa@accustandard.com
www.accustandard.com

CATALOG NO. S-13907-BLK-250ML

EXPIRATION: Nov 9, 2006

DESCRIPTION: Isooctane Control Blank

LOT: B5110053

SOLVENT: N/A

This product is guaranteed accurate to + 0.5% of the Certified Analyte concentration through the Expiration Date on the Label.

See reverse for additional certification information.

Component	CAS #	Purity % MFG	Prepared Concentration[1]	Certified Analyte Concentration[2]
Isooctane	540-84-1	99.9	N/A ±0	N/A

Please note: AccuStandard follows the U.S. conventions in reporting numerical values, on both certificates and labels.

A comma (,) is used to separate units of one-thousand or greater.
A period (.) is used as a decimal place marker.

1. All weights are traceable through National Institute of Standards & Technology, Test No.
2. Certified Analyte Concentration = Purity x Prepared Concentration
3. A product with a suffix (-1A, -2B, etc.) on its lot# has had its expiration date extended and is identical to the same lot# without the suffix.

Certified by: R. Cooper

This product was manufactured to meet the quality system requirements of ISO 9001

QR-ORG/INO-001
Rev. 11/02

CERTIFICATION REPORT

1. **Intended Use:** The product covered by this Certificate is designed for Calibration or for use in Quality Control procedures for the specified chemical compounds listed on the reverse side. This product can be used for Identification and/or Quantification. This product can also be used as a Reference Material to validate analytical procedures, subject to the conditions under Section 8.

2. **Raw Materials:** Reference Standards are prepared from the highest quality starting materials with defined purities. All analytes and solvents are obtained from pre-qualified vendors and then analyzed or evaluated prior to use according to IS09001 requirements.

3. **Manufacturing:** AccuStandard, Inc. manufactures its products under an ISO 9001 certified quality system. Balances used in the manufacturing process are calibrated regularly. All weights are traceable through the National Institute of Standards and Technology (NIST), Test No, 822/254480.

4. **Homogeneity Assessment:** Homogeneity of the finished product is assessed by analyzing sample batches or by other methods consistent with the intended use of the product and by procedures that comply with the ISO 9001 Quality System.

5. Stability **Assessment:** AccuStandard, Inc. guarantees the stability of this solution through the expiration date stated on the label, when handled and stored according to the conditions stated on the label. To ensure a uniform solution, mix the contents of the sealed container thoroughly prior to use. Care should be taken not to contaminate the contents of the original container.

6. **Analytical Quality Control:** Products are tested by validated analytical methods covered under the company's ISO 9001 Quality System.

7. **Uncertainty Statistics and Confidence Limits:** The maximum Uncertainty stated on the face of this certificate has been calculated in accordance with the EURACHEM/CITAC Guide –Quantifying Uncertainty in Analytical Measurement - Second Edition. The Uncertainty given is the Expanded Combined Uncertainty and represents an estimated Standard Deviation equal to the positive square root of the total variance of the uncertainty of components. The Expanded Uncertainty is U which is $U_c(y)$ *K, where K Is the coverage factor at the 95% confidence level (K=2). The Expanded Uncertainty is based on the combination of uncertainties associated with each individual operation involved in the preparation of the product.

8. **Legal Notice and Limit of Liability:** This product is for research use only. No warranty for any particular application Is expressed or implied. Due to their hazardous nature, they should be handled by trained personnel. The company's liability will be limited to replacement of product or refund of purchase price. Notice of claims must be made within thirty (30) days from date of delivery.

U.S. DEPARTMENT OF THE INTERIOR
MARCH 3, 1849

NATIONAL OCEANIC AND ATMOSPHERIC ADMINISTRATION
NOAA
U.S. DEPARTMENT OF COMMERCE

DEPARTMENT OF ENVIRONMENTAL CONSERVATION
NEW YORK STATE