UNIVERSITÉ DU QUÉBEC À MONTRÉAL

A SUB-ACUTE STUDY TO EVALUATE THE USE OF ACUTE PHASE PROTEINS IN MALLARD DUCKS (*Anas platyrhynchos*), AS INDICATORS OF LONG-TERM HEALTH AFTER INGESTION OF CRUDE OIL

THESIS

PRESENTED

AS PARTIAL REQUIREMENT

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LYNN MILLER

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ÉTUDE SOUS-AIGÜE POUR ÉVALUER L'UTILISATION DES PROTÉINES DE PHASE AIGUË CHEZ LES CANARDS COLVERTS (*Anas platyrhynchos*), COMME INDICATEURS DE LA SANTÉ À LONG TERME APRÈS L'INGESTION DE PÉTROLE BRUT

THÈSE

PRÉSENTÉE

COMME EXIGENCE PARTIELLE

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PAR

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AVANT-PROPOS

Les déversements d'hydrocarbures sont dévastateurs pour l'environnement à plusieurs niveaux. Plusieurs facteurs entrent en ligne de compte, particulièrement concernant la faune. L'effort de réhabilitation nécessite une expertise, des installations et de nombreuses personnes qui souhaitent gérer tous les aspects de la réponse. Tous les oiseaux qui ont été capturés ont été évalués et admis au processus de réadaptation. Le problème s'est posé lorsque ces animaux sont retournés à l'état sauvage. Sharpe (1996) a attiré l'attention sur l'apparence du faible taux de survie qui suit la réhabilitation de plusieurs espèces d'oiseaux. Si, en tant que rééducateurs, nous voulons vraiment faire le travail qui nous intéresse, la simple survie n'est pas la réponse à tout ce processus. La faune que nous nettoyons et que nous réhabilitons doit être en mesure de retourner à sa vie et de pouvoir vivre comme des animaux sauvages, ce qui incluse le fait de donner naissance à une progéniture en santé. Ce projet de recherche est axé sur le développement d'un outil de triage additionnel qui sera utile au cours de la phase précoce lors d'un déversement de pétrole. Il faut d'abord explorer l'évolution de la réponse des animaux en phase aigüe à travers les profils protéiques, et lier ensuite ce processus biochimique au résultat à long terme. C'est avec un certain soulagement teinté de tristesse que je peux affirmer que cela a été un projet de recherche qui a très bien réussi. L'électrophorèse des protéines peut être utilisée comme outil pour guider l'effort de redressement. Il reste évidemment beaucoup de travail à faire. Nous avons besoin des électrophérogrammes de base et de la quantification des groupes de protéines trouvées dans ma technique pour de nombreux oiseaux aquatiques généralement touchés. Donc les oiseaux admis à un nettoyage lors des opérations de déversement, pourront bénéficier de mes recherches. Cette prestation peut être l'euthanasie, mais il y aura une approche beaucoup plus humanitaire pour venir en aide aux oiseaux sur le plan toxicologique.

L'entreprise de cette recherche prend de multiples facettes, avec en premier lieu, l'installation et les oiseaux. J'ai eu la chance de pouvoir faire ce travail à la station Delta Waterfowl Research, Delta Beach, au Manitoba. J'ai fait l'aller-retour pendant quatre ans, passant mes étés (et un hiver) auprès du troupeau de canards à mener mes recherches. J'ai travaillé tous les aspects du projet à partir de la prise en charge du troupeau de canards Delta, à la gestion d'une installation spécialisée, la supervision des techniciens d'été, et toutes les activités quotidiennes liées à ma recherche. Je n'aurais pas pu réaliser ce travail sans l'équipe de la station de la sauvagine de recherche Delta. L'installation est de classe mondiale et les connaissances que j'ai acquises auprès des spécialistes travaillant à la station sont immenses.

Pour m'avoir aidée à explorer l'électrophorèse capillaire des protéines capillaires comme offrant le meilleur outil potentiel pour ce projet, je suis reconnaissante de l'appui technique que j'ai reçu de la part de Sylvie Lemieux de l'UQAM. J'ai pu profiter de cette base pour le laboratoire du Dr Cameron Skinner à l'Université Concordia, de raffiner et de l'utiliser pour explorer les protéines exprimées par mes canards tout au long de la période de recherche dans son ensemble. Dr Skinner et son équipe m'ont apporté une aide précieuse et inestimable.

J'ai pu envoyer des échantillons au Dr Grégory Bédécarrats au Département des sciences animales et avicoles de l'Université de Guelph en Ontario. Il a gracieusement réalisé en utilisant sa radio-immuno essai pour arriver à confirmer qu'il y a un changement sous-jacent dans le cycle de prolactine des colverts femelles qui reçoivent une dose d'hydrocarbure élevée.

Un grand nombre de personnes ont manifesté leur soutien et leur confiance dans mon travail. Je tiens à remercier tout particulièrement mon mari, Michel Béland. Il m'a

soutenue dans tous les aspects de cette recherche; finances, expertise informatique, soutien moral, et il a été à mes côtés lors des hauts et des bas qu'engendre la recherche - je vous remercie. Mes superviseurs, qui n'ont jamais montré de découragement, ont été à mes côtés pendant toutes ces années à m'aider à donner vie à ma passion - je vous remercie, Phil et Diana. J'ai également reçu le soutien financier de la Fondation Delta Sauvagine, de la Société québécoise pour la protection des oiseaux, le Oiled Wildlife Care Network en Californie, ainsi que le Dr Philip Spear. Merci.

Plusieurs personnes ont manifesté un intérêt particulier et m'ont grandement aidé pendant toutes ces années: ma fille, Danielle Miller Béland, M. Phil Kline (décédé), Michel Boisvert, Shirley Desrosiers et le Dr Charlie Blummer, pour n'en nommer que quelques-uns. Et je tiens à souligner la précieuse contribution Patricia Hennesey, qui a repris ma thèse pour m'aider à l'améliorer, ce que je ne n'aurais pu accomplir sans son soutien et son dévouement.

FOREWORD

Oil spills are devastating to the environment at many levels. There are many factors that are involved in an oil spill response, especially when wildlife is involved. The rehabilitation effort requires expertise, facilities and many willing people to manage all the aspects of the response. All captured birds are evaluated and admitted to the rehabilitation process. The problem arises once these animals are returned to the wild. Sharpe (1996) focused attention on the apparent poor post rehabilitation survival in many bird species. If we as rehabilitators are truly doing the job we care about, for birds to simply survive is not the answer to this whole process. The wildlife that we clean and rehabilitate must be able to return to their lives and continue as truly wild animals, which includes producing normal healthy off-spring. This research project focused on developing a new triage tool to aid in the early phase of the oil spill response. First, by exploring the evolution of the animal's acute phase response through the protein profiles, and then by linking this biochemical process to the long term outcome. It is with relief tinged with sadness that I can say this has been a very successful research project. Protein electrophoresis can be used as a tool to guide the recovery effort. Of course there is still work to do; we need the baseline electropherograms and the quantification of the protein groups found using my technique for many of the commonly impacted waterfowl. The birds then admitted to a spill 'clean-up' operation can benefit from my research. That benefit may be euthanasia, but it will be a far more humane approach to toxicologically damaged birds.

To undertake this research required many facets. First was the facility and birds. I was very fortunate to be able to do this work at Delta Waterfowl Research Station, Delta Beach, Manitoba. For four years, I commuted, spending summers (and one

winter) there, conducting my research with the Delta Waterfowl Foundation duck flock. I worked all aspects of the project from the care taking of the flock, to managing a dedicated facility, supervising the summer technicians, and all daily activities related to my research. I could not have done this work without the Delta Waterfowl Research station team. The facility is world class and the knowledge I gained by being able to turn to the people at the station is immeasurable.

To explore whether protein capillary electrophoresis can offer the best potential tool for this project, I am grateful to the technical support I gained at UQAM from Ms Sylvie Lemieux. I was able to take this base knowledge to Dr Cameron Skinner's lab at Concordia University, refine and use it to explore the proteins expressed by my ducks throughout the whole research period. Dr Skinner and his team's help were invaluable.

I was able to send specimens to Dr Grégory Bédécarrats, Animal and Poultry Science Department, University of Guelph, Ontario. He graciously performed prolactin radio-immuno assays to help confirm that there is an underlying shift in the cycle of prolactin in the high oil dose female mallards.

There are so many people who have shown their support and faith in my work. I want to especially thank my husband, Michel Béland. He has supported me in all aspects of this research; financially, computer expertise, moral support, and has been there through the many ups and downs that research engenders – thank you. My supervisors, never despaired openly, were there for me through these years of trying to bring my passion to life – thank you Phil and Diana. I have also had financial support from Delta Waterfowl Foundation, the province of Québec's Society for the Protection of Birds, Oiled Wildlife Care Network in California and Dr Philip Spear. Thank you.

There are also so many people who have taken special interest and have helped enormously through these years; my daughter Danielle Miller Béland, Mr. Phil Kline (deceased), Michel Boisvert, Shirley Desrosiers and Dr. Charlie Blummer to name a few. Finally, Patricia Hennesey has taken my thesis and worked with me to improve it tremendously; I could not have done it without her dedicated help.

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RÉSUMÉ

Les techniques actuelles utilisées lors de la réhabilitation d'oiseaux mazoutés capturés lors d'un déversement de pétrole donnent des résultats avec un haut taux de réussite. Cependant, des études sur la survie des oiseaux après la réhabilitation suivant un déversement de pétrole, ont mis en doute leur taux de survie après leur remise en liberté (Sharpe, 1996; Goldsworthy et al, 2000; Newman et al, 2003.). L'électrophérogramme de protéinés est une méthode couramment utilisée pour aider à évaluer de nombreuses fonctions physiologiques au sein de la médecine humaine et vétérinaire. Au moyen de l'utilisation de l'électrophorèse capillaire dans le développement de l'électrophérogramme, une technique qui nécessite une taille d'échantillon très faible tout en donnant un grand nombre d'informations, des profils ont été développés pour des canards colverts (*Anas platyrynchos*). Des canards colverts se sont fait administrer par voie orale des faibles, moyens ou élevés volumes de pétrole brut léger Brent en volumes similaires à ceux des déversements d'hydrocarbures, sous la présence d'un groupe de contrôle positif. Les traitements ont eu lieu en juillet et les oiseaux ont été suivis pendant un an. Des échantillons de sang ont été recueillis au cours de l'année englobant le cycle de reproduction annuelle, et des électrophérogrammes de protéines ont été développés. Cela a permis l'identification à la fois positive (augmentation) et négative (diminution) des protéines liées à la réponse du corps à un stress de la phase aigüe (immédiatement après l'évènement) à la phase chronique. La quantification relative de ces pics de protéines et leurs réponses ont lié des protéines individuelles avec d'importants résultats biologiques pour les groupes exposés au pétrole. Les profils des protéines liées à ce résultat ont également été réparties par sexe. D'autres résultats ont montrés les tendances vers une liaison paire réduite, la baisse de fertilité, l'augmentation de la production d'œufs et des niveaux accrus d'agitation. La prolactine, une hormone quantifiée à plusieurs reprises au cours de la recherche, a augmenté de façon significative chez les femelles recevant la dose de l'huile élevée lors de la phase post reproductive en phase avec tous les autres oiseaux étudiés. L'utilisation de profils protéiques et la quantification des protéines individuelles offrent de précieux renseignements sur l'issue possible des oiseaux affectés par le pétrole par rapport aux oiseaux normaux et en santé. Cette étude a abouti à l'élaboration d'un autre outil de triage afin d'aider à l'évaluation du stress toxique à l'ingestion de l'huile.

Mots clés: paire liaison, électrophorèse capillaire, prolactine, déversement de pétrole, protéines de phase aigüe

ABSTRACT

Current techniques used during the rehabilitation of oiled birds captured during an oil spill have a high degree of success. However, studies exploring the survival of birds after oil spill rehabilitation have questioned their post-release survival. The protein electropherogram is a commonly used method to aid in evaluating many physiological functions within human and veterinary medicine. Using capillary electrophoresis to develop the electropherogram, a technique which requires a very small sample size while giving a great deal of information, protein profiles were developed for mallards (*Anas platyrynchos*). Mallards were dosed orally with either low, medium and high volumes of light crude Brent oil in volumes similar to oil spill exposures, and compared to a negative control group. Oil dosing was conducted in July and the birds were followed for one year. Blood samples were collected over the year encompassing the annual breeding cycle, and electropherograms were evaluated. This allowed for the identification of both positive (increased) and negative (decreased) protein responses during the acute phase (immediately following the event) to the chronic phase. Partial quantification (i.e. percentages) of these protein peaks; transthyretin, albumin, alpha 1, alpha 2, beta globulins and gamma globulins, along with two unidentified peaks, and their response served to link individual proteins with significant biological outcomes for the oil dose groups. The proteins linked to this outcome were also gender specific. Other results included trends toward reduced pair bonding, lowered fertility, increased egg production, and increased agitation levels. The hormone prolactin was quantified at several times points over the course of the research and found to be significantly increased in the high oil dose females in the post reproductive phase as distinct from all other birds in the research. The use of protein profiles and the quantification of individual proteins offer valuable insight into the potential outcome for oil impacted birds when compared to normal birds. This study has contributed essential preliminary information leading towards the development of a new triage tool to aid in the evaluation of toxic stress from the ingestion of oil.

Key words: Capillary electrophoresis, pair bonding, prolactin, oil spill, acute phase proteins

INTRODUCTION

The current approach to handling captured oil-exposed wild birds involves a multi-tiered rehabilitation process that is physiologically stressful for the birds. Once captured, birds are evaluated to decide if they are suitable candidates for rehabilitation. Those that are suitable are stabilized with fluids to restore them to normal hydration, and then fed. When stable, those that are oiled or have lost waterproofing are washed following the established criteria for water temperature, water hardness, non-toxic compounds such as methyloleate or canola oil can be used as a pre-treatment when needed to soften tarry petroleum products, and Dawn® dish detergent (Berg, 2003). This results in birds with cleaned feathers and restored water proofing. The next step in the rehabilitation process is to house the birds from days to weeks, to ensure that they are clean, healthy, and fully waterproofed. The return of birds to the wild is then dependent on the release site, which must either have recovered from the oil spill, or be a suitable distance from it to ensure the birds do not return and become re-oiled.

Survival data from historical and recent spills are sketchy; however, what data do exist indicate that these birds have a limited survival period after handling (Sharpe, 1996; Environment Canada, 1996; Schmidt, 1997; Newman *et al.*, 2003) even though the rehabilitation of oiled birds appears to be useful in restoring some birds to releasable condition. For example, Tri-State Bird Rescue, Delaware, U.S.A. reports a release rate of between 45 and 90% depending on the species and weather conditions (personal communication, L. Frink). Given the high cost of handling oiled birds, the limited

technical and human resources, and the fact that many birds are severely affected before undergoing the stressful cleaning procedure, the currently applied rehabilitation strategy merits improvement. If we could initially measure a biological parameter or parameters, which would predict long term health, then it would be possible to better choose the subsequent course of treatment, including euthanasia when appropriate.

The main objective of the present investigation was therefore to develop a technique that is practical and inexpensive to add to the evaluation criteria of the birds admitted during an oil spill response. Acute phase proteins were evaluated as possible predictors of long-term health status in birds. Acute phase proteins are well documented and characterized in the human and agriculturally-oriented veterinary literature. The induction of acute phase proteins has a known time line that can be followed from days to weeks. In the field, where sample collection and storage may present difficulties in maintaining appropriate storage temperatures, these proteins will remain stable even through temperature fluctuations. Since protein electrophoresis and analysis of many acute phase proteins are routine tests in hospital and veterinary laboratories, the oil spill response teams could rapidly arrange with a local hospital or veterinary laboratory to quickly run the assays.

The test species chosen for this research was the mallard (*Anas platyrhyncos*), whose natural habitat frequently occurs where oil spills are most likely to be found. In North America, mallards are the most abundant duck species and are very tolerant of human activity (Drilling *et al.,* 2002). Due to the mallard's tolerance of human handling, stress-

induced infections such as aspergillosis are less likely to develop (Tseng, 1999; Carrasco, 2001).

Both behaviour and reproduction were examined because they are essential to the survival of the rehabilitated individuals and populations. Additionally these parameters are measureable and quantifiable with references to normality in the literature (Caldwell and Cornell, 1975).

CHAPITRE I

LITERATURE REVIEW

Oil, the basis for much of our economic and social development over the past century, has been a natural part of the environment since the start of its formation during the Carboniferous period. It has now become an integral part of our modern world. However, by its very nature we have difficulty in containing it, and limiting its impact once free of its containment.

1.1. Historical background about oil spills

As shipping evolved in the late 19th century from sail and steam to petrochemical-powered vessels, spills and oily waste became a common sight around harbors and coastlines. Oil was often deliberately spilled 'to calm the waters' and indeed the 30' vessel Detroit carried oil for this purpose on the first crossing of the Atlantic Ocean by a petrochemical driven boat during the summer of 1912, as reported in the newspaper the Detroit News of June 26th, 1912. Little attention was given to the environmental impact, especially as these spills were generally small and the recognition of the damaging environmental effects of oil spills was yet to be quantified.

1.1.1. The first oil tanker spill

The crude oil from the wreck of the Torrey Canyon led to the first massive public response to oil spills and their effect on seabirds and the environment. At 8:50 a.m. on the 18th of February, 1967 the Torrey Canyon grounded on the Seven Stones Reef, off the western coast of Cornwall, England, ultimately spilling her whole cargo of 117,000 tons of Kuwaiti crude (Baker, 1976). Appeals for funding and help appeared in many public sources (British Birds, 1967). Many books and publications explored and analyzed the

disaster (Cowan, 1968; Petrow, 1968). Estimates of the number of birds killed by this spill begin at 30,000 (Bourne, 1968). However, evidence from mark and recapture experiments, using dead birds released at the spill site and recovered on beaches, place this figure closer to 100,000 birds. Of the nearly 8,000 birds brought in alive, only 100 were still alive 6 weeks later (Petrow, 1968).

1.1.2. The media response to the plight of oiled birds

Since then, the media has captured the public's attention through the many images depicting the plight of oiled birds. Often, the first view the public has of an oil spill event is the photo on the front page of the newspaper, the television report or the in-depth report in magazines such as the National Geographic (January, 1990) (Figure 1.1).

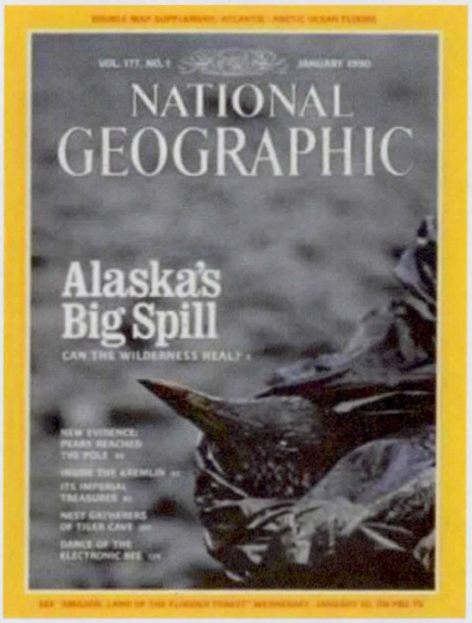


Figure 1.1 The front page of the National Geographic for January, 1990

1.1.3. Oil responses - the Canadian situation

The Canadian Wildlife Service (CWS), as lead agency in the response to the environmental impact during an oil spill, also promotes awareness through its Hinterland Who's Who pamphlet 'Oil pollution and birds' (1992). This has been followed up further with a policy in 2000, detailing the role that CWS will play in the event of oiling of birds, especially those species at risk (Environment Canada, 2000). The three main areas of response are;

1. Knowing and providing information on the migratory bird resource and species at risk (under CWS jurisdiction) in the area of a spill (this includes damage assessment and restoration planning after the event).
2. Minimizing the damage to birds by deterring unoiled birds from becoming oiled.
3. Ensuring the humane treatment of captured migratory birds and species at risk by determining the appropriate response and treatment strategies, which may include euthanasia, or cleaning and rehabilitation.

Thus, the need to mount an oil spill response is driven through public pressure, increased knowledge of appropriate techniques and legislative policy. However, further investigation needs to be undertaken to ensure a thorough understanding of the impact of an oil spill on the avifauna and given the current state of knowledge, what is the most valid and practical response for this group of animals.

1.2. Oil impacts on birds and the rehabilitation effort

1.2.1. Background

Oil impacts birds at many levels. Initially, plumage is fouled through contact with a slick or spill. In the early evolution of the event, the bird will try to clean and restore its water proofing. As the bird tries to preen the oil from its feathers it unfortunately spreads the oil over a greater percentage of its plumes and further jeopardizes its thermoregulatory ability (Jenssen and Ekker, 1991). During this period it is also inhaling any volatile components from the oil. During preening it ingests biologically significant quantities of the oil (Hartung, 1964). Experimentally, it has been shown that a bird can preen 50% of the oil on its feathers in a five to six day period (Hartung, 1963), although much of this activity often ceases after three to four days as the bird begins to become dehydrated, emaciated and obviously ill (personal communication L. Frink, Tri-State Bird Rescue). Leighton (1994) produced a report which touched on many aspects of the impact that oil has on the avian body. In summary Leighton's report looks at the following factors: external contamination leading to loss of water-repellency, insulation and flight, embryo toxicity, systemic toxicity including depressed growth and emaciation, adrenal gland enlargement, perturbations in thyroid hormones and corticosterone, liver damage, osmoregulatory impacts, decreased absorption of nutrients, reproductive losses, immune suppression and anaemia. This report is further supported by results of other researchers (Table 1.1).

Table 1.1 A summary of pathological findings reported in oiled birds submitted for pathological examination.

Oil type	Species	# of birds	Pathology	Reference
crude	common murres	13	weight loss/emaciation	Khan and Ryan, 1991
	thick-billed murres	2	necrosis in liver and duodenum, renal tubular degeneration	
	herring gulls black guillemots Leach's petrels	?	increased thyroxine, corticosterone and ACTH[a]	Peakall *et al.*, 1981
	herring gulls	24	weight loss	Peakall *et al.*, 1985
	herring gulls	24	hemolytic anaemia	Leighton *et al.*, 1985
	herring gulls	72	decreased growth hemolytic anaemia raised Cytochrome P-450	Lee *et al.*, 1985
mixed origin	mixed species	241	emaciation and haemorrhagic gastro-enteropathy	Jauniaux *et al.*, 1997
heavy	rhinoceros auklets	44	weight loss	Oka and Okuyama, 2000

a. Adrenocorticotropic hormone

Historically, oiled birds were often left to die or were killed, and the earliest efforts in oiled bird rehabilitation from records dating back to 1942 were dismal failures (Berkner, 1979; Newman *et al.*, 2003). Many techniques have been tried, from the use of solvents and cleaning agents (Odham, 1971; Newman *et al.*, 2003), to iron powder (Orbell *et al.*, 1999), and specially designed automated bird washing machines (Mazet *et al.*, 2002).

1.2.2. Development of a professional wildlife rehabilitation response to oil spills

With the establishment of major rehabilitation centers (International Bird Rescue Research Center, Long Beach, California, established in 1971 and Tri-State Bird Rescue and Research, Inc, Newark, Delaware, established in 1976) specializing in oil spill responses, the use of a multidisciplinary approach has improved basic bird care. Established guides (Tri-State guide, 1990; Beaulieu and Fitzgerald, 1996; Daigle and Darveau, 1995; Berg, 2003; Mazet, 2003) now include protocols for;

- capture and handling to minimize further stress or injury,
- euthanasia criteria,
- immediate care to minimize further impact of oil and oily substances,
- warming or cooling (depending on season and location) while bird stabilizes and begins to rehydrate,
- washing the bird,
- drying and re-establishing waterproofing,
- maintaining adequate diet and ensuring weight gain,
- releasing into a clean environment.

These guidelines are put into use and tailored to the spill, species involved, environment, and public and legislative pressures. Each spill is different, yet the response is still based on the same principles (Appendix 1).

From the early 1970's there have been improved survival rates for birds entering the rehabilitation facility, with up to 100% of the birds admitted being released (Edmondson, 1981; Tri-State reports, 2002; Mazet *et al.*, 2002). Much of the scientific work of discovery has been championed by the Oiled Wildlife Care Network (OWCN) which was founded in the wake of the Exxon Valdez oil spill by legislative efforts in California (Newman *et al.*, 2003). It is administered at the University of California, School of Veterinary Medicine, Davis, California, to promote scientific based recovery efforts and research for oiled wildlife. This team also attends and aids with oil spill responses (Table 1.2).

Several studies suggest that the rehabilitation process may be very successful for some species (Golightly *et al.*, 2002). Western gulls followed after rehabilitation for oiling showed normal dispersal and survival compared to unoiled birds. Pelicans also tend to do well after rehabilitation (Anderson *et al.*, 1996). There has been continued work aimed at ensuring that the protocols for the rehabilitation of oil-affected birds utilize the most efficient and safe methods to manage the patients (Jenssen and Ekker, 1989; White, 1991; Mazet *et al.*, 2002).

Table 1.2 Oiled Wildlife Care Network spill responses in which more than 25 live birds were recovered and rehabilitated (Newman *et al.*, 2003).

Name And Location Of Major Spills*	Date of Spill	# Birds
Clark Pipeline, Huntington Beach	Oct 1996 – Nov 1996	35
Cape Mohican, San Francisco Bay	Oct 1996 – Nov 1996	58
Ballona Creek, Long Beach	Jan 1997 – Feb 1997	160
Torch/Platform Irene, Lompoc	Sept 1997 – Oct 1997	53
Monterey Mystery, Santa Cruz	Oct 1997 – Nov 1997	505
Kure, Eureka	November 1997	484
Pt. Reyes Mystery #1, Pt. Reyes	Nov 1997 – Dec 1997	303
Carson, Carson	January 1998	153
Pt. Reyes Mystery #2, Pt. Reyes	Dec 1997 – Mar 1998	635
Command, San Mateo City.	Sept 1998 – Nov 1998	76
Wintersburg Channel, Huntington Beach	Dec 1998	50
Golden West, Huntington Beach	Jan 1999 – Feb 1999	35
Calloway Canal, Bakersfield	June 1998	25
Stuyvesant, Eureka	September 1999	644
Trona, Trona	June & Sept 2000	29
Stockdale, Bakersfield	Oct 1999	155
Luckenbach, Pt. Reyes to Monterey	Nov 2001 – Jan 2003	1,095
* Overall, over 40 spill responses and more than 4,300 live birds collected for care.		

One other factor must also be noted when examining survival rates. Oil is not a simple product, but rather a mix of many compounds reflecting its origins and subsequent processing (Miller *et al.*, 1982; Jokuty *et al.*, 1989; Fingas, 2001). Thus each spill's survival success or failure rate must also be examined in light of the chemical properties of the oil itself (Hartung and Hunt, 1966).

1.2.3. Survival data in birds following oil spill exposure

Sharpe (1996) examined the band returns reported for birds banded in the course of normal biological surveys (control birds) versus the band return data collected on birds admitted to a rehabilitation facility for cleaning in the aftermath of an oil spill event (Figure 1.2). The oiled birds, once cleaned and released, showed a significant decrease in long term survival when compared to the control birds. This led to concerns about the value of rehabilitation techniques and the lack of understanding of the real impact these birds endure after such an event (Environment Canada, 1996; Anderson, 2000; Briggs, 1996; Briggs, 1997; Newman, 1999 and 2000).

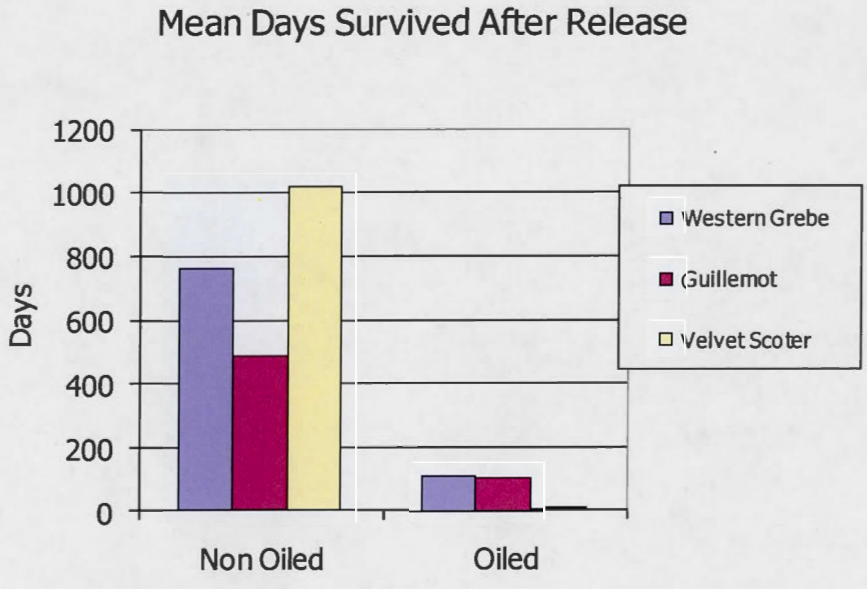


Figure 1.2 The number of days for bands to be recovered between non-oiled birds (control) vs. oiled, rehabilitated and released birds (*adapted* from Sharpe, 1996).

Cleaning is a well-established process, and Sharpe (1996) noted that the data presented showed no difference in band return days in birds treated prior to the institution of the most efficacious oiled bird rehabilitation process compared to those techniques in practice before the early 1990's. There are other issues involved, potentially those relating to the toxic impact of the petroleum products.

1.2.4. Oil toxicity in birds

Research on mallards and Pekin ducks (*Anas peking*) highlighted the impact that the toxicity itself can have (Hartung and Hunt, 1966). The impacts resulting from the ingestion of various oils and oil products on these birds included lipid pneumonia, decreased red blood cells, hematocrit and hemoglobin levels, increased liver enzymes, nephritic changes and adrenal gland enlargement. Khan and Ryan (1991) described the condition of common murres following a crude oil spill off the coast of Newfoundland in January 1990. Post-mortem examination of birds showing evidence of oil contact revealed no fat stores, low hematocrit and intestinal necrosis. Some birds showed significant liver changes including congestion, fatty degeneration, and dissociation of the hepatocytes. All birds in this study had kidney function impacts including renal tubular degeneration and necrosis. Of the 15 birds presented for rehabilitation, only two were subsequently released. Oka and Okuyama (2000) found dead oiled rhinoceros auklets (*Cerorhinca monocerata*) with severe nutritional exhaustion, low liver weights and empty stomachs.

Anderson *et al.* (2000) followed the survival of American coots (*Fulica americana*) post rehabilitation after being impacted by San Joaquin crude oil. Decreased survival, failure to regain normal body condition and behavioural changes, including increased preening activity, were all seen in this group of oil-impacted birds.

Fry and Addiego (1987) explored the health parameters of several species of seabirds following two oil spills in the central California region. Their findings indicated perturbations within the serum protein profiles consistent with an acute phase response. The other factor noted was a hemolytic anemia which in some cases was severe (10% red blood cell loss). Initially the raised total protein levels were thought to be indications of dehydration, however, rehydration therapy failed to change them. Further investigation of the protein profiles of common murres indicated a loss of the pre-albumin peak, with raised levels of alpha, beta and gamma globulin proteins.

1.2.5. Impacts of oil spills in birds at the population level

Trying to evaluate the real impact of a spill presents many problems. Surveying birds to gain an understanding of population status is a useful technique, but one that needs to take into consideration geographical and temporal issues which may also affect assessment of true population levels. Once these issues were addressed, Esler *et al.* (2000, 2002) concluded that by 1997, the harlequin duck (*Histrionicus histrionicus*) population impacted by the 1989 Exxon Valdez spill still had not fully recovered. The population had not reached its former levels nine years after the spill, a result in contrast to a

commonly held belief that oil spills, while impacting birds initially, have little long term impact at a population level. Golet *et al.* (2002) found a similar result in surveying the pigeon guillemot (*Cepphus columba*) populations in this region. These differences were also seen in some blood biochemistry values between birds from non-oiled and oiled areas. These included hepatic cytochrome P4501A (CYP1A), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) enzymes, which all remained elevated when compared to pigeon guillemots from non-oiled regions.

1.2.6. Impacts of oil spills on reproduction in birds

Following the rehabilitation of oiled brown pelicans (*Pelecanus occidentalis*), observations and band returns indicated that these birds had not only significantly lowered survival rates, but those that survived showed no breeding activity (Anderson *et al.*, 1996). Of interest, these birds did not return to the breeding colony during the reproductive period. In Magellanic penguins (*Spheniscus magellanicus*) a similar picture emerged in birds that were oiled and rehabilitated (Fowler *et al.*, 1995) although, those birds that did return to the breeding colony and remained paired were able to produce chicks. Thus there is compelling evidence that ingested petroleum products cause reproductive impairment even when rehabilitation has been successful.

Research into the reproductive aspects of birds impacted by oil shows some significant changes in hormonally-based processes. Ovarian development is delayed and male reproductive abilities are depressed when mallards are fed oil-laced food (Cavanaugh and Holmes, 1982, 1987). Some of these impacts may also be related to low plasma prolactin

concentrations (Cavanaugh *et al.*, 1983) as well as depressed levels of estradiol and progesterone (Cavanaugh and Holmes, 1982). Peakall *et al.* (1981) noted elevations in plasma corticosterone and thyroxine levels in nestling herring gulls and black guillemots after one dose of either a crude oil or its aromatic fraction. The corticosterone level responded immediately to the dose event while the thyroxine levels were raised some six days later. This increase persisted for two weeks. Further work by Peakall *et al.* (1985) confirmed earlier results and highlighted the impact oil ingestion has on weight loss.

Following the Prestige oil spill off the coast of Galicia, Spain, in 2003, the European shag (*Phalacrocorax aristotelis*) colonies impacted by the oil were monitored for reproductive perturbations. When compared to non-impacted colonies, those colonies influenced by the spill showed a 50% reduction in breeding activity (Velando *et al.*, 2005). It is unclear whether this can be attributed to sublethal oil exposure or low food availability in the wake of damage from the spill.

1.2.7. Immune function impacts in oiled birds

Immune suppression of seabirds undergoing rehabilitation is always an issue. The stress of the rehabilitation process can induce secondary disease invaders, which contribute to higher mortality and morbidity (Mazet *et al.*, 2002). One area which may be overlooked is the role that the petrochemicals themselves play in inducing immune function stress (Briggs *et al.*, 1996, 1997). The mechanisms of immune-mediated stress may also contribute to poor post-release survival. This may be a factor in the increased

seroprevalence of avian malaria seen in jackass penguins (*Spheniscus demersus*) in South Africa (Graczyk *et al.*, 1995) that had been oiled and rehabilitated.

1.2.8. Impacts of oil in young birds

Ducklings are also vulnerable to the impact of ingestion of oil. Mallard ducklings were fed No. 2 fuel oil, a refined petroleum product, during the first eight weeks of development (Szaro *et al.*, 1981). Growth was depressed in the birds with diets containing 5% of the petroleum product. Liver hypertrophy and splenic atrophy were noted along with evidence of renal damage. Also noted were some behavioural changes in those ducklings.

1.2.9. Limitations to oiled bird rehabilitation

The impact of petroleum products both on and in the avian body often leads to the death of the bird. However, once an oil spill response is mounted, those birds entering the rehabilitation process have a high probability of release at a later date. In major spill events, rehabilitation priority is often given to species of concern and those individuals with minimal secondary problems, such as fractures and wounds. Even if small numbers of oiled birds are captured, triage ensures that only those individuals that are likely to benefit most from the rehabilitation process are admitted. At present, triage is often based on overall condition and some common biochemical and hematological tests including a hematocrit (hct) to measure packed cell volume (pcv) and the proportion of the buffy coat

(white cell layer), and total solids as measured by a refractometer. These procedures are described in Appendix A, a case study of the rehabilitation process based at the Tri-State Bird Rescue and Research facility, Fort George, Louisiana, during the Deepwater Horizon oil spill, in the Gulf of Mexico, off the coast of Louisiana in 2010.

However, the present triage protocols may not be enough to ensure that the rehabilitated birds are capable of long term survival and successful reproduction. Therefore other parameters should be evaluated for inclusion in the evaluation process during the admission of oiled birds. The changes in the acute phase proteins may be the best option for ensuring the rehabilitation of only those birds that are able to return to their lives in the wild as expected for their species, and reproduce successfully.

1.3. Acute Phase Proteins in the context of oil spill responses

An oil spill event where birds are oiled is a potentially toxic assault. Physiological responses result in difficulties linked to thermoregulation, nutrition and hydration. The resulting shift in homeostasis would be expected to induce an acute phase response with the attendant changes in levels of acute phase proteins.

1.3.1. An overview of the acute phase response and the subsequent induction of acute phase proteins.

The use of human acute phase proteins as markers in a clinical study was first described in 1930 by Tillet and Francis. The induction or suppression of acute phase proteins is a part of the organism's attempt to restore homeostasis (Heinrich *et al.*, 1990). This complex response (Figure 1.3) to an assault on the body, whether it is from an infection, trauma, neoplasia, stress or inflammation, can result in a wide range of change in levels of some of the proteins, specifically those known as the acute phase proteins (Heinrich *et al.*, 1990). The inflammatory process and subsequent acute phase response requires precise regulation to ensure an appropriate response, which is not compounded by immaturity, immunosuppressive disease, genetics or stress (Klasing, 1998). The acute phase proteins arise from stimulation of the hepatocytes in the liver (Miller *et al.*, 1951) by cytokines including interleukin (IL) 6-β, interleukin (IL) 1 and tumor necrosis factor - alpha (TNF-α) (Figure 1.4).

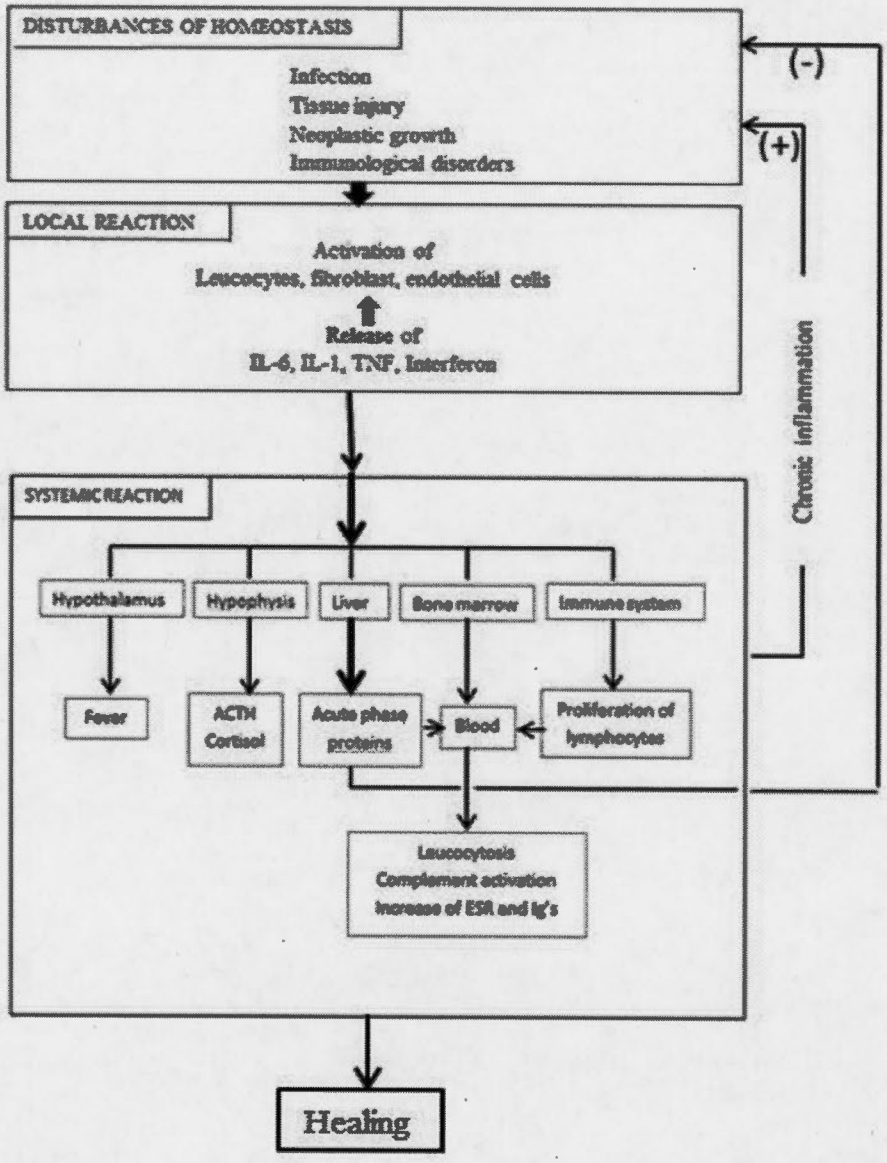


Figure 1.3 An overview of the acute phase response in an organism (Heinrich *et al*,. 1990).

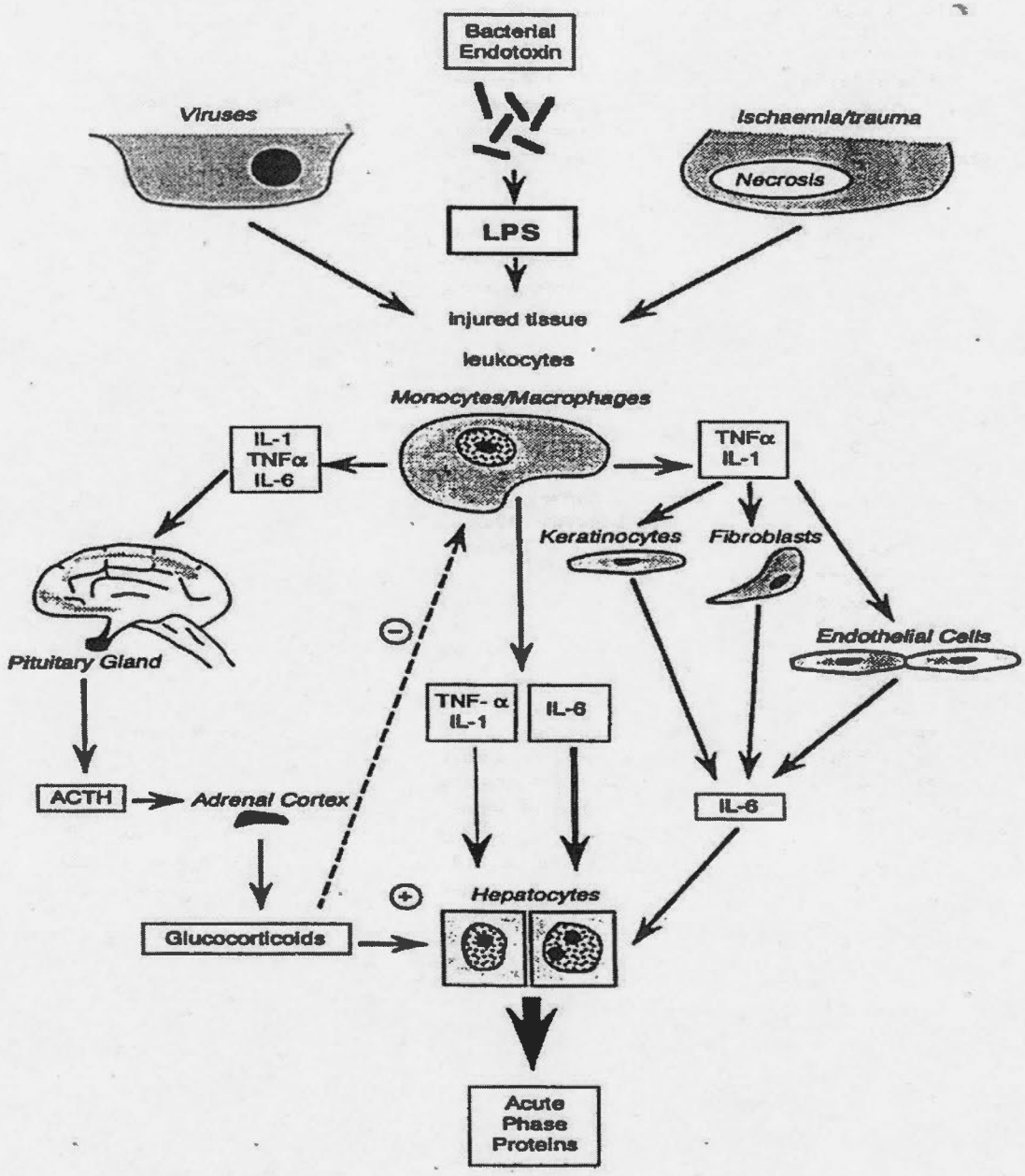


Figure 1.4 Schematic representation of the processes involved in the induction of acute phase proteins by hepatocytes (*adapted from* Heinrich *et al.*, 1990, *in* Gruys *et al.*, 1994).

1.3.2. An overview of the cytokine response

The cytokines are produced by lymphocytes, macrophages, fibroblasts and endothelial cells (Bornstein, 1982; Marinkovic *et al.*, 1989; Heinrich *et al.*, 1990, Gruys *et al.*, 1994) and are a relatively short lived group of proteins when compared to that of the acute phase proteins. The cytokine IL 6 and the acute phase protein α1-acid glycoprotein (α1-AG) response were monitored in research on chickens injected with *E. coli* lipopolysaccharide compared to the control birds which were injected with phosphate buffered saline (PBS) (Nakamura *et al.*, 1998). The rise in the cytokine IL 6 in both the control and test birds is rapid, with a peak response seen at 3 hours post injection (Figure 1.5). The return to baseline levels, rapidly in the case of the PBS injected control group (12 hours), and somewhat longer in the test birds (7 days). As the acute response of the IL 6 peaks and starts to return to baseline levels 3 hours post-injection, the α1-AG levels (Figure 1.6) (Nakamura *et al.*, 1998) begin to rise and persist at increased levels for 4 days before returning to baseline levels at day 7.

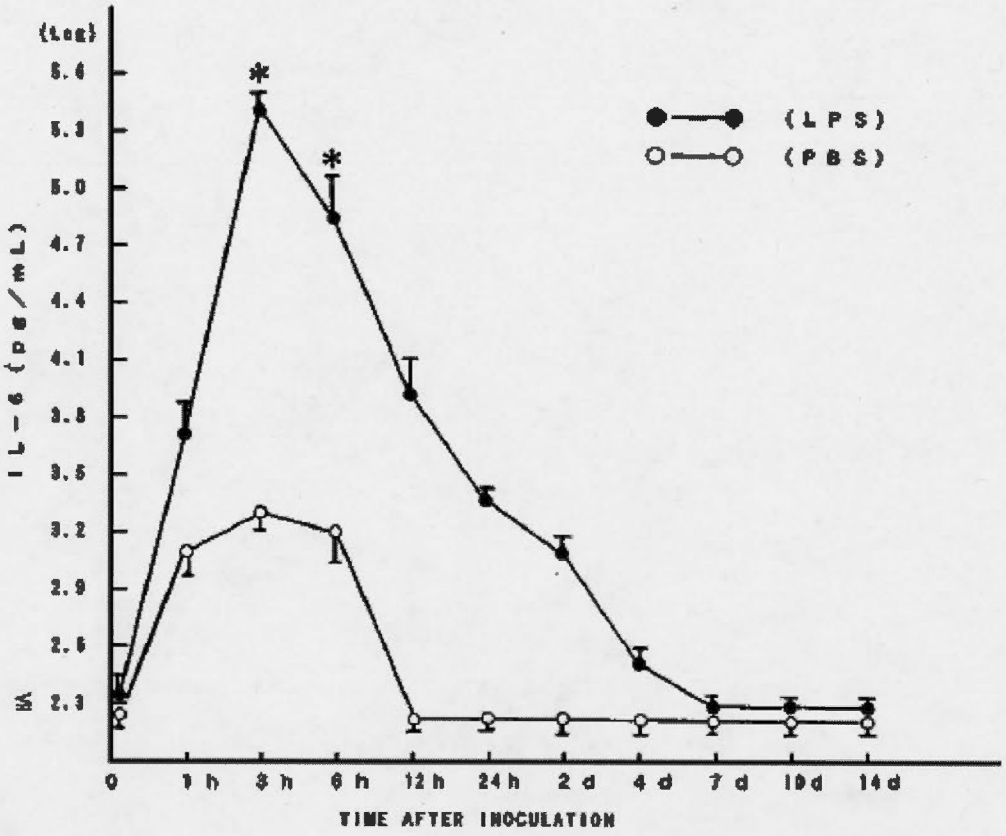


Figure 1.5 Interleukin (IL)-6 concentration in the sera of chickens inoculated with *Escherichia coli* lipopolysaccharide (LPS) and PBS (control). The asterisks designate that the levels of LPS group were significantly ($P < 0.05$) higher than those of the PBS group at indicated time points. Vertical bars represent the SD. (*from* Nakamura *et al.*, 1998).

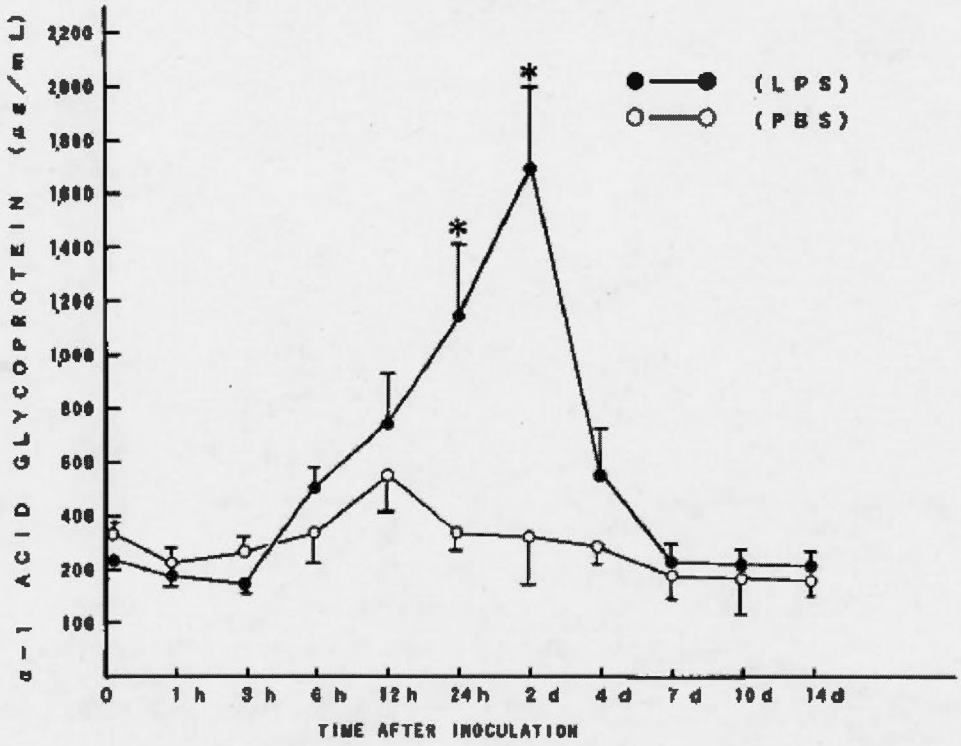


Figure 1.6 The acute phase protein α1-acid glycoprotein (α1-AG) concentration in the sera of chickens inoculated with *Escherichia coli* lipopolysaccharide (LPS) and PBS (control). The asterisks designate that the levels of LPS group were significantly ($P < 0.05$) higher than those of the PBS group at indicated time-points. Vertical bars represent the SD. (*from* Nakamura *et al.*, 1998).

1.3.3. An overview of the acute phase proteins

Serum protein electrophoresis is based on the separation of protein components by electrical charge. The quantification and proportions of the protein fractions greatly add to the clinical picture, often showing up before any clinical signs, and remaining elevated or depressed once clinical symptoms have resolved, thus indicating an underlying

pathology. As a response to an inflammatory condition, many acute phase proteins are either induced (positive acute phase proteins) or suppressed (negative acute phase proteins) over time, as can be seen in the generalized response as illustrated in Figure 1.7 (Gabay and Kushner, 1999). It is this prolonged response over a set time frame which can be potentially of use in determining the degree of insult suffered in an oil spill response, where birds may not be admitted until some days to weeks after oiling occurs.

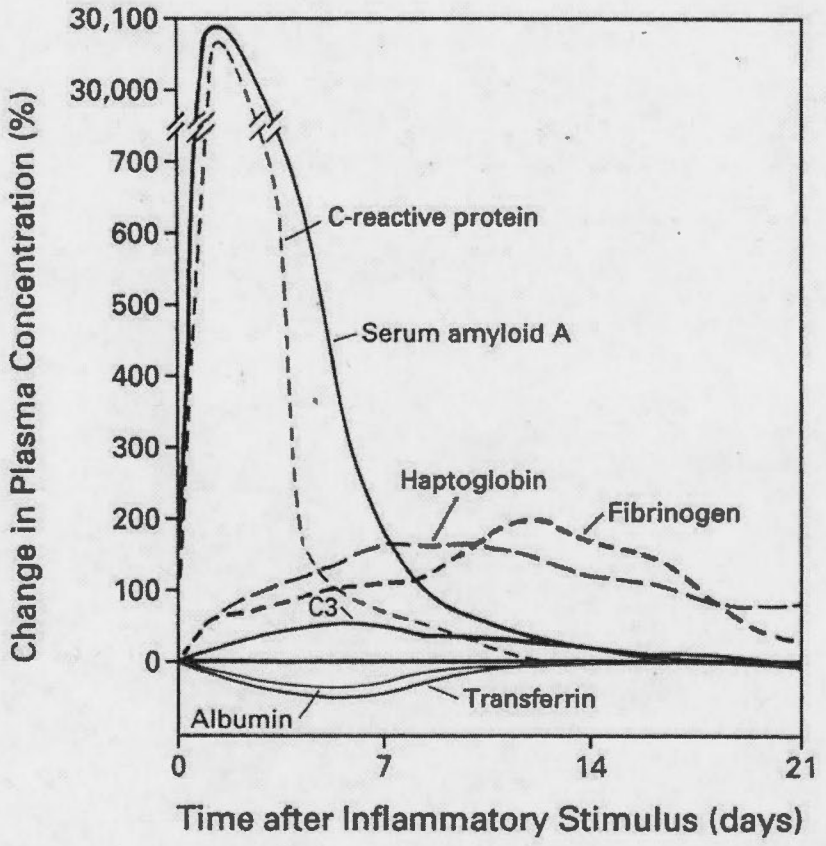


Figure 1.7 Generalized patterns of change in plasma concentrations of some acute phase proteins in response to a moderate inflammatory stimulus in humans (Gabay and Kushner, 1999).

The increase or decrease in a specific protein post-trauma (disease, injury, toxic insult) is not consistent throughout the animal kingdom, and thus each species, sex and age class may have its own unique set of responses, which need to be identified to understand the diagnostic role that they may each have (Apanius, 1983; Goldfarb *et al.*, 1986; Kushner and Mackiewicz, 1987; Kent, 1992). This point is illustrated (Table 1.3) in comparing the acute phase proteins in two well-studied mammalian species; humans and rats (Heinrich *et al.*, 1990).

Seasonal activities also may result in some variation within species, however, examination of the plasma proteins of American kestrels *(Falco spaverius)* indicated no significant difference between the albumin:globulin levels in laying and non-laying hens and male birds (Gauthier *et al.*, 1983).

Another area of concern is the potential for an acute phase induction when a bird is handled by humans, essentially a predator from the bird's perspective. This was explored by a paper detailing the potential for acute phase protein induction in response to acute physical stress in pigs (Hicks *et al.*, 1997). There was no apparent induction of an acute phase protein response to physical stressors, such as handling and transport, which may be useful if this function is conserved across species. If birds respond in the same manner, it eliminates the potential for obscured results as birds enter the rehabilitation process with increasing stress responses related to close interactions with humans. Any research examining acute phase proteins should take this potential response to handling into account in the research protocol.

Table 1.3 Acute phase proteins in humans and rats in response to a stressor (Heinrich *et al.*, 1990).

	Increase				
Species	**10 - 100-fold**	**2 - 10-fold**	**≤ 2-fold**	**No change**	**Decrease**
Human	C-reactive protein Serum amyloid A	α_1-Proteinase inhibitor α_1-Acid glycoprotein α_1-Antichymotrypsin Fibrinogen Haptoglobin	Ceruloplasmin C3 of complement α_2-Antiplasmin C_1-inactivator	α_2-Macroglobulin Haemopexin Serum amyloid P Prothrombin	Inter-α-antitrypsin Transferrin α_1-Lipoprotein Prealbumin Albumin
Rat	α_2-Macroglobulin α_1-Acid glycoprotein	Fibrinogen Haptoglobin Cysteine proteinase inhibitor	α_1-Proteinase inhibitor Ceruloplasmin Prekallikrein Haemopexin C-reactive protein	α_1-Macroglobulin Antithrombin III Serum amyloid P Prothrombin	α_1-Inhibitor 3 Transferrin Prealbumin Albumin

1.3.4. Acute phase proteins in humans

Serum proteins have long been used as important indicators in assessing the health of a human subject (Kushner and Mackiewicz, 1987). Protein electrophoresis patterns are well established aids in the detection and differential diagnosis of many disease states as well as toxic responses (Keren, 1988). Table 1.4 and Table 1.5 give an overview of the diagnostic and biological role played by some select proteins and the levels induced in an acute phase response.

Engler (1995) discussed the use of several acute phase proteins as a "biological tool" for the clinician in human medicine. Specifically this paper cited the use of C-reactive protein, haptoglobin (Hp) and alpha-1 acid glycoprotein as being useful in assessing inflammatory responses in patients. C-reactive protein is also cited as being of use as a predictor of the response to a therapy (Blackburn, 1994) or in cases of complications, delayed recovery or recurrence of infections (Johnson *et al.*, 1999). Many other researchers (Kaysen, 2000; Black *et al.*, 2004) have also used C-reactive protein as an aid in establishing the degree of acute phase response that their patients are undergoing. However, this is always used with several other biological indicators.

Table 1.4 Major protein patterns (Albumin [Alb], α1, α2, β1, β2 and γ) with diagnosis in human medicine (Keren, 1988).

Pattern[a]	Alb	α1	α2	β1[b]	β2[c]	γ
Acute Phase	D[d]	I	I	D	I	D
Chronic inflammation	I or N	N	N	N	I or N	I (broad)
Chronic liver disease	D	D or N	D or N	I or N	I or N	I (broad)
Protein loss pattern	D	D	I	D or N	D or N	D
Monoclonal gammopathy	D or N	N	N	I or N	I or N	I or N or D[e]
Estrogen effect	D or N	I	I	I or N	I or N	N
Hemolysis	N	N	D	N	N	N
AIAT deficiency	N	D	N	N	N	N

a. These are general patterns, individual variations will occur.

b. β1 is largely transferrin

c. β2 is largely C3

d. I = increased, D = decreased, N = normal

e. With myeloma there is often a suppression of polyclonal immunoglobulins while the monoclonal protein will increase the total gamma region. Note that a monoclonal immunoglobulin is not restricted to the gamma region. They frequently occur in the beta region and have been reported in the alpha region, though rarely.

f. A1AT – alpha 1 anti-trypsin

Table 1.5 Characteristics of selected acute phase reactants (from Johnson *et al.*, 1999).

Acute phase reactant	Normal serum concentration	Increase during acute phase response	Biologic function
C-reactive protein	<1 mg/dL	1000X	• activates classical complement pathway and acts as opsonin • binds autogenous debris and facilitates clearance • inhibits platelet aggregation • modulates PMN function
Serum amyloid A	0.5 mg/dL	1000X	• apolipoprotein that associates with HDL3 and may interfere with cholesterol metabolism • amyloid A deposition inhibits platelet activation and neutrophil oxidative burst
Serum amyloid P	3 mg/dL	1X	• structurally and functionally homologous to CRP • concentration remains constant during inflammation
Coagulation factors			• blood coagulation
fibrinogen	200-450 mg/dL	2-4X	• wound healing
Complement proteins			
C3	130 mg/dL	1.5X	• complement cascade
C4	65 mg/dL		
Protease inhibitors			
α-1-antitrypsin	200-400 mg/dL	2-4X	• inhibits antitrypsin
Metal binding proteins			
ferritin	10-150 ng/mL	2-4X	• binds iron
haptoglobin	100-300 mg/dL		• binds hemoglobin
ceruloplasmin	15- 60 mg/dL		• binds copper, scavenges free radicals and superoxide ions

Albumin, a negative acute phase protein, is proving to be a useful tool in predicting mortality in kidney disease patients whose albumin levels continue to decrease after treatment (Lorie and Lew, 1990; Kaysen *et al.*, 2000; Kaysen, 2000). Hypoalbuminaemia is also a function of inadequate protein intake and starvation (Kaysen, 2000). However, when the albumin level was persistently below 40g/L (study of 12,000 hemodialysis patients), the patient had a high probability (>66%) of death occurring, often within six months (Kaysen *et al.*, 2000). The role of albumin as an acute phase protein as a marker of mortality was also confirmed in a study which linked the critical level of 40g/L albumin with the presence of kidney failure, resulting in death in the patients (Lorie and Lew, 1990).

Several of the acute phase proteins remained elevated years later in people who had survived the initial acute phase response after the ingestion of rapeseed oil contaminated with aniline (Bell *et al.*, 1995). These acute phase proteins included C-reactive protein, alpha-1-antitrypsin, fibrinogen and ceruloplasmin. These results indicate that the initial toxic insult had profoundly impacted their livers' ability to recover.

1.3.5. The use of serum protein electrophoresis in veterinary practice

The veterinary world also uses the serum proteins as an aid in diagnosis and as a prognostic indicator (Kent, 1992; Cray, 1995, 1997, 2000; Cray and Tatum, 1998; Koutsos and Klasing, 2001; Murata *et al.*, 2004). Harr (2002) detailed the use of proteins in avian species as useful indicators of health status. This paper noted the use of gel

electrophoresis as the most useful in quantifying the range of protein groups found in avian species; transthyretin or the prealbumin fraction, albumin, α-1-antitrypsin or α-1-globulins, α-2-macroglobulins (also known as α-2-globulins), fibrinogen, β-lipoprotein, transferrin, complement, and vitellogenin (β-globulin fraction), the immunoglobulins and complement degradation products (γ-globulins). This common protein screening allows for early diagnostic evaluation.

Cerón *et al.* (2005) have detailed the use of acute phase proteins to aid in diagnosis of events in dogs and cats. They have also compiled the biological functions of the main proteins used in veterinary medicine diagnosis (Table 1.6). This guide is useful in identifying the processes involving an acute phase protein response, as the body reacts to a stressor and mobilizes innate systems to aid in restoring homeostasis. A review by Eckersell and Bell (2010) also explored the roles the acute phase proteins play in veterinary medicine, specifically comparing the canine and bovine responses. Species differences exist and must be used in conjunction with known normal ranges.

Table 1.6 The biological function of the acute phase proteins used in veterinary medicine (Cerón *et al., 2005).*

Protein	Main Biological Functions
C-reactive protein	When bound to bacteria, promotes the binding of complement, which facilitates bacterial uptake by phagocytes; it has been considered as a primitive form of antibody specifically interacting with cell membrane
	Induction of cytokines
	Inhibition of chemotaxis and modulation of neutrophil function
Serum amyloid-A	Chemotactic recruitment of inflammatory cells to sites of inflammation
	Down regulation of the inflammatory process (inhibition of myeloperoxidase release and inhibition of lymphocyte proliferation)
	Involvement in lipid metabolism and transport
Haptoglobin	Involvement in host defense responses to infection and inflammation; acts as a natural antagonist for receptor-ligand activation of the immune system
	Binding of free hemoglobin (a toxic and proinflammatory product resulting from hemolysis)
	Bactericidal effect in infected wounds by binding hemoglobin and limiting the availability of hemoglobin iron for bacterial growth
	Inhibition of granulocyte chemotaxis and phagocytosis
Alpha-1-acid glycoprotein	Anti-inflammatory and immunomodulatory agent with antineutrophil and anticomplement activity; increases the secretion of interleukin-1 receptor antagonist by macrophages
	Drug binding to numerous basic and neutral lipophilic drugs and also acidic drugs, such as phenobarbital
Ceruloplasmin	Transport of copper needed for wound healing, collagen formation, and maturation
	Protection of cells and tissues against oxidant compounds generated by phagocytes in the course of clearing microorganisms or tissue debris
	Reduction in the number of neutrophils attaching to endothelium

Cray (1995, 2000) compared psittacine health to changes in six major protein groups (Figure 1.8) using data from gel electrophoresis. Each protein group can be identified and quantified. From these changes in relative protein abundance, a differential diagnosis table was prepared to aid veterinarians when confronted with sick parrots (Cray and Bossart, 1995)(Table 1.7).

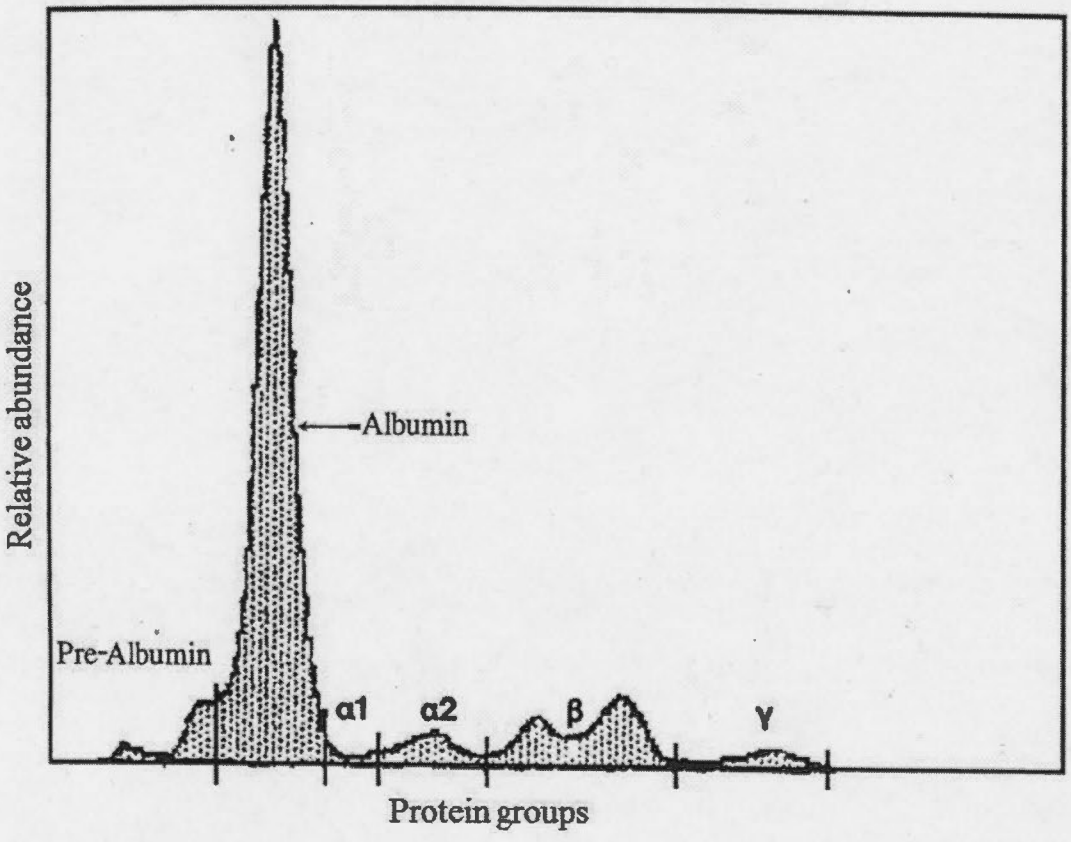


Figure 1.8 A representative normal electropherogram for a psittacine (from Cray, 1995).

Table 1.7 Protein fraction changes in psittacine disease (*from* Cray and Bossard, 1995).

Disease	Pre-Albumin	Albumin	Alpha	Beta	Gamma	A/G Ratio
Acute Chlamydiosis	unchanged	mild to moderate decrease	unchanged or mild increase	mild-moderate increase	moderate-marked increase	moderate-marked increase
Aspergillosis	unchanged	unchanged or mild decrease	unchanged or mild increase	mild-moderate increase	mild-moderate increase	mild decrease
Mycobacteriosis	unchanged	unchanged or mild decrease	unchanged or mild increase	unchanged or mild increase	mild-moderate increase	mild decrease
Egg Related Peritonitis	unchanged	unchanged or mild decrease	unchanged or mild increase	unchanged or mild increase	mild-moderate increase	mild decrease
Chronic-Active Hepatitis	unchanged	mild to moderate decrease	unchanged or mild increase	mild-moderate increase	mild-moderate increase	mild-moderate decrease
Acute Nephritis	unchanged	mild to moderate decrease	unchanged or mild increase	mild-moderate increase	unchanged or mild increase	mild-moderate decrease

Within these patterns of altered protein levels, the major avian acute phase proteins were identified and evaluated. Using avian plasma, Cray (1997) also identified the major protein fraction in which the acute phase proteins are to be found in an electropherogram (Table 1.8). Each protein is essential to one or many biological functions. The major functions are noted with each acute phase protein identified in Table 1.8.

Table 1.8 The fractions where major acute phase proteins are found in avian plasma electrophoresis (*adapted from* Cray, 1997) with their overall biological function.

Protein Fraction	Protein	Function(s)	Reference
Alpha	Alpha lipoprotein	Lipid transport	Bruss, 2008
	Alpha-1-antitrypsin	Protease inhibitor	Gettins, 2002
	Gc globulin	Vitamin D binding and transport	Cooke and Haddad, 2009
	Alpha-2-macroglobulin	Proteinase inhibitor	Sottrup-Jensen, 1989
	Haptoglobin*	Binds free hemoglobin	Dobryszycka, 1997
Beta	Fibronectin	Structure to support wound healing	Grinnell, 1984
	Transferrin	Iron transport	Ching-Ming Chung, 1984
Gamma	Beta lipoprotein	Lipid transport	Bruss, 2008
	Complement	Immune system	Janeway *et al*, 2001
	Immunoglobulins	Immune system	Janeway *et al*, 2001

*Wicher and Fries(2006) report Haptoglobin is not found in chickens.

The acute phase protein response in veterinary research also demonstrates the course of the event over time. The viral Infectious Bursal Disease results in a 20% mortality rate in chickens (Merck, 1991). When chickens were challenged with this virus and were monitored for α1-acid glycoprotein and albumin, all showed an acute phase response with levels of α1-acid glycoprotein peaking at day five and returning to normal at day fourteen, while a negative peak for albumin was seen at day four, it then rebounded to above normal levels at day ten (Inoue *et al.*, 1997).

1.3.6. Acute phase response in other vertebrate species

The Atlantic loggerhead sea turtle (*Caretta caretta*) protein profiles show identifiable protein groups (Gicking *et al.*, 2004) (Figure 1.9). There are some differences identified, with the beta and gamma globulins often forming one peak in this technique. A transthyretin peak was also seen in one individual in this research as noted in the profile #2 in Figure 1.9. The protein peaks were quantified to account for gender and age differences (Table 1.9).

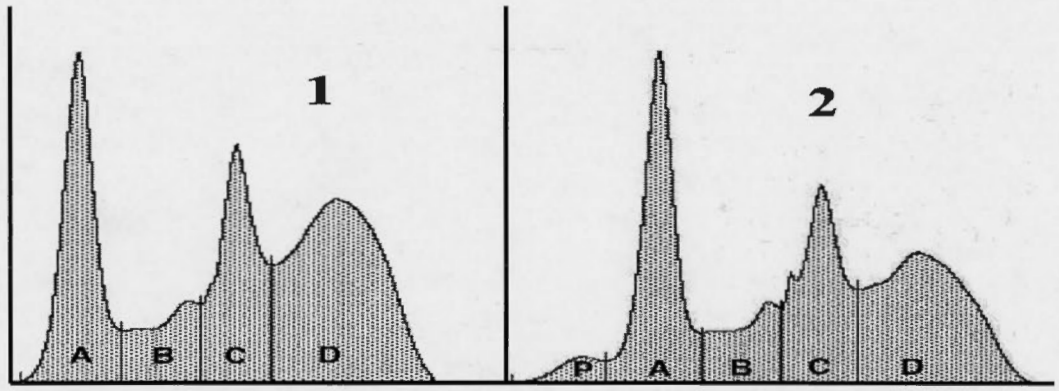


Figure 1.9 Representative plasma protein electrophoretograms of the loggerhead sea turtle, *Caretta caretta.* Image (1) The dark vertical lines from left to right denote the breaks between the albumin (A), Alpha globulin (B), Beta globulin (C), and Gamma globulin (D) fractions. Image (2) with a pre-albumin (P) band (*adapted from* Gicking *et al.*, 2004).

The maximum and minimum values for the sample size (N) and the age and gender of the animals tested provided insight into the range of the major proteins peaks in the loggerhead sea turtle. Also recorded is the albumin to globulin (A:G) ratio, which is a widely used indicator for conditions affecting the production of the various proteins from disease to nutritional status. This ratio is widely used in both human and veterinary medicine, providing another parameter to explore physiological parameters.

Table 1.9 Plasma protein fractions identified in loggerhead sea turtles, *Caretta caretta* (*from* Gicking *et al.*, 2004).

Group	Total Protein (g/dL ± SD)	Albumin (g/dL ± SD)	Alpha (g/dL ± SD)	Beta (g/dL ± SD)	Gamma (g/dL ± SD)	A:G
All (N=29)	4.3 ± 0.72	1.0 ± 0.17	0.48 ± 0.10	0.80 ± 0.20	1.94 ± 0.62	0.33 ± 0.10
Minimum	2.9	0.7	0.3	0.51	0.77	0.17
Maximum	5.4	1.3	0.81	1.3	3.0	0.63
Adult Males						
(N=7)	4.6 ± 0.33	1.1 ± 0.11	0.54 ± 0.12	0.99 ± 0.17	1.97 ± 0.27	0.32 ± 0.05
Minimum	4.2	0.92	0.43	0.74	1.58	0.23
Maximum	5.2	1.25	0.81	1.31	2.48	0.
Adult females						
(N=7)	4.4 ± 0.75	0.97 ± 0.13	0.49 ± 0.05	0.81 ± 0.14	2.1 ± 0.64	0.30 ± 0.62
Minimum	3.3	0.79	0.42	0.56	1.1	0.18
Maximum	5.4	1.2	0.56	0.98	2.98	0.38
Juvenile males						
(N=8)	4.1 ± 0.66	0.96 ± 0.19	0.46 ± 0.11	0.78 ± 0.13	1.8 ± 0.61	0.33 ± 0.10
Minimum	3.5	0.71	0.34	0.59	1.27	0.17
Maximum	5.4	1.23	0.66	1.05	2.93	0.45
Juvenile females						
(N=7)	3.9 ± 0.78	1.0 ± 0.17	0.44 ± 0.06	0.60 ± 0.07	1.9 ± 0.76	0.38 ± 0.15
Minimum	2.9	0.77	0.36	0.51	0.77	0.25
Maximum	5.2	1.28	0.54	0.73	2.88	0.63

An acute phase protein response was also seen in snakes (personal observations) that were implanted with abdominal tracking devices at the Toronto Zoological Park, Toronto, Ontario, Canada. Schwantes (1976) confirmed the presence of haptoglobin in 17 of 24 species of snakes. This protein showed similar electrophoretic mobility to human haptoglobin.

Studies conducted by Jensen *et al.* (1997) demonstrated that the major acute phase proteins amyloid A, C-reactive and amyloid P are also found in salmonid fishes.

1.3.7. Acute phase proteins in response to a toxic event

Research into avian acute phase response proteins is still in the early stages when compared to the data available in the mammalian literature. However, work is underway to understand and document the process and the potential uses for the monitoring of health, especially in economically important species. Adler *et al.* (2001) followed the acute phase proteins hemopexin and α1-acid glycoprotein after intraperitoneal injection of various inflammatory agents in chickens. Both proteins showed an appropriate response by twelve hours post injection, with an increase in the α1-acid glycoprotein and a decrease in the levels of albumin, a negative acute phase protein, which continued until twenty four hours post injection. By 336 hours, levels slowly returned to normal for the α1-acid glycoprotein, and rose above the normal levels for albumin. This implies a longer period for induction of the acute phase reactants' activities than previously noted in mammalian responses.

Research has also drawn attention to the use of acute phase proteins as potential biomarkers of impact of other xenobiotics including pesticides (Saleh *et al.,* 1996). Serum collected from healthy, unexposed rats was incubated with several insecticides (lindane, endrin, endosulfan, fenvalerate, toxaphene, and heptachlor) and industrial pollutants (trichlorophenol and polychlorinated biphenyls) in an *in vitro* experiment. Protein profiles were monitored in these incubated serum samples using Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Fast Protein Liquid Chromatography (FPLC) techniques. The research documented changes in the pre-albumin, albumin and transferrin bands. These are either positive or negative acute phase proteins. In an *in vivo* experiment, rats administered lethal levels of the pain medication acetaminophen responded with the indication of mRNA for several of the acute phase proteins including beta-fibrinogen, α1-acid glycoprotein and α-tubulin (Tygstrup, 1998). These changes were greater than those seen for non-lethal levels of the drug.

Haptoglobin and C-reactive protein have been widely used as indicators of tissue damage caused by inflammation, infection or trauma. C-reactive protein was also seen to rise in pharmacological toxicities (Eckersall e*t al.*, 1998); however, it must always be considered that these are not specific markers but are guides to the level of tissue damage and potential prognosis.

Fibrinogen, classified as an acute phase protein (Kaneko, 1980), is increased in acute inflammatory disorders. In avian species with a confirmed bacterial infection, Hawkey and Hart (1988) found that hyperfibrinogenaemia was present in 63.2% of cases, representing a wide range of avian species. When combined with either fibrinogenaemia or heterophilia, the evidence to support an infection increased to 77%. Fibrinogen was also noted to rise over a period post dosing with the control (saline dose) and test (oil) rhinoceros auklets (*Cerrorhinca monocerata*) (Newman *et al.*, 1999). It peaked on day five and decreased to expected normal levels by day twenty-eight. This response potentially indicated that the stress of captivity and handling had affected these birds, rather than the toxic assault. Newman (personal communication) however, confirmed the potential use of fibrinogen as a biomarker in common murres impacted by oil from the Patricia spill in California. The fibrinogen levels for normal murres were 264 ± 132mg/dl compared to those oiled and rehabilitated birds 435 ± 278 (p= 0.002). The birds with significantly raised fibrinogen levels died subsequently during the oil spill response.

Hp has been studied in mammalian systems and occasionally monitored for its usefulness in avian systems. Seiser *et al.* (2000) found no significant differences between Hp levels in pigeon guillemots (*Cepphus columba*) from oiled and unoiled areas eight years after the Exxon Valdez spill. It may be that Hp has limited use in avian systems as the test kits used for this determination are often those targeting mammalian Hp, which is functionally different from the avian form (Mackiewicz, 1993). Avian proteins have not been well studied, however, assumptions have been made when the absence of these proteins is noted in comparison to mammalian models. This 'absence' may be attributed

to the inability to detect a given avian protein due to subtle but important changes in the molecules (Goldfarb *et al.*, 1986). Musquera *et al.* (1979) noted that chicken Hp shows narrow species specificity and only binds to reptile and avian hemoglobins. Thus using mammalian Hp test kits would be of little use. This is also the case with another acute phase protein, hemopexin (Goldfarb *et al.*, 1986).

The Exxon Valdez oil spill initiated many levels of response, often driven by the media. The numbers of animals impacted included an estimated 300,000 – 640,000 birds, 3,500 – 5,000 sea otters (*Enhydra lutris*), with 200 harbour seals (*Phoca vitulina*) known dead as the result of the oil impact (Etkin, 1998). Duffy *et al.* (1994, 1996) examined a number of biochemical parameters from the blood of several species of Alaskan mammals, including both sea and river otters, impacted with crude oil from the Exxon Valdez spill of 1989. Of note were increased levels of the acute phase proteins Hp, α_1-acid glycoprotein, and fibrinogen in animals from oiled areas of Prince William Sound (Duffy *et al.*, 1994, 1996), while albumin was reduced as it acts as a negative acute phase protein. Bodkin *et al.* (2002), examining abundance of the sea otters from this region, found indications that the population from an oil impacted area had not recovered 11 years after the Exxon Valdez spill. Prey abundance does not seem to be the major problem arising from the long term spill effects, but rather that other factors are impacting this population, as evidenced from elevated cytochrome P450 levels in the blood of the resident otters.

1.3.8. Acute versus chronic changes in acute phase proteins

The time line in the evolution of an acute phase response is not well defined and depends on many factors, including the stressor and then its impact in perturbing the homeostasis of the organism, and the organism itself. However, in general, the acute phase period covers the first 4 to 5 days post impact (personal communication C. Cray, Division of Comparative Pathology, University of Miami, Miller School of Medicine, Miami, FL), with a possibility of extending slightly longer. The organisms' acute phase response, through the increase or decrease in its acute phase proteins, has then fulfilled its role in protecting the body and protein levels begin to return to the normal ranges for that species (Figure 1.7). However, what happens if the damage becomes chronic? From discussions with Dr. Cray, the chronic phase should be fully instituted by three weeks after the initial assault which resulted in changes in the levels of acute phase proteins. This chronic response is seen in cattle with inflammation (Figure 1.10) (Horadagoda *et al.*, 1999).

Catching birds in the wake of an oil spill can be challenging if they are still able to fly or lightly oiled. The 'golden hour', a term coined by Lynne Frink (founder of Tri-State Bird Rescue and Rehabilitation, Delaware, USA), is the time when a bird can be caught and still has a chance for recovery in rehabilitation. However, the acute phase protein period may have passed. The changes need to be followed over several weeks to months to have an understanding of how the body does cope with the initial event.

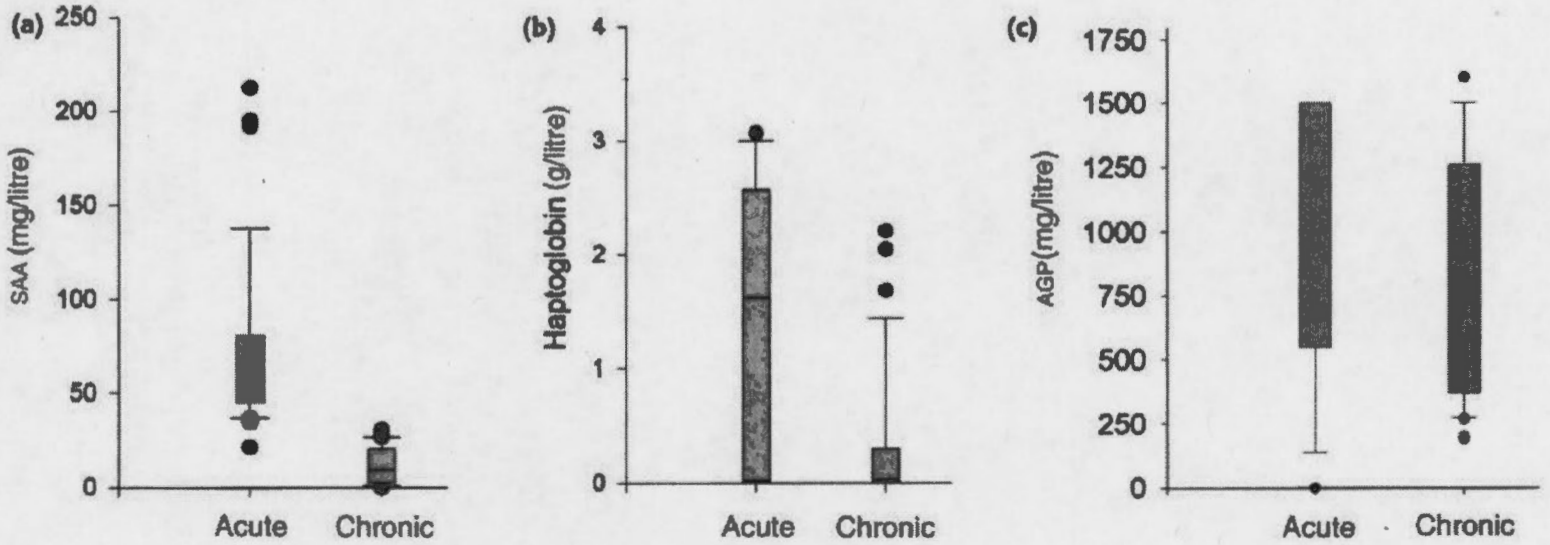


Figure 1.10 Box plots comparing the distribution of: (a) serum amyloid A (SAA); (b) haptoglobin; (c) α1-acid glycoprotein in 31 cattle with acute inflammation and 50 cattle with chronic inflammation. The median is marked with a line, the box shows the 25th to 75th percentile, the whiskers show the 10th and 90th percentiles and outliers are marked with closed circles (*adapted from* Horadagoda *et al.*, 1999).

1.3.9. Perspectives for use of acute phase proteins as biomarkers in oil spill responses

It would be very convenient to have a definitive answer, for instance, that increases in a specific protein will always indicate a fatal outcome. This expertise is already an integral part of human medicine. Cray's research (1995, 1997) has aided with psitticine diagnostics by preparing a differential diagnosis tool (Table 1.7). There are some data to support that avian inflammatory proteins do respond to stressors. However, until we attempt to quantify the overall protein changes through the acute phase and into the chronic phase in an avian model, we will not know where to look for individual biomarker proteins.

1.3.10. The use of capillary electrophoresis (CE) in avian research

The use of capillary electrophoresis (CE) in avian studies has few references. CE has been used to quantify the proteins from a captive group of Spanish imperial eagles (*Aquila adalberti*) (García-Montijano *et al.,* 2002). This technique was also used to separate the products used to determine gender in 58 avian species using the avian sex determining chromosome, the CHD gene (Lee *et al,*. 2010). CE has also been used to quantify the antibiotic thiamphenicol in avian blood samples (Kowalski, 2007). To date, however, this technology has not been explored to evaluate its efficacy in optimizing quantification of protein changes for health status and identification of shifts in homeostasis linked to toxic stressors.

1.4. The avian model - the mallard and its annual cycle

1.4.1. Introduction

The mallard (*Anas platyrynchos*), the most common duck species belongs to the Order Anseriformes, Family Anatidae. It is a surface feeding duck inhabiting fresh and salt water wetlands (Jorde *et al.*, 1983).

The annual cycle of the mallard, whether in captivity or in free living wild populations, has essentially the same elements geared to reproduction and resource acquisition. This section will cover the activities of the population of mallards in the northern hemisphere and specifically the North American population which breeds in Canada and overwinters, primarily, in the United States. However, the goal of this section is to explore the events for the wild population, beginning with the period covering the start of the experimental year. This annual cycle is linked to several endogenous and exogenous factors as can be seen in Figure 1.11 from Bluhm (1992) and will be discussed in relation to the activities of the mallards in North America.

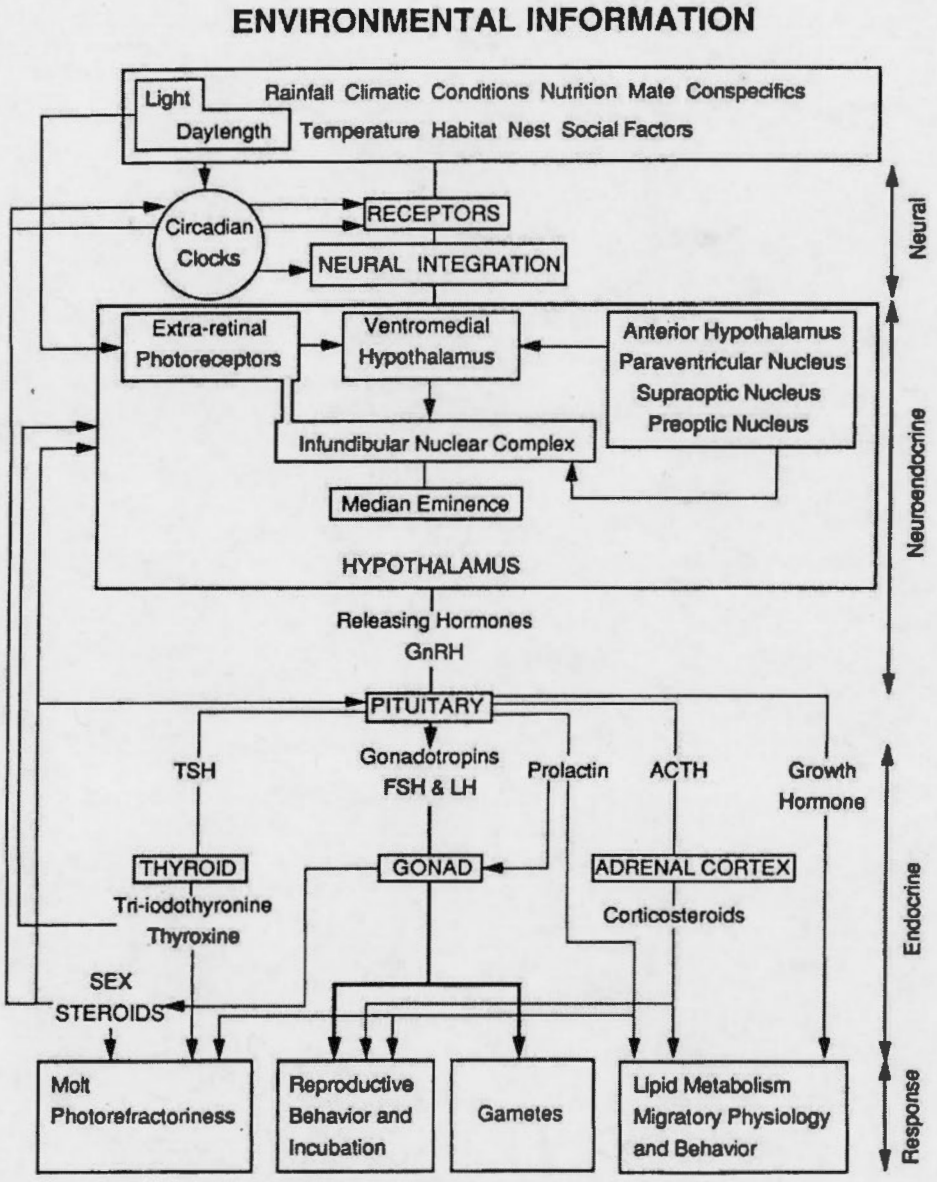


Figure 1.11 A generalized scheme showing the transduction of environmental factors into neuroendocrine and endocrine secretions which regulate waterfowl reproduction and associated factors (Bluhm, 1992).

1.4.2. August – September

1.4.2.1. Location of birds

By this time of the year the flocks of mallards are all gathered in areas of abundant food resources, water and cover (Jorde *et al.*, 1983; Sugden and Driver, 1980). Diet is extremely varied and may depend on the region as agricultural practices can result in waste grains such as corn being available. However, the mainstays of the mallard diet include gastropods, invertebrates, crustaceans, worms, many varieties of seeds and plant matter, and roots and tubers (Bellrose, 1976; Gruenhagen and Frederickson, 1990).

1.4.2.2. Reproductive cycle

Almost all of the previous year's reproductive behaviours have ceased. There may be a few late broods cared for by female mallards, however, this is rare and over the several years of observations made in the Delta Marsh region, Manitoba, I only saw one brood of newly hatched ducklings. The rarity of this observation was echoed by the director of the Delta Marsh Bird Observatory, Ms. Heidi Den Haan. However if conditions are exceptionally favorable, with abundant food and rainfall, some fall breeding has been recorded in the northern prairies (Krapu *et al.*, 2001).

Through this period of photo-refractoriness the testes regress, potentially due to negative feedback via testicular androgens on the hypothalamo-hypophyseal system (Haase, 1983). Ovarian tissue has also regressed completely at this stage (Johnson, 1961). Prolactin levels are also at their lowest or basal levels in drakes (Haase *et al.*, 1985) and hens (Bluhm *et al.*, 1983; Boos *et al.*, 2007).

1.4.2.3. Moult

The mallard is a strongly sexually dimorphic species (Figure 1.12). The males have begun to moult into their alternate (breeding) plumage by the end of September. This is represented by the typical dark-green head, with a narrow white neck-ring leading to a chestnut-brown breast. The body upper-parts are brownish-gray with grayish under-parts. The rump and under-tail coverts are black. The tail combines white outer tail feathers with strongly recurved black central tail feathers. The wings flash an iridescent blue to violet-blue speculum on the upper-side of the secondaries. The bill can be yellow to olive in colour, while the feet and legs are red (Drilling *et al.*, 2002). This particular breeding colouration may be crucial in female mate selection, with females rejecting atypical colouration (Klint, 1978). Female mallards replace their wing feathers, becoming non-flighted at this time (Palmer, 1976).

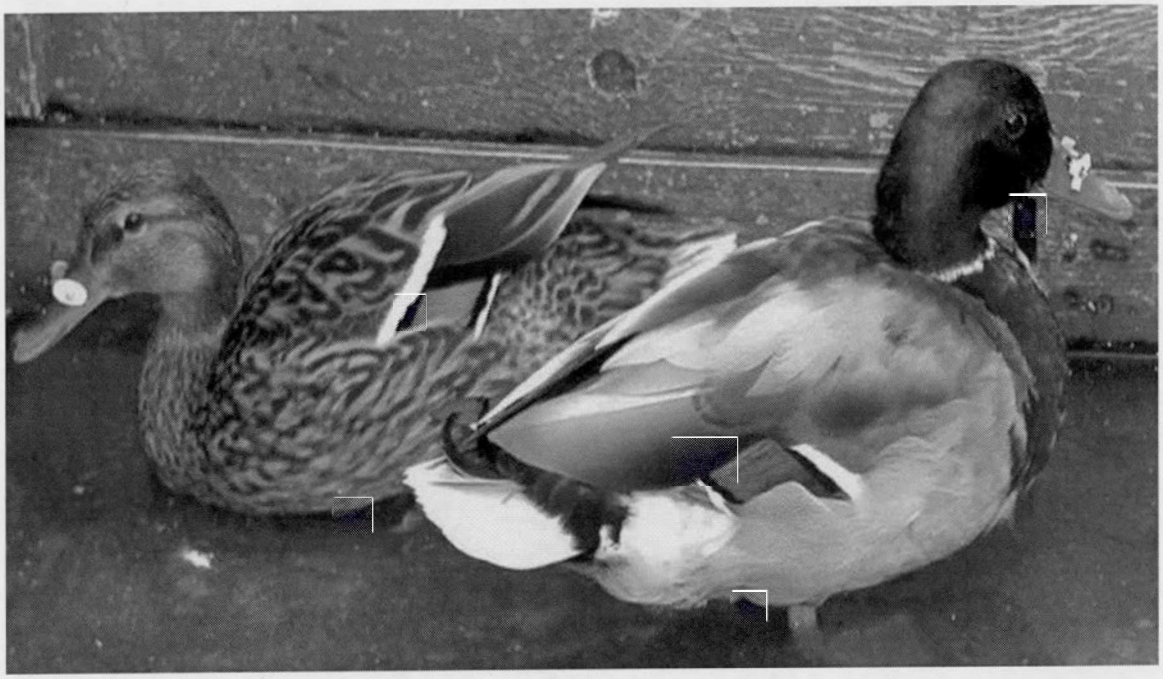


Figure 1.12 The male (right) and female (left) mallard in alternate or breeding plumage.

1.4.2.4. Onset of Breeding Behaviour

The male-male sexual displays increase in frequency during this period (Johnsgard, 1960). These major displays are described by Lorenz (1951) as the 'Gruntwhistle', 'Head-up-tail-up' with the associated 'Nod-swimming' and the 'Down-up' and are among the ten primary male displays (Lorenz, 1958) (Figure 1.13).

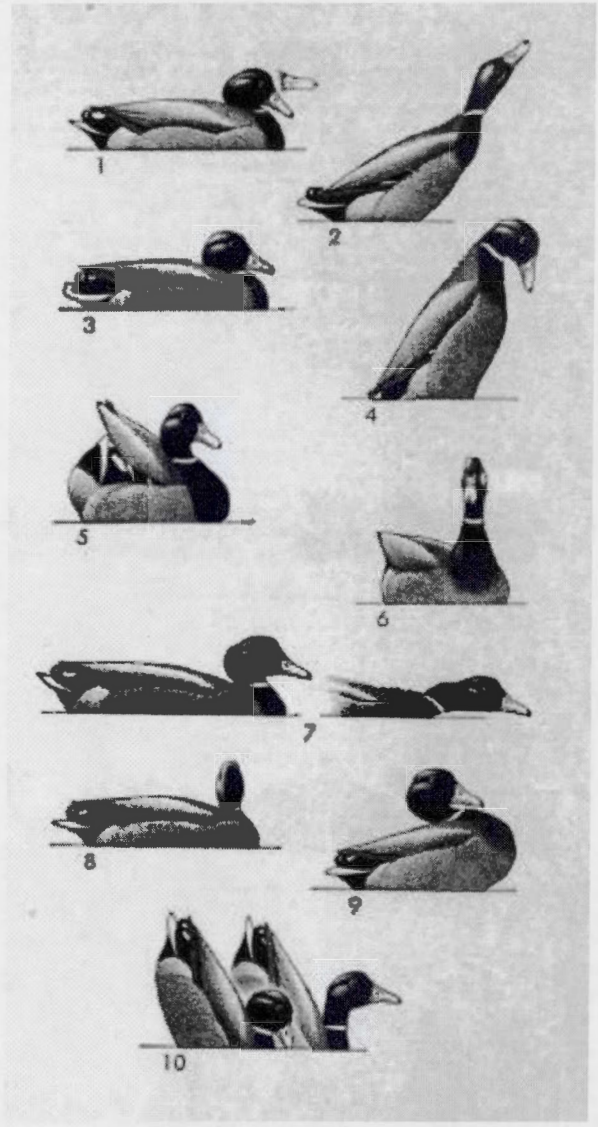


Figure 1.13 Ten courtship poses which belong to the common genetic heritage of surface-feeding ducks are shown here as exemplified in the mallard: (1) initial bill-shake, (2) head-flick, (3) tail-shake, (4) grunt-whistle, (5) head-up – tail-up, (6) turn towards the female, (7) nod-swimming, (8) turning the back of the head, (9) bridling, (10) down-up (Lorenz, 1958).

Pair bonding begins with many new bonds being formed (Rowher and Anderson, 1988), or resumption of a previous pair bond (personal communication F. Rowher) as females chosen males (Lorenz, 1951). The inciting display along with copulatory behaviours seals and maintains the bond (Bossema and Kruijt, 1982). Johnsgard (1980) described the relationship between the frequency of these male-male displays and the percentage of pair bonded birds in the mallards, indicating that the courtship poses described by Lorenz (1958) decline as previous pair-bonds are re-formed or new pair-bonds begin (Figure 1.14).

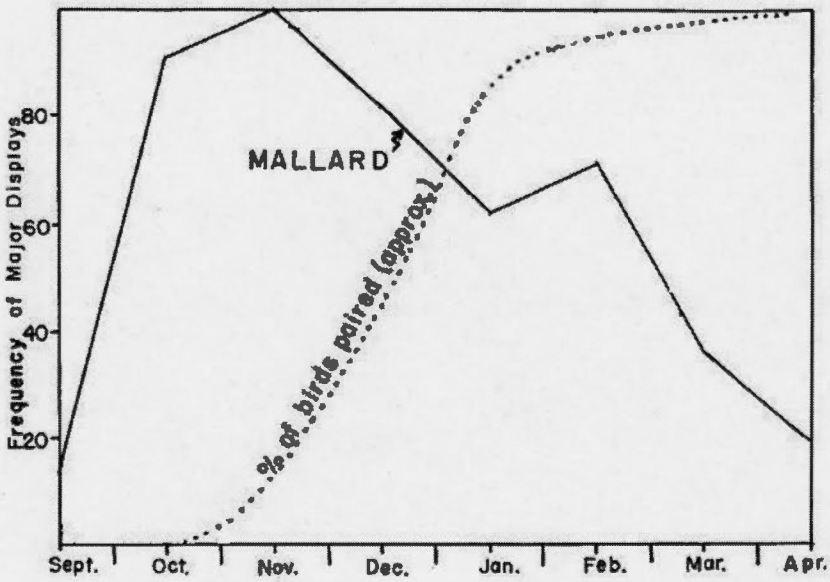


Figure 1.14 Relation of approximate period of pair bond formation in mallards to the relative frequencies of major male displays. *Adapted from* Johnsgard (1960).

1.4.3. October – November

1.4.3.1. Location of birds

The timing of the fall migration is spread over several months and is somewhat dependent on the weather. Severe storms may hasten this process, however, the primary factor in pushing this move may be related to the loss of access to food and water during the freezing of open water bodies (personal observation; reviewed in Drilling *et al.*, 2002).

Their migration takes the birds south to areas along the Gulf of Mexico, from Florida to Texas. The sex ratio in the migrating flocks is almost equal with a male to female ratio from 56:46 (Petrides, 1944) to 52:48 (as reviewed in Drilling *et al.*, 2002) reflecting a slight male dominance. However, there are now significant numbers of birds staying further north, in areas where open water is found all winter long. These flocks are predominantly unpaired males (personal observation).

1.4.3.2. Reproductive cycle

Male mallards show a bimodal pattern of testosterone peaks with the second peak occurring through this period (Paulke and Hasse, 1978). This testosterone peak is potentially linked to the reproductive behavioural displays through this period, and the initiation of pair bonds (Rowher and Anderson, 1988). Other evidence links beak colour to pairing success. Peters *et al.*, (2004) explored the impact on the level of circulating carotenoids in male mallards challenged with sheep red blood cells to initiate a non-pathogenic based immune response. The increased level of antibodies was mirrored by a decrease in carotenoids and testosterone. Female mallards may be able to discern those males with higher testosterone through the increased ornamentation of the beak coloured by correspondingly higher carotenoid levels (Omland, 1996a,b). About 55% of the females leaving for the wintering grounds in Louisiana are already paired (Johnson and Rohwer, 1988). Johnsgard (1960) recorded that 90% of the female mallards leaving the Ithaca region of New York were pair bonded. Although the testes are still regressed, copulation was recorded as occurring with the highest frequency in October and November (Johnsgard, 1961). This behaviour is perhaps closely related to the newly formed pairing or re-pairing of the birds, aiding in cementing the pair bond.

1.4.4. December to January

1.4.4.1. Location of birds

Once the paired birds have arrived in the overwintering regions, it is time to simply forage and maintain the established pair bond. For those birds that are not yet paired, the opportunity to find other un-paired birds is increased with many birds arriving from regions all over the North American breeding grounds. This may contribute to the genetic movement of changes within the population across the continent.

1.4.4.2. Reproductive cycle

This period continues to see pair bonding occurring (Figure 1.14). Once day length begins to increase, the gonads respond by increasing in size and activity (Höhn, 1947).

1.4.4.3. Moult

The female mallard begins her basic moult, replacing all her body feathers. In contrast to the males, female mallards are more subdued, with a similar plumage all year long. The upper body is covered with a broken streaky pattern of buff, white,

gray or black on brown feathers. The under-parts are pale coloured tending towards a whitish mottled appearance. She has a speculum similar to that of the male, bounded with more white than the male. The bill is orange and splotched black when younger and more speckled with black with aging. The legs are orange-red (Drilling *et al.*, 2002). The wing feathers, however, are not replaced until her brood fledges (Palmer, 1976).

1.4.5. February – April

1.4.5.1. Location of birds

Migration begins with flocks of mallards moving northward. The arrival back on the breeding grounds is dependent on there being open water, so this process can begin in February in the southern regions of North America and extend to April in the northern prairies. These flocks may contain up to 200 birds, however, the pairs disperse over the next days, with many hens returning to their natal site or close to the previous year's successful nesting sites (Lokemoen *et al.*, 1990).

1.4.5.2. Reproductive cycle

Male mallards actively defend their chosen territory, although this behaviour may be more directed to protecting their mates (Goodburn, 1984). Active sperm production begins by the end of February and peaks at the end of April (Johnson, 1966) as the testicular recrudescence responds to lengthening days (Johnson, 1961).

1.4.6. April – May

1.4.6.1. Reproductive cycle

By mid-April in the prairie region, nesting and preparation for egg lay is well underway (Krapu and Doty, 1979) with luteinizing hormone rising from early in the month in both males and females (Figure 1.15) (Donham, 1979). Luteinizing hormone decreases rapidly in the female at the onset of incubation, however it remains elevated in the males. This supports observations that they have no involvement in brooding the eggs (Goldsmith and Williams, 1980) and that prolactin is implicated in incubation. Male sperm production peaks in April and then falls precipitously to zero at the end of May (Johnson, 1966).

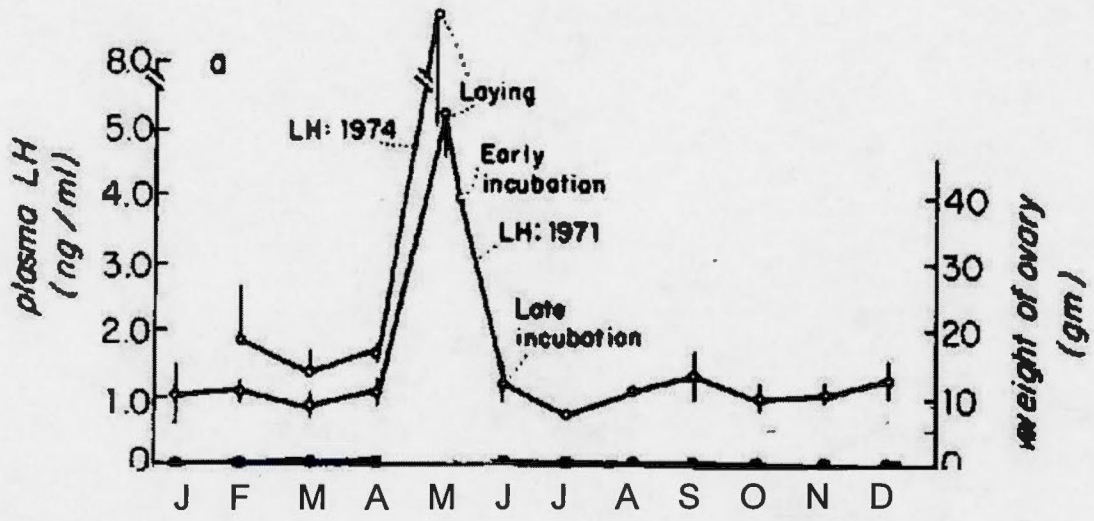


Figure 1.15 Plasma luteinizing hormone (LH) levels (ng/ml) in female mallards and ovarian weight (gm), month by month in 1971 and 1974 (*adapted from* Donham, 1979).

Once the nest site is chosen, the hen prepares a nest bowl formed of vegetation and lined with down feathers plucked from her breast area. She will also pull vegetation over the nest, forming a cover to add protection for her nest and herself (Caldwell and Cornell, 1975; Palmer, 1976). The male stands guard over the hen as she lays her 7 to 10 eggs, although records indicate this may range from 6 to 15 eggs (Bellrose, 1976; Bent, 1923; Palmer, 1976). Egg production is somewhat dependent on weather conditions, with egg production delayed when the daytime temperatures are below 10°C.

Male mallards, after arriving with a very good body mass, lose a significant amount of weight during this period. This is reflective of the high level of activity involved in pair bond maintenance, nest searching and territorial defense (Krapu, 1981). Female mallards that have arrived in good body condition will initiate nesting some 15 days earlier than those with lower body mass (Devries *et al.*, 2008). Weight gain in the hens of some 100g may simply reflect maturation of the reproductive organs (Krapu, 1981) and perhaps the presence of an egg in the oviduct.

The local environmental conditions have little influence on the clutch size in mallards as lipid reserves are acquired prior to arrival on the nesting grounds (Krapu, 1981). Female mallards use their lipid stores during egg deposition and incubation, loosing some 25% of their body fat stores at this period. These lipid reserves in the hens are also positively correlated to clutch size. If re-nesting is attempted, the required lipids are acquired in their diet, perhaps a more difficult proposition later in the breeding season and thus reflective of the smaller clutch sizes reported. Proteins however are acquired concurrently with egg lay by the ingestion of invertebrates. The other essential resource is water, however there is little to indicate that water conditions impact clutch sizes (Pietz *et al.*, 2000). When the mallards leave the nest during egg lay and incubation, foraging consumes a significant amount of time to ensure adequate nutritional intake for reproduction.

1.4.7. June – July

1.4.7.1. Location of birds

Having left the hen to incubate and care for the ducklings, male mallards gather together in the moulting grounds (Gilmer *et al.*, 1977). By mid-June all the male groups have assembled, and the predominantly male-male groups remain together through their moult into their basic plumage (Johnson, 1961; Palmer, 1976). This plumage closely resembles that of the female. During this period of about 25 days, the male is flightless.

The females finish their parental duties as the ducklings reach approximately 6 weeks of age. At this stage the females abandon the ducklings, with 39% remaining on the breeding grounds (Gilmer *et al.*, 1977), the rest joining other females and males in protected areas, as they go into post-reproductive wing feather moult (Palmer, 1976).

1.4.7.2. Moult

Male mallards moult into their basic or eclipse plumage once they separate from their mates and join non-breeding male flocks. This plumage is similar to that of the female, but the males may retain remnants of a solid dark-greenish crown and warm brown breast (Drilling *et al.*, 2002). Towards the end of this period the female mallards will begin their primary feather moult (Palmer, 1976).

1.4.7.3. Reproductive cycle

At this stage, luteinizing hormone has dropped to baseline levels in the female mallards (Donham, 1979). Prolactin, which increases during egg lay, also peaks at the end of egg production, remaining elevated through the period of incubation (Bluhm *et al.*, 1983; Hall and Goldsmith, 1983). Once the eggs have hatched, prolactin falls sharply towards baseline levels (Goldsmith and Williams, 1980) leading to speculation that tactile stimulation via the brood patch is required (Hall and Goldsmith, 1983). Oring *et al.,* (1988) speculated that prolactin returns to baseline levels once the precocial young are reaching thermal independence. Boos *et al.,* (2007) followed prolactin levels in mallard hens, finding baseline levels were restored at 6 weeks post hatch. Therefore, it can be expected that by the end of July, both luteinizing hormone and prolactin in mallard hens are at baseline levels.

1.4.8. Summary

Mallards are the most common dabbling duck, with a wide distribution, both natural and introduced to boost local populations (USFWS, 1991). With two major migrations annually and a breeding season impacted by many factors such as suitable territory, weather and availability of required nutrients, the wild mallard faces many challenges. Another challenge is its vulnerability to oil spills. As dabbling ducks dependent on both inland and marine shorelines, they are often found at sites of oil spills and are a common species admitted to oil spill rehabilitation centers.

1.5. Hypotheses and Objectives

The need to understand the complexity of oil spills and the impact on wildlife is important. Oil and oil products are not uniform in composition, therefore there is not one solution for all situations either. For those oiled birds admitted to a rehabilitation facility there are important questions to ask; do we clean and rehabilitate all oiled animals or euthanize some of them, or are there other tools to help decide the best course for that individual? By understanding the level of impact on each bird, we have the opportunity of using a triage system that is truly geared towards evaluating the potential for the individual to overcome the loss of water-proofing, starvation and dehydration, and also the toxic assault due to oil products within the body.

Hypothesis 1.

The protein profile in mallards shows a dose-dependent acute phase response after crude oil ingestion.

Specific objectives

1) To develop and optimize a method using capillary electrophoresis technology to identify and quantify serum proteins in mallards.
2) To expose birds to a range of oil doses to examine the possibility of a dose-response relationship.
3) To determine if there is an observable acute phase into a chronic phase response in mallards dosed with crude oil.

Hypothesis 2.

Crude oil ingestion in mallards negatively impacts subsequent reproductive effort.

Specific objectives

4) To evaluate male-female pair bonding success following crude oil ingestion.
5) To evaluate the reproductive success as measured by egg production, fertility and hatchability.

One might define a fully rehabilitated bird as continuing a life cycle typical of its species after oil exposure. This would include reinsertion into the population, typical behaviour and reproductive success. Ideally, tests such as acute-phase protein profiles would provide an indication of the potential for complete, long-term rehabilitation. The eventual goal of this line of investigation is the development of specific acute-phase protein ranges for both normal and oil-impacted avian species which can act as an additional step in the triage protocol for birds admitted during an oil-spill response to a wildlife rehabilitation center.

CHAPITRE II

METHODS AND MATERIALS

Mallards in captivity are well studied, so that baseline data are readily available (CCAC, 1993). They are also resistant to stress-related diseases including aspergillosis. This fungal disease causes losses in pelagic avian species within an oil spill response (Beaulieu, 1996; Mazet *et al*, 2002; and Tseng, 1999) by growing over the trachea, lungs and air sacs, effectively suffocating the bird. These reasons contributed to choosing mallards as the research species. This research required ethical permits which were approved by UQAM's Animal Care Committee and the Canadian Wildlife Service (Scientific Permit CWS00-M021).

2.1. Animal housing facility

Several areas in the Delta Waterfowl facility, Delta Marsh, Manitoba, Canada (50°11'1.77"N, 98°18'37.94"W) were used for this research. Table 2.1 shows the housing areas used throughout the year and the activities in each area.

Table 2.1 Housing, time frame and research activities

Year	Dose group	Housing	Time frame	Activities
2001	Female	Breeding pens	August	Dosing with oil
				Blood collection
		Outdoor aviary	September	Flock Rest time
		Indoor aviary	October to December	Flock Overwintering
2002		Indoor aviary	January to early April	Pair-bonding activities
		Indoor aviary	April to May	Oil dose groups
		Breeding pens	May to June	Second clutch
		Outdoor aviary	July to September	Flock Rest time
	Male	Breeding pens	August	Dosing with oil
				Blood collection
		Outdoor aviary	September	Flock Rest time
		Indoor aviary	October to December	Flock Overwintering
2003		Indoor aviary	January to early April	Pair-bonding activities
		Indoor aviary	April to June	Oil dose groups
		Outdoor aviary	July	Flock Rest time

2.1.1. The breeding pens

The breeding pens were set up in a 30.8 meter by 6.2 meter outdoor aviary. The floor sloped to a channel in the middle which allowed the flow of water from one end to the other along the 30.8 meter length. A unidirectional flow of water continuously

moved through the wet zone along the centre of the aviary (Figure 2.1). The plywood sided breeding pens were constructed within the aviary, centered on the middle of the water channel (Figure 2.2). Each individual breeding pen measured 2.3 meters by 1.23 meters and was covered with a wire mesh top. The breeding pens were set up with a dry zone where food and nesting boxes could be located. They were also flushed daily with high volume water flows, ensuring continual cleaning. Each breeding pen was cleaned every 2 days. The pen arrangement also ensured birds could be housed from the Control to High dose groups along the water flow gradient to ensure no possible upstream contamination during the dose period.

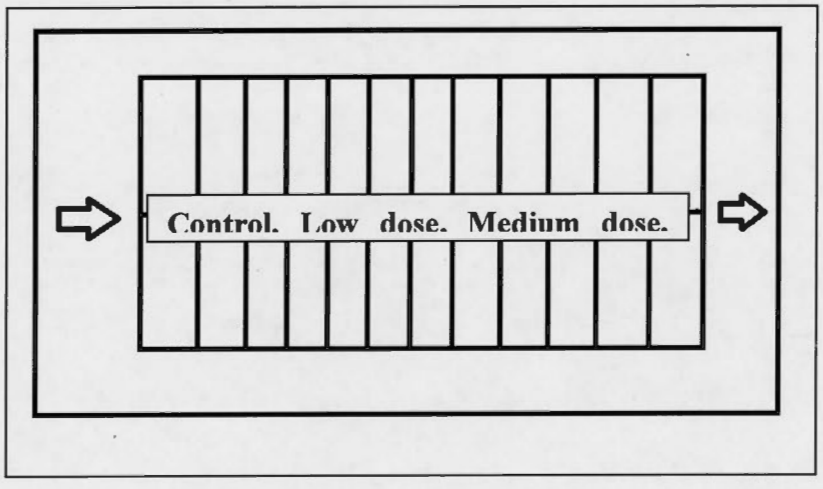


Figure 2.1 Diagram of the breeding pens showing the direction of water flow (arrows) and the dose group layout.

Figure 2.2 Duck breeding pens at Delta Waterfowl Research Station

2.1.2. The outdoor aviaries

These aviaries were constructed with a concrete waterproof base. The sides and roof of heavy-gauge wire mesh were supported by steel posts. The aviary floor area covered 30.8 meters by 6.2 meters (Figure 2.3) with a height of 2.4 meters. The aviaries had flooded floors and drainage ports to allow for an overflow system that continually removed the top layer of water. This flushing continued 24 hours per day. The water level was held at approximately 30cm deep along the sides to a 38cm deep central channel running the length of the aviary. The water was pumped from Lake Manitoba and passed through the aviaries, discharged into outdoor ponds and ultimately into Delta Marsh. Each aviary had a minimum of 3 platform pull-out areas

holding at least one gravity feeder. All of the birds had access at all times to the Delta waterfowl diet of commercially prepared high protein duck pellets (Ward and Batt, 1973), wheat, commercial poultry vitamin and mineral mix, oyster shell and grit. The feeders were filled as needed and cleaned daily to ensure no build up of wet pelleted materials. The aviaries were drained, hosed clean and refilled weekly.

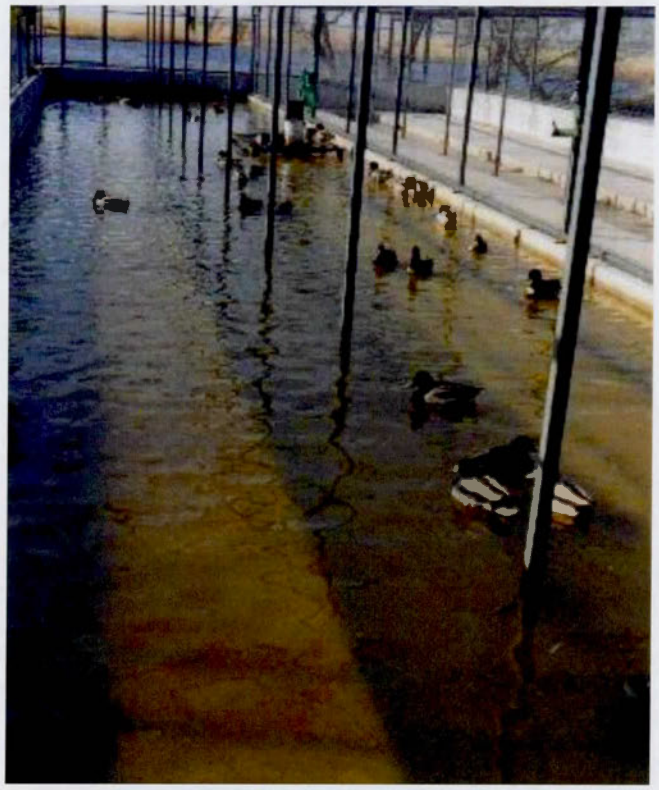


Figure 2.3 Outdoor aviary at Delta Waterfowl Research Station

2.1.3. The indoor aviaries

The building, the Gaylord Avicultural Laboratory, was a purpose-built facility opened in the early 1990's to house waterfowl. The indoor aviaries (Figure 2.4) were 8.62 meters by 12.92 meters and considered adequate space for at least 160 wild mallards (CCAC, 1993). Day-night cycles were maintained close to those in the local region which ranged from 8 hours on the shortest day to 12 hours by mid March. The indoor aviary lights were then set to increase day length artificially to 16 hours per day.

Temperatures were maintained at >5° C using industrial heaters. Each indoor facility had flooded floors (minimum depth 16cm) with a deeper channel (40cm) running down the length of each side of the building (Figure 2.5). Water was continually pumped from Lake Manitoba through the aviaries, discharged into outdoor ponds and ultimately into Delta Marsh. These aviaries were drained, hosed clean and refilled weekly.

Figure 2.4 Indoor aviary showing two of the four pens used to house waterfowl over the winter at Delta Waterfowl Research Station.

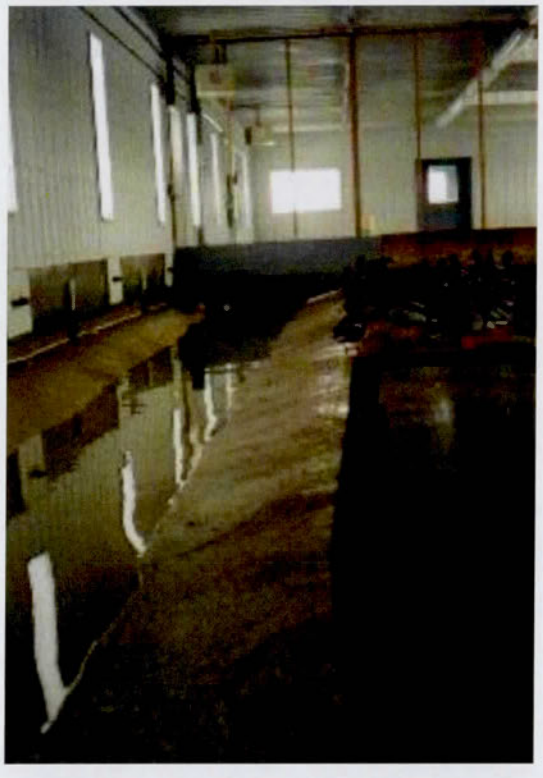


Figure 2.5 The deep water channel used along the sides of all indoor aviaries

Water flow was constant from sources at each corner of the indoor aviary, with outflow pipes to ensure constant water height and quality. Each indoor aviary had multiple raised (1.2 meter by 2.46 meter) platforms of wire mesh-covered frames. Multiple gravity grain feeders were placed on these platforms. All birds had access at all times to the Delta waterfowl diet (commercially prepared high protein duck pellets (Ward and Batt, 1973), wheat, commercial poultry vitamin and mineral mix, oyster shell and grit). The feeders were filled as needed and cleaned daily to ensure no build up of wet pelleted materials.

2.1.4. The blood collection area

A work area was established in an air-conditioned indoor room in the main waterfowl facility of the Gaylord Avicultural Laboratory. A table covered with foam pads and towels provided a stable platform to restrain the ducks for the phlebotomy. The adjoining bench top held the digital balance, blood collection supplies and record-keeping materials.

2.1.5. The nest boxes

Nest boxes, based on the design by Ward and Batt (1973) (Fig. 2.6) were constructed as an octagon, such that the upper plywood sections overhung the lower ones to add

weather protection to the interior. Each box was constructed of 0.6cm thick plywood, and the dimensions were an overall length of 40.6cm and height of 32cm, with the opening measuring 15.9 cm by 14 cm. Straw was stuffed inside each box for nesting material.

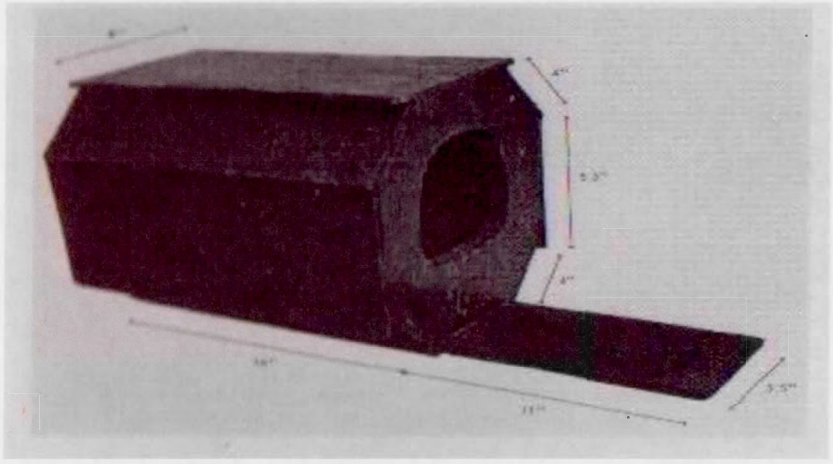


Figure 2.6. Duck nesting box designed by Ward and Batt (1973).

2.1.6. Artificial incubation and egg care

Artificial incubation of the eggs was carried out in a Humidaire® automated incubator (Figure 2.7) while hatching was carried out in a separate Humidaire® unit (Figure 2.8). Both units were thoroughly cleaned with hot soapy water, well rinsed and then turned on for 2 weeks prior to the expected onset of egg lay in the hens. The temperature and

humidity were monitored and recorded daily using the units' maximum/minimum thermometers and hygrometer. This allowed the units time to stabilize and ensure they were both functioning within the specified range required for incubation and hatching of mallard eggs. Once stable, the temperature was maintained at 37.5°C with a relative humidity of 70%. The incubator unit's automated mechanism rotated all racks through 90 degrees hourly.

Candling of eggs was carried out in a purpose-built darkened room. The bench held a recessed light tray allowing for a tray of eggs to be rapidly examined. Any egg that appeared abnormal was then candled individually with a single candling unit, essentially a flashlight.

Figure 2.7 Humidaire® incubator for the artificial incubation of eggs.

The Humidaire® hatching unit (Figure 2.8) ensured that temperature and humidity were maintained to optimize hatch using Delta Waterfowl's established standard temperature of 37.5° C and relative humidity of 70% (Ward and Batt, 1973). Each egg was placed in a small square covered unit to ensure that as it hatched, each duckling could be identified with an individually numbered foot web clip (Figure 2.9).

Figure 2.8 Hatching unit

Figure 2.9 Toe tag clipped into the foot web to identify individual ducklings.

2.2. Establishing the oil dose groups

In mid-July 2001, forty mature female mallards were randomly selected from a well established flock housed at the Delta Waterfowl facility. These birds were aged between four and six years, and had spent most or all of their lives at this facility. Many of these birds were born in captivity from wild eggs collected as part of a government program to rescue eggs from destructive farming activities, or had parents hatched from these programs. Each bird had a Delta Waterfowl leg band with a unique identification number. These birds were randomly divided into four groups of ten birds, identified as belonging to the control, low, medium or high dose group. Within each of these four groups, five birds were randomly assigned to one of two

subgroups. The subgroups were assigned to alternate days for blood sampling to allow longer recovery periods. Each subgroup was given different coloured leg bands for easy identification.

In 2002, the same protocol was applied to establishing a series of forty male mallards randomly selected to be placed in each of the dose groups; control, low, medium and high (Table 2.2). These males were mature birds, ranging from 4 to 7 years in age. These birds all had Delta Waterfowl bands with their own unique number. Again, the dose groups were divided into two subgroups of five birds per dose group and colour banded for easy identification.

Table 2.2 The randomly selected ducks were randomly assigned to a dose group and then to a sub-group.

Oil dose group	Sub Group 1	Sub Group 2	Total
Control	5	5	10
Low	5	5	10
Medium	5	5	10
High	5	5	10

2.3. Housing of the groups throughout the year (Table 2.1).

2.3.1. July and August

In mid-July, the selected birds were moved to the breeding pens. They were placed in numerical order of both dose group and sub-group along each side of the paired breeding pens that centered on the water channel. The birds were placed two per pen and one individual alone on each side of the paired pens. The control birds were placed in the first six pairs of pens from the primary water inflow point. Then the low, medium and high oil dose birds followed along the downstream water flow. This allowed for adjustment to this housing arrangement prior to the next phase of the research. The birds were housed in the breeding pens during the period of dosing and until regular blood collection was finished, approximately one month later.

2.3.2. September

As soon as the blood collection was completed for this stage, all birds were moved to a large outdoor aviary until early fall (Figure 2.3). The birds remained as a single sex group with all control and dose groups together for this rest period.

2.3.3. October to early April

The next move was to house the birds indoors for the winter. All of the birds in the different oil dose groups were housed together in one of the indoor flight aviaries. In the case of the female dose groups, 56 normal male mallards randomly selected from the Delta flock were introduced to this aviary in October 2001, to provide a choice of mates for the treated females. The male dose groups mallards had 56 normal mallard hens introduced to their indoor aviary the following October 2002.

2.3.4. April and May

All of the research mallards were moved into one of four indoor aviaries prior to egg laying activity. These aviaries then housed only those individuals from one dose group, their mates and extra potential mates. The allocation of each aviary to a dose group was done by blind drawing to ensure no bias in assigning an aviary. All aviaries were fitted with the same number of raised platforms (6), feeders (6), and a total of ten nesting boxes placed on the raised platforms. All nesting boxes were stuffed with fresh straw prior to placing them within each aviary.

2.3.5. May and June

In 2002, all of the mallard hens with their mates, or randomly chosen males where the hen was unpaired, were moved to the outside breeding pens once they were reconstructed after the thaw.

In 2003, the entire male dose groups were maintained in the indoor aviaries until egg lay had ceased. The change in protocol was necessary as the facility was to be closed and depopulated by August 2003.

2.3.6. July to September

With all reproductive activity finished, all birds were released into the outdoor aviaries as a single sex group and maintained until the experiment was terminated.

2.4. The fate of the eggs

2.4.1. Eggs

In both 2002 and 2003, all eggs laid in the indoor flight pens were collected on a daily basis over a six week period. Egg lay began as soon as all birds were separated into their individual dose group flight pens. This ensured that all eggs could be identified to the oil dose group. Each egg was dated and labeled with its dose group in pencil on the shell. In accord with the established practice at Delta Waterfowl Research Station, eggs were held at room temperature for one week. The eggs were then placed in the Humidaire® incubator.

During 2002, all eggs with viable embryos were incubated to hatch. However, in 2003, all the eggs collected from the male dose groups were incubated to day 18 and placed in a refrigerator at 6°C.

Candling (Figure 2.10) was carried out weekly in the first three weeks in 2002 and for the first two weeks in 2003 to ensure infertile or infected eggs were removed and refrigerated immediately. In the final week, candling was done daily to ensure that any internal pipping indicating that hatch was imminent was noted and the egg transferred to the hatching unit. Internal pipping was seen as movement or evidence of a beak in the airspace of the egg. Where embryo development had ceased, these

eggs were also placed in the refrigerator for later ageing of the embryo and visual examination for obvious problems.

If there was mortality in the later stages of incubation, those eggs were transferred as soon as embryo death was confirmed. Removal of these eggs was critical to ensure that they did not explode due to gases generated with bacterial growth and thus jeopardize the whole incubator (personal communication P. Ward, former director Delta Waterfowl).

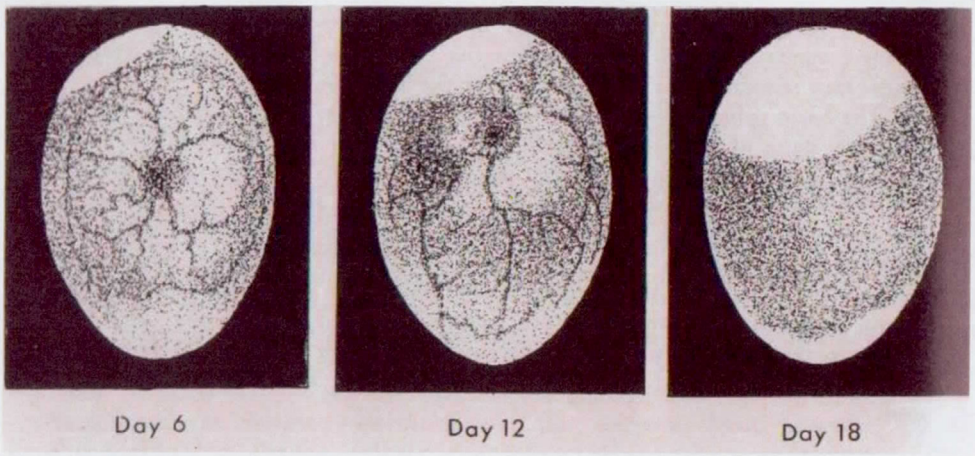


Figure 2.10 Images for days 6, 12 and 18, used in candling for the evaluation of embryological development in mallard eggs. (Ward and Batt, 1973).

In 2002, all of the eggs that had not hatched or were removed from the incubator for the reasons previously stated, were subsequently opened and the embryo aged using the diagram in Caldwell and Snart (1974) (Figure 2.11).

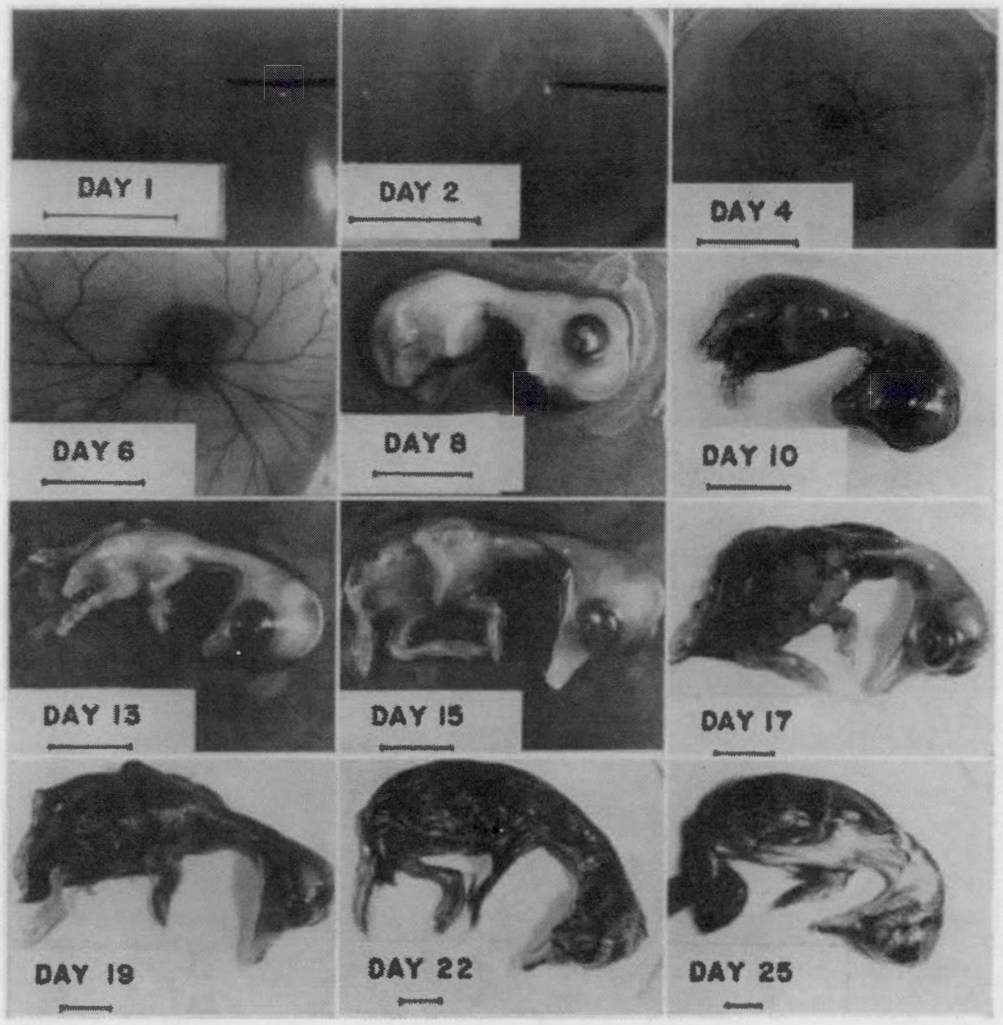


Figure 2.11 Photographic index to determine the age of mallard embryos. The line below the age designation represents 1 cm (*from* Caldwell and Snart, 1974).

Once the breeding pens were ready in June, all of the hens were placed outside in the breeding pens with their chosen mates. Un-paired hens were assigned a single male mallard. The hens were left to lay and incubate any second lay and nesting attempts. Once the nest hatch had occurred, all unhatched eggs were opened and the embryos if any, aged.

In 2003, the male mallards were the oil dose subjects, the mallard hens were all clean, all eggs were collected as per the female mallard dose groups and incubated. Incubation was halted at day 18 by placing the eggs into a 6° C refrigerator. All the eggs were then opened for examination and aging based on their development (Caldwell and Snart, 1973). This was necessary since this research project was finishing this season and there was no reason to allow hatching and subsequent euthanasia of any ducklings.

Where visually obvious deformities were noticed, data were collected including a description of the deformity, embryo age and dose group. Many of both the normal and abnormal embryos were then fixed in excess 10% buffered formalin. This process was undertaken to ensure any further investigation would have the necessary representative samples to explore this phenomenon.

2.4.2. Ducklings

At hatch, all of the ducklings were toe tagged with unique identification numbers using small web clips (Figure 2.12). These ducklings were then reared in predominantly same oil dose group units. Their weights and observational notes were recorded on a daily basis, usually between 8 and 10 am. Once old enough, at approximately three weeks of age, they were sexed by cloacal examination and fitted with uniquely numbered Delta Waterfowl leg bands.

These birds were maintained at the Delta Waterfowl research station for one year. Data were collected for further studies into the impact of dosing with Brent crude oil on the F1 generation. This data set included the maternal dose group, hatch date, weights collected over several weeks of growth, and any mortality.

Figure 2.12. Toe tagged newly hatched mallard duckling

2.5. The Test Oil

Brent crude oil was supplied by Dr. Merv Fingas, Director of the Environmental Emergencies Science and Technology Section, Environment Canada, Ottawa, Canada. This oil was chosen as the test oil for this study because it is shipped world-wide (personal communication M. Fingas, director, Environmental Emergencies, Environment Canada), and has been the product found in some oil spills. Wildlife oil spill responders have noted few issues in managing this type of oil with respect to the mortality rates in care. They survive the rehabilitation process and are released in good health with few apparent problems (personal communication Jay Holcombe, Director, International Bird Rescue and Research Center).

Brent crude oil comes from North Sea drilling operations. Brent blend is a light crude oil, which contains approximately 0.37% of sulphur, placing it in the class known as sweet crude. It is primarily used in the production of gasoline and middle distillates. In general, it is refined in the Northwest Europe region however, it can also be refined in North America and the Mediterranean region, depending on market prices. Therefore, this oil can be found in many of the major shipping areas of the world.

The American Petroleum Institute (API) lists Brent crude oil with a gravity of around 38.06 degrees and specific gravity of around 0.835, which indicates that this oil is lighter or less dense than water and will therefore float on water.

Although some laboratory studies have been performed on this oil, the exact components are proprietary information and are not available at the time of preparing this thesis. In common with all crude petrochemicals, Brent Crude has a combination of components including heavy metals and BTEX (Benzene, Toluene, Ethylbenzene and Xylene) which are small carbon chain volatile hydrocarbons.

The metals found in Brent Crude blend include Vanadium (V) (6ppm) and Nickel (Ni) (1ppm) (Oil and Gas Journal, 1999). The V content noted in this analysis indicate that the dose given in this experiment is equivalent to 0.12mg/20ml in the high dose group, or a range of dose equivalent based on the weight range in the high test groups (male and female) to 0.108mg to 0.144mg/kg. These dose levels are several orders of magnitude below 84.2mg/kg Vanadium pentoxide (V_2O_5) and 44.8mg/kg Sodium metavanadate ($NaVO_3$) where observable impacts were recorded in mallards (Rattner *et al.*, 2006). Ni toxicity was not reported by Brewer *et al.* (2003) where no observable impact was seen in mallards dosed with Ni coated shot pellets. These authors also reported that no observable toxic effects occurred even though significant levels of Ni were detected in the blood, liver and kidney.

The main volatile organic components (VOC's) of oil which are also found in Brent Crude include Benzene, Ethylbenzene, Toluene and Xylenes (BTEX). Environment Canada's Emergencies Science Division (ESD), River Rd, Ottawa, Ontario analyzed

this crude oil (ESD, 1996, 1997) to develop the basic data with the technology available at that time. The table 2.3 gives an excerpt with a field added to indicate the likely time period under field conditions that the VOC's would persist (personal communication Dr. Bruce Hollobone, Chemist, Environment Canada). The research was carried out under controlled conditions where the crude oil was maintained in a closed system at 80°C. Using the standardized weight loss points of 0, 14, 26, and 40%, the weight loss data is then linked to the ppm of the VOC in the sample. This is then linked to the time estimated for this loss to occur under field conditions. These data are variable and give an indication of the time that these compounds could still be found in a particular oil sample after exposure to the environment.

Brent Crude oil has the primary volatile components found in all petrochemical crude oils and avoids the highly toxic heavy metals that can be found in crude oils found in other geographic regions.

Table 2.3 Excerpted data from ESD Brent Crude publication (1996, 1997) showing the Volatile Organic Compounds (VOC's) and their evaporation percentage loss and ppm in a closed system. This is then related to the time estimated for this event under field conditions which may be highly variable.

Volatile Organic Compounds	Weight	ppm	Time range
Benzene	0	2280	0
Benzene	14	921	~1 - 2
Benzene	26	0	~5 - 10
Benzene	40	0	~20+
Toluene	0	6851	0
Toluene	14	5915	~1 - 2
Toluene	26	220	~5 - 10
Toluene	40	0	~20+
Ethylbenzene	0	2715	0
Ethylbenzene	14	2959	~1 - 2
Ethylbenzene	26	380	~5 - 10
Ethylbenzene	40	0	~20+
Xylenes	0	8703	0
Xylenes	14	9711	~1 - 2
Xylenes	26	2290	~5 - 10
Xylenes	40	0	~20+
C3-benzenes	0	11923	0
C3-benzenes	14	14025	~1 - 2
C3-benzenes	26	5680	~5 - 10
C3-benzenes	40	0	~20+
Total BTEX*	0	20550	0
Total BTEX	14	19577	~1 - 2
Total BTEX	26	2890	~5 - 10
Total BTEX	40	0	~20+
Total VOCs	0	32473	0
Total VOCs	14	33602	~1 - 2
Total VOCs	26	8570	~5 - 10
Total VOCs	40	0	~20+

BTEX - benzene, toluene, ethylbenzene, and xylenes

2.6. Dose rates and dosing process

Dosing was done over a two day period to mimic the slow steady ingestion of oil resulting from preening that would occur during an oil spill. To simplify the actual dosing process, each group was given a fixed amount of oil: 2ml, 10ml and 20ml. Therefore, the actual dose per kilogram of bird varied slightly from individual to individual. The total volume was divided into six doses with three doses given daily via gavage (Table 2.3). In addition to the oil, Tropican® parrot hand raising powder (R. C. Hagan Inc.) was prepared as a paste, and used during the gavage process with 1ml delivered, followed immediately by the oil, and then another bolus of paste. The control birds were given 6ml of the Tropican® paste.

Table 2.4 The oil dose per bird volumes and the total volume of Brent Crude Oil and Tropican®

Oil dose group	Volume of each	Volume of each	Total volume oil	Total volume
Control	0	6	0	36
Low	0.33	5.67	2	34.02
Medium	1.67	4.33	10	25.98
High	3.33	2.67	20	16.02

The dosing was done in the early morning, midday, and late afternoon. The birds were observed several times during the days of dosing and the day after dosing to ensure no adverse impacts were seen resulting in a distressed bird. The only event of

note was a very slight oil sheen seen coming from several medium and high dose group pens when the male mallards were the dosed birds. This indicated that some oil had passed intact through the gastro-intestinal tract and the total absorbed dose was slightly lower.

2.7. Blood sampling

In the mallard, of the three common sites for blood collection in birds, the brachial or wing vein was the most easily accessible (Figure 2.13). It provided a rapid collection point, minimizing the time required to collect samples, thus reducing the stress for the birds. The jugular site in these birds is covered with feathers, making it difficult to visualize the vein and the medial meta-tarsal (leg) vein is small and would not provide the volume of blood required in a timely manner.

A baseline sample of blood was collected one week prior to oil dosing. Blood sampling was begun the day after the dosing was completed, with samples collected every 24 hours for two weeks, then for a further two weeks at two day intervals. Samples were also collected every time the birds were moved and at termination. All samples were taken between 10:30am and 12 noon, except for the collection on the day of termination, when all birds were sampled between 8:00am and 10:00 am.

The birds were captured in the breeding pens, transported in plastic boxes with multiple air holes and an insert of plastic matting, ensuring that the birds remained dry and free of any faecal contamination. Each transport box weighed the same, so that taring could be performed rapidly prior to weighing each animal.

Figure 2.13 Blood sampling mallards, using the brachial wing vein

The birds were wrapped in towels, and positioned on a padded surface, dorsal side down, with the head covered, and right wing extended. Feathers were swabbed with small amounts of rubbing alcohol (70% isopropyl alcohol) over the elbow joint allowing for good visualisation of the brachial vein. Blood was collected using a single use, sterile 25G 5/8" needle (B-D® PrecisionGlide ®Needle) and either a 3ml or 5ml syringe (Terumo®), depending on the volume needed. Blood sample volumes

were based on the recommendations by the Canadian Council for Animal Care (1993). With the procedures in place, each bird was handled for only 10 minutes or less on the day of sampling.

Blood samples for serum were collected in a plain sterile BD Vacutainer®, and blood for plasma was collected in a sterile BD Vacutainer® tube containing the anticoagulant K_3EDTA. To ensure the anticoagulant had minimal dilution impact, blood volumes were kept proportionately high in these containers. Gentle mixing was done immediately. All blood samples were immediately placed in a rack over ice to provide initial cooling. The plasma samples were also used to prepare a capillary tube (Fisherbrand Micro-Hematocrit Capillary Tubes, sealed on one end using Fisherbrand clay sealant) for a hematocrit as well as a blood smear. As soon as the plain blood tubes samples had clotted, all tubes were centrifuged at 1000 RPM for 10 minutes in a bench top centrifuge. The serum and plasma were separated using a single-use plastic pipette, aliquoted into 0.5ml plastic vials and frozen at -32° C. They were later transferred to -80°C for long term storage. The haematocrit tubes were centrifuged in a bench top hematocrit centrifuge pre-set to 12,000 RPM for five minutes. The tubes were then read using a hematocrit reader to give the % of red blood cells in the sample.

2.8. Euthanasia and tissue preparation

All of the birds were euthanized over a 4 day period. All euthanasia's were carried out before 10 a.m., to minimize diurnal hormonal value changes (personal communication P. Spear). The birds were randomly captured from the flock, placed in the transport boxes, weighed, blood sample collected, and placed into a CO_2 chamber. Cooling of the bodies was begun immediately by placing them into a freezer. Only 8 to 15 bodies were prepared daily, depending on technical assistance, thus ensuring rapid processing of the animals. All the necropsies were carried out and tissue samples collected in anticipation of further studies.

All of the birds were opened ventrally and small tissue samples (~0.5g) collected from the lower lobe of the liver, lower portion of the heart, spleen J-I junction, lower duodenal loop with pancreas, thyroid glands, and reproductive organs. These samples were all placed in 10% buffered formalin. The ovary or both testes were excised and weighed. One testis was fixed in 10% buffered formalin, and the other was frozen on dry ice. Brain tissue was collected from all birds, and managed depending on the reproductive performance of the bird. Specimens representing birds that had been reproductively active or inactive, were divided into one of the two sample groups, ensuring representative tissue from both outcomes. Brain samples for histopathology were prepared by excising the whole head, removing as much organic material as possible: bill, skin and muscle. The head was then placed in a large container of

buffered formalin. The formalin was replaced with fresh formalin a week later for long term storage. The brain tissue for freezing on dry ice was prepared by removing the bone over the frontal and parietal region of the skull and removing the intact brain.

2.9. Capillary electrophoresis

In preparing to evaluate the protein profiles in the mallards, both gel and capillary electrophoresis techniques were explored. Both techniques are well validated within human laboratory technology with gel electrophoresis having a wider application in non-human research. Essentially these techniques involve the separation of the major protein groups with some individual proteins being represented by a single peak, however, many peaks will often represent many proteins that have similar characteristics and elude together. These two techniques also have differences that impact the appearance and elution times for some proteins. However, the overall outcome of both electropherogram techniques can give essential information regarding the status of the individual subject.

According to the scientific literature, the analysis of acute phase proteins in duck serum has not been carried out previously. Therefore, preliminary studies were performed to establish optimal conditions of analysis. The preliminary work was done on normal mallard serum samples to ensure reliability and reproducibility of the

technique. The serum samples were analyzed using a Beckman Capillary Electrophoresis unit (P/ACE MDQ Capillary Electrophoresis System, from Beckman Coulter Canada, Mississauga, Ontario, Canada) in the laboratory of Dr. Cameron Skinner, Chemistry Department, Concordia University, Montreal, Quebec, Canada. Based on techniques described in several papers (Chen *et al*, 1991; Jenkins and Guerin, 1995, 1997; Jenkins *et al*, 1995; Gao, *et al* 2008; Gao, *et al* 2009), a borate run buffer was developed to optimize protein peaks. Several other capillary electrophoresis run buffers were explored, including sodium and potassium phosphate buffers, which failed to give a clean electropherogram. While exploring the borate buffer system several parameters, including borate concentration and pH, were tested to ensure the most reliable results. Runs were performed using a concentration range covering 70mM to 150mM borate buffer, with 100mM borate buffer giving the cleanest peaks. Publications from other researchers indicated they used a range of borate buffer concentrations (50mM, 75mM and 100mM) with a range of calcium lactate concentrations (0.2mM, 1mM) (Jenkins and Guerin, 1995, 1997; Jenkins *et al*, 1995). These were evaluated, but did not give the desired results. Finally the addition of EDTA to the Borate buffer was evaluated. At 2mM EDTA the peaks were maximized and well separated. A pH range from pH 7.0 to pH 10.0 was tested with the 100mM borate, 2mM EDTA buffer. The best results were found at pH 9.7 with clear peaks and a maximized signal. For this work with mallard serum, optimal

results were obtained using the following run buffer; 100mM borate with 2mM EDTA at pH 9.7.

The serum samples required diluting to ensure the quantity of the protein did not overwhelm the capacity of the capillary electrophoresis unit to separate each protein group into discrete peaks. The dilution of the sample is dependent on the total amount of protein in the sample. For the avian samples, a 1:10 dilution was found to give optimal results based on the mean protein concentration in male mallards of 38mg/ml and 42mg/ml in female mallards (Fairbrother *et al.*, 1990). The diluent for the serum samples was explored using water, the run buffer and phosphate buffered saline. The phosphate buffered saline (PBS) pH 7.4 (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl) as published by Sambrook *et al*, 1989, was found to offer the most reliable results. This isotonic diluent avoided the issues faced with other diluents. The use of the run buffer, a typical diluent for many capillary electrophoresis techniques, resulted in signal return issues including a loss of peaks and general flattening of the protein peaks. Water, while appearing to preserve the individual peaks, resulted in a baseline shift at the sample front.

The effect of the internal diameter of the capillary (50μm, 75μm, 100μm) was also tested and the best results were found using a 50μm internal diameter capillary cut to the standard cassette specifications resulting in a capillary length of 60cm.

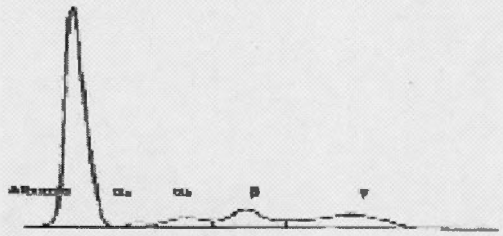
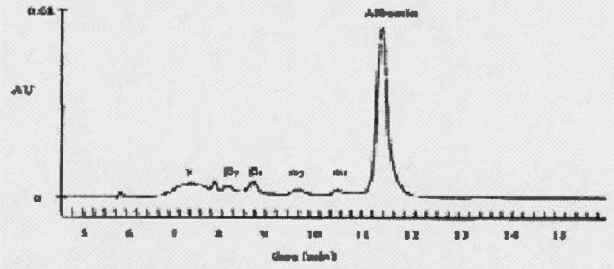
Samples may be collected under less than ideal conditions in some field sites. These may include little access to refrigeration or freezers, or equipment failure that results in thawing of stored samples. A series of runs were performed to explore the integrity of the protein groups under less than ideal conditions. One pooled sample of mallard serum was thawed, aliquoted into six sample vials and refrozen. Once re-frozen at -80°C for a minimum of 2 hours, five vials, then four vials, three vials, two vials and finally one vial were thawed and refrozen. All vials were then thawed, along with a sample vial that had never been thawed, diluted 1:10 in PBS and samples analyzed using the standard run conditions. Analysis of each major protein group indicated no significant loss of protein during the freeze-thaw process over 6 cycles.

A commercial capillary electrophoresis unit offers a wide range of functions, including allowing the set up of multiple samples, loaded at the same time in the sample trays, but not analyzed until many hours later. It is however, critical to ensure protein group integrity and maintenance of the proteins over time. Using a cycle of twenty minutes per sample, three samples were prepared from the same pooled mallard serum, diluted with three different diluents (PBS pH 7.4, borate buffer and water) and set to repeat runs over twelve hours. Although the PBS pH 7.4 had proved to be the most reliable diluent, it was valuable to ensure that there were no benefits to be gained in using another solution. There was no significant loss of peak presence or integrity over this sequence of runs. Every run was monitored using Sebia® Human

capillary electrophoresis control serum treated exactly as all the duck samples. The control serum was run every 10 samples and monitored for changes or loss of peak integrity by overlaying each trace and reviewing the resulting graphic visually.

Based on standard protein profiles in human and veterinary medicine and avian research, the standard protein profile of the mallard developed for this research (Figure 2.14) can be putatively identified. Examples used to aid in this process can be seen in Figure 2.15, Figure 2.16 and Figure 2.17. In general, standard gel electrophoresis results in the pattern of proteins reading from left to right; albumin, α1 globulins, α2 globulins, β globulins and γ globulins as seen in Figure 2.15 and Figure 2.17. However, this profile of a capillary electropherogram is reversed to represent a standard gel electropherogram view. In capillary electrophoresis, the elution begins with gamma globulin and finally albumin and transthyretin (Figure 2.14). The major factors that are involved and the resulting standard protein profile development in gel and capillary electrophoresis are noted in Table 2.5. Where corresponding protein patterns are known from this well established technology, the putative protein peaks are identified in Table 2.5.

Table 2.5 Factors that differentiate standard Gel Electrophoresis from Capillary Zone Electrophoresis developed in consultation with Dr. C. Skinner, Biochemistry Dept., Concordia University, Montreal.

Stages in Electrophoresis	Gel Electrophoresis	Capillary Electrophoresis
Sample preparation	Sample usually denatured and mixed with a high concentration of SDS[a] which binds to the protein at a uniform amino acid to SDS ratio producing a fixed negative charge per amino acid.	Sample proteins can be separated in their native state or can be denatured. There can be some SDS in the running buffer but usually it is dilute and does not bind at a constant SDS/amino acid ratio but is used to help neutralize some band spreading effects, minimize wall adsorption and to assist proteins in achieving a more uniform quaternary structure. The protein's charge is dictated by its amino acid sequence and the pH environment.
Electropherogram	The gel restricts the movement of the denatured protein/SDS complexes e.g. larger complexes are more restricted therefore the separation is based on molecular weight. Smaller proteins migrate faster/farther while heavy proteins are slower/nearer the start line. The distance - molecular weight relationship is, to a good approximation, logarithmic.	Proteins separate based on their net charge/volume ratio. The usual high pH eliminates positive proteins. The electropherogram usually displays a baseline disturbance around the time when the EOF[b] (including neutral molecules) comes through and is followed by the negative species. The first out will have low charge/volume ratios, later migrating species will have larger charge/volume ratios. Small anions, e.g. metabolites and inorganic anions, often trail behind the proteins.
Measuring Proteins	The gel is stained and placed in a detector. Using protein standards it is possible to calibrate the migration distance/time to molecular weight. Quantification can be made using staining and density detection, dynamic range, accuracy and precision are poor.	Using a, primarily UV absorbance detector, proteins are quantified using their absorbance. Absence of background absorption from the gel, freedom from staining, fixed path-length & optics results in a larger dynamic range, better accuracy and precision. Species are quantitated using protein standards, either pure reference proteins or certified reference materials/standards.
Standard electropherogram appearance with normal human serum protein profiles (Lehman *et al.*, 1997)		

a Sodium dodecyl sulfate

b Electro-osmotic flow

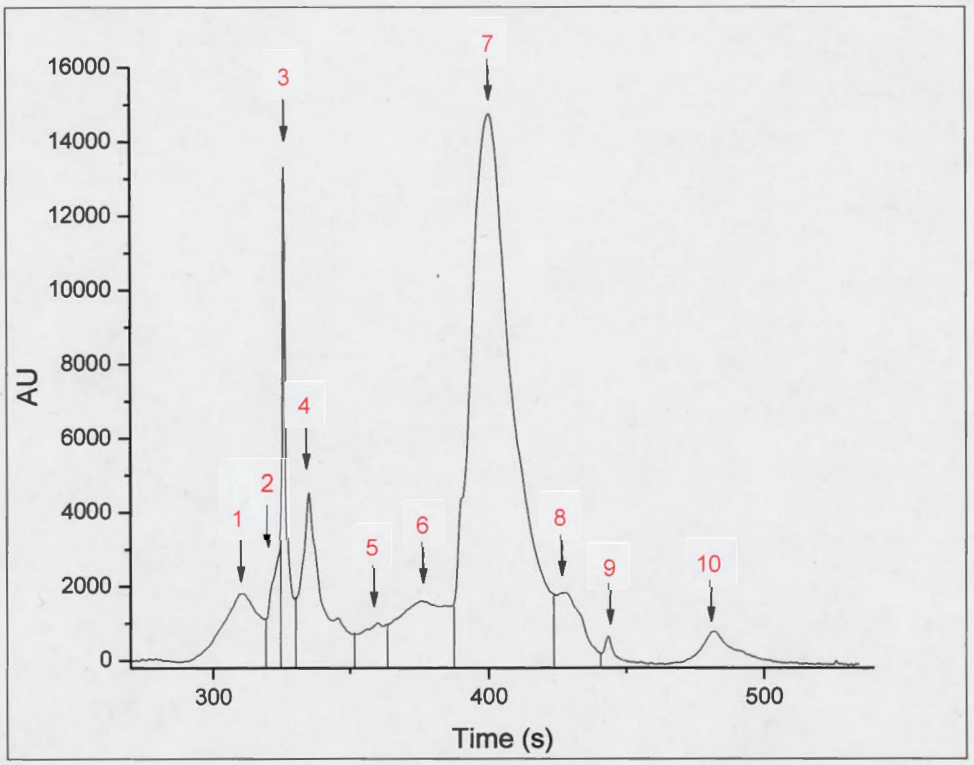


Figure 2.14 Trace of a normal mallard serum CE with peaks and their regions numbered 1 – 10 in Table 2.4. The time for the passage of the protein through the capillary is noted in seconds (s).

Table 2.6 The protein groups corresponding to the protein peaks in Figure 2.14

Peak	Putative protein group(s)
1	gamma (γ) globulin
2,3,4	beta (β) globulins
5	alpha 2 (α2) globulin
6	alpha 1 (α1) globulin
7	albumin
8	transthyretin (prealbumin)
9	unknown
10	unknown

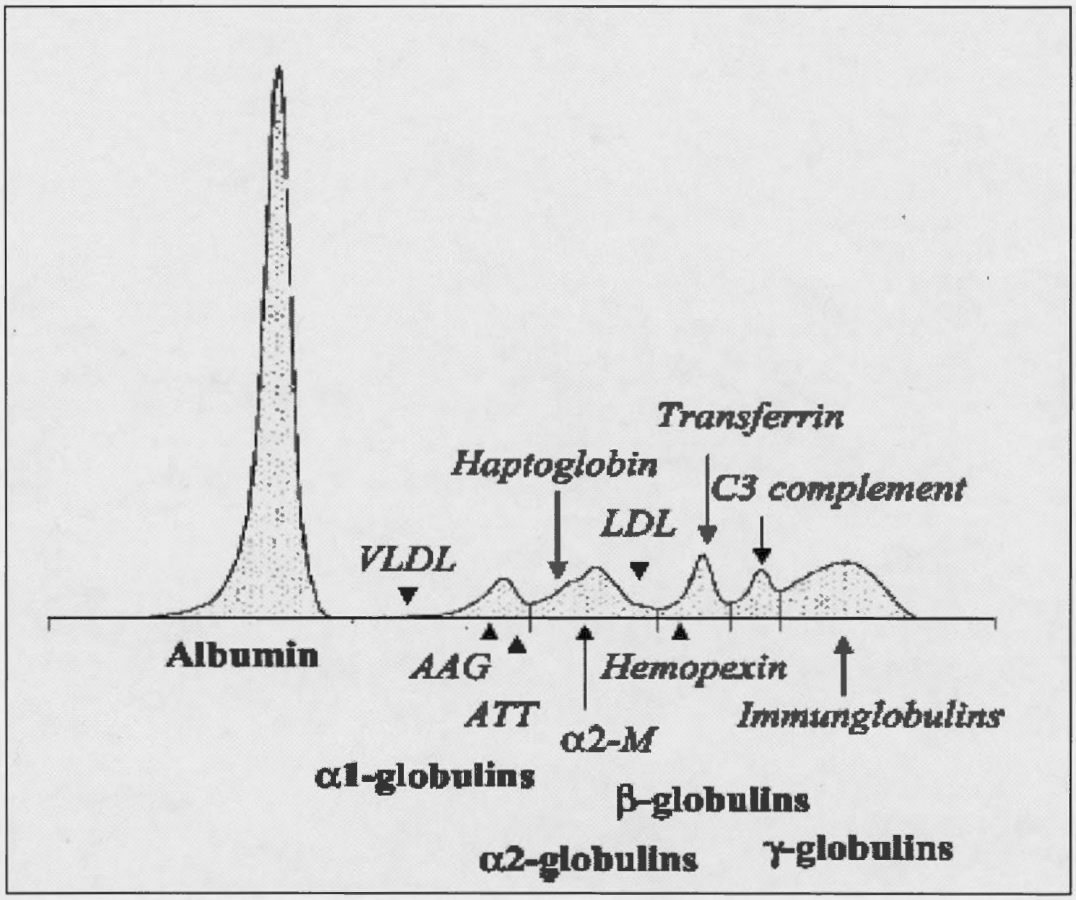


Figure 2.15 A normal human serum capillary electropherogram with the major protein peaks identified (Gay-Bellile *et al.*, 2003). The CE trace is reversed or normalized for viewing as for a conventional gel electropherogram.

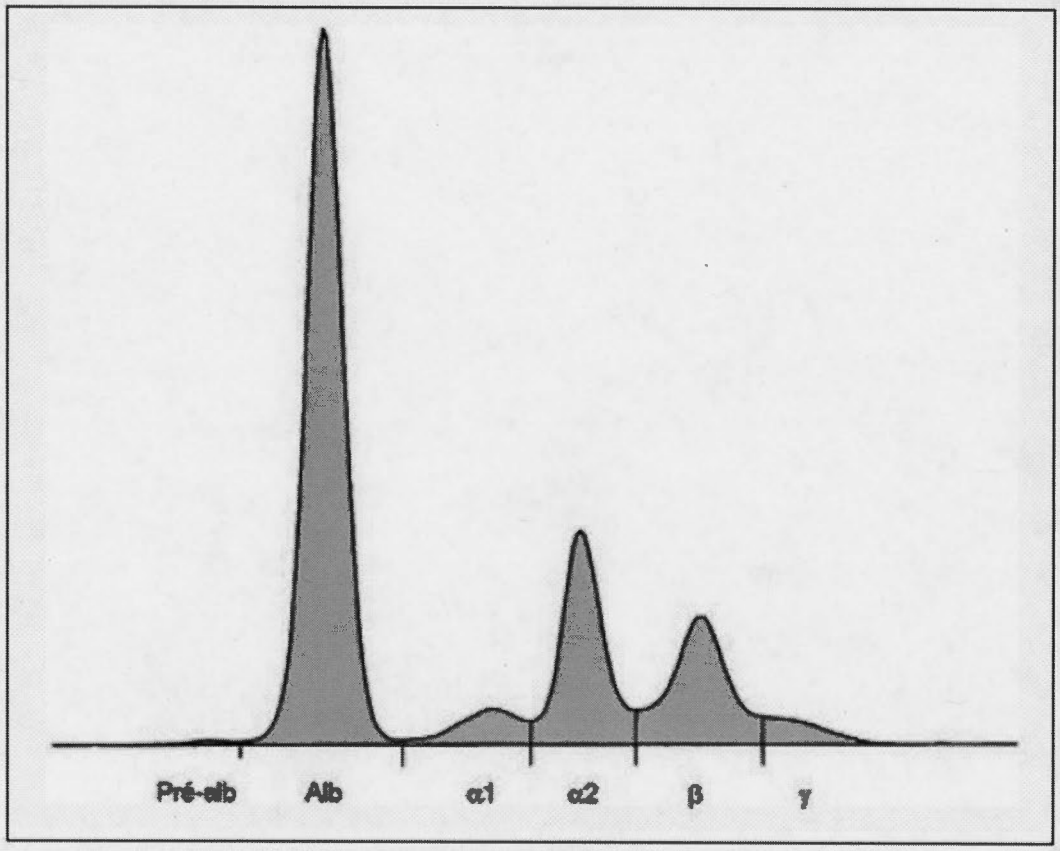


Figure 2.16 Example of plasma gel electrophoresis patterns of a Bar-headed Goose (Anser indicus). The major peaks noted are, (left to right) pre-albumin, albumin, α1, α2, β, γ globulins (from Roman et al, 2009).

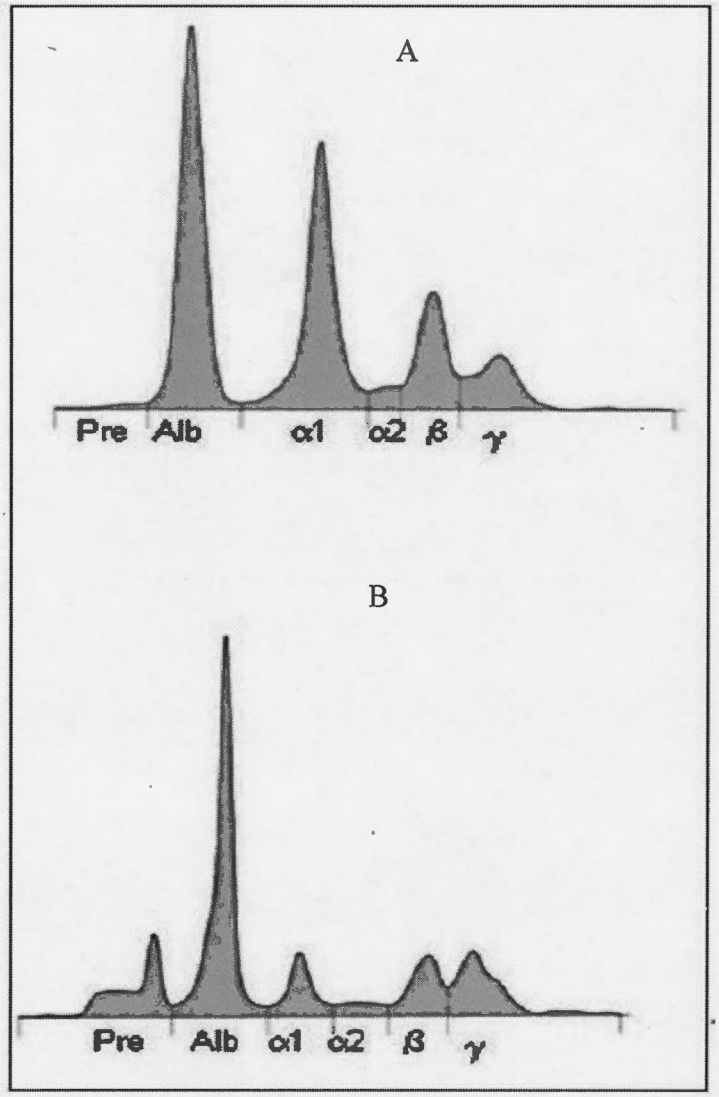


Figure 2.17 Example of plasma gel electrophoresis patterns of a Black Kite (*Milvus migrans*). Image A shows the electrophoretic pattern using agarose gel electrophoresis. Image B shows the pattern using capillary zone electrophoresis with the pattern normalized to that of the standard gel electropherogram. The major peaks noted are, (left to right) pre-albumin, albumin, and α1, α2, β, and γ globulins (adapted from Roman et al., 2013).

Interpreting the data in Figure 2.14 is based on these and other resources (Archer and Battison, 1997; Cray, 1997, 2000; Cray and Bossart, 1997; Cray and Tatum, 1998; Jenkins and Guerin, 1997) resulting in the putative identification of the peaks detailed in Table 2.4.

All samples were run and absorbance wavelength data collected from 190nm to 256nm. The data were imported into Origin® 8, software developed by the OriginLab® Corporation, Northampton, MA. Wavelength data from 190 to 200nm were summed and integrated to transform each sample's results into abundance of each peak of interest. This also allowed the development of tables of percentage abundance for the peaks of interest (Figure 2.14) and tabulated in the Results section. In doing these analyses, the control group results are used as the basis for comparing the oil dose group results. This is because an oil spill is a real world situation where one bird cannot be tracked, and the only way we can determine the degree of change brought about from the oil spill is to compare these results with other clean individuals at a comparable time in their annual cycle.

2.10. Behavior

2.10.1. Pair bonding

In April in both 2002 and 2003, the pair-bonded birds were evaluated in terms of the following behaviours: proximity, inciting behaviour, and head pumping (McKinney, 1992). These pairs were captured and removed to their new assigned indoor pens according to their dose group. Once all pairs had been identified and removed, all unpaired females in 2002, and unpaired males in 2003, were caught, identified and released into their assigned pen according to their oil dose group. Finally all unpaired birds were caught and randomly assigned to a pen, ensuring an equal number of males in 2002 and females in 2003 per pen and that there was still excess of non-dosed birds available for any breeding possibility.

2.10.2. Second clutch attempt

With the female oil dose groups, a second clutch opportunity was made possible once the outside individual breeding pens were reconstructed after the thaw. Known pairs were moved to these pens, and observations of the remainder of each group were undertaken to ensure no pairs were overlooked. The pairs' band numbers and other identifications were recorded and they were then moved to the appropriate outside pen. Unpaired oil dose group birds were randomly assigned a mate and moved to the

outside pens. At this stage, the birds were encouraged to attempt a second nesting by placing fresh nesting material and a nest box within each breeding pen. Observations continued noting if a second clutch was attempted. If so, the male was removed once incubation was well under way. The female was left to incubate and hatch the eggs herself.

2.10.3. Nest boxes

Once the four indoor aviaries were prepared and assigned to the four dose groups, nest boxes were added. A total of ten nest boxes per aviary per test group were placed on several of the pull-out platforms. Straw was added to each of these boxes, literally stuffed into the box as a wad of material. These boxes were then investigated by the female mallards, and organized into a suitable nesting environment. A normal nest included a sculptured base with the hen actively moving straw to form a solid structure. The hens then lined the bowl with down plucked from their breast feathers. Observations were made on all nests used by the hens in this study.

2.10.4. Blood sampling issues

Based on comments during August 2002 by technicians on this project that they were having more and more difficulty catching the dosed males, especially those in the high oil dose group, it appeared that these birds were undergoing behavioural changes. These apparent changes included what appeared to be hyper-responsive behaviours with the birds startling easily. A hyper-responsive behavior was manifested by greater difficulty in actually catching the bird, handling it and placing it in the transport box. Once inside the box, the bird often continued to make thrashing movements. Also noted were frenetic movements, characterized by twitching and obvious movement, even when restrained. In some birds, the heart beats could be heard. Although not quantitatively measured, it is of interest to note that in high oil dose animals, their heart beats were often audible during venepuncture as recorded in the day reports. Hyperventilation, a classic sign of stress, was noted frequently. Open mouth breathing, referred to as 'gullar fluttering', is also seen as a mechanism of cooling in avian species, however the incidence of hyperventilation was noted to be more common in the oil dosed groups as recorded in the day notes. All of these issues were reflected in the attempts to obtain the blood sample, which gave this research a measureable value.

To quantify these behavioural changes, a scoring system (Table 2.7) was instituted, identifying the number of attempts taken to obtain the blood sample. A score of one

or two indicated a normal venepuncture. A score of five indicated a bird showing extreme agitation.

Table 2.7 Scoring system used to quantify agitation level in mallards during the venepuncture.

Score	Venepuncture	Agitation
1	1	normal
2	2	normal
3	3	some
4	4	moderate
5	5	extreme

This system was used primarily in the 2002/2003 seasons with the male oil dose groups. However, several of the day notes from 2001 reflect difficulty with handling the high oil dose female mallards. The scoring system was therefore used on the female dose groups as the birds were handled one last time at the termination of that phase of the research.

2.11. Prolactin determination

Serum samples were sent to Dr. Gregoy Bedecarrats, Department of Animal and Poultry Science, University of Guelph, Guelph, Ontario, Canada for prolactin determination using radio-immuno assay (Bédécarrats *et al.*, 1990). At this time, this laboratory was the only available facility in Canada with an assay targeted for avian prolactin.

2.12. Statistics

Data were analyzed using SigmaStat version 2.2 and JMP IN version 4.0 SAS Institute, California. In the case of protein analysis by CE, the data treatment is described in Annex B. Briefly, protein data were transformed to achieve normality of residues and tested by multifactor ANOVA's. For the other results, parametric tests were performed on data shown to be normally distributed (Shapiro-Wilks test for goodness of fit) with equal variance (Bartlett's test for heterogeneity of variance). Two-way comparisons were conducted with the Student's t-test while multiple comparisons were first tested with analysis of variance (ANOVA) followed by specific pair-wise tests. Tests used for specific comparisons are indicated in each case in the Results section. Specific comparisons to the control group were tested by the Dunnett's test. Data sets that did not comply with the assumptions of parametric statistics were compared using either the Wilcoxon test or the Kruskal-Wallis Chi^2

approximation. Where observed data were compared to expected (control) values, the Fisher exact test was employed due to low sample numbers. Statistical analyses were carried out with assistance from Mr. Bertrand Fournier, Service de consultation en analyse de données (SCAD), University of Québec at Montreal.

CHAPITRE III

RESULTS

3.1. Behavior

Behavioural changes that have deleterious effects on the ability to reproduce, to meet nutritional demands or to avoid predation will ultimately decrease the probability of survival of the individual bird.

Oil has numerous recorded deleterious effects on birds. This section presents behavioural changes that were observed as a result of the defined oil challenge given in this work. An understanding of the profound impacts on the individual in the short term will allow a better appreciation of the effects that oil pollution may have on avian populations in the longer time frame.

This data leads through the chronological sequence of recorded anomalies from my research, from agitation to breeding behavioural changes. Much of the early data gathered were initially observational notes, but subsequently resulted in a scoring system to scale agitation levels in these birds. The next major behavioural issue arose from the poor pair bonding ability of the dosed birds, both in the female and male streams. The final notes cover the female dosed groups' second clutch and the lack of nest building and parental care behaviours.

3.1.1. Agitation

Agitation during the collection of blood was first noted during the early post-dosing period in the female dose group. Observations were subsequently quantified and recorded on the difficulty of collecting blood in the male dose group. This observation led to the scoring system as described in the materials and methods.

The data were first presented from the male dose groups; on days 14 and 334 post oil dosing, and the female data for 414 days post oil dosing. The male behavioural issues were most pronounced at the day 14 post-oil dosing period (Figure 3.1). The female mallards were only evaluated on the one day at 414 days post-oil dosing, using this system (Figure 3.3).

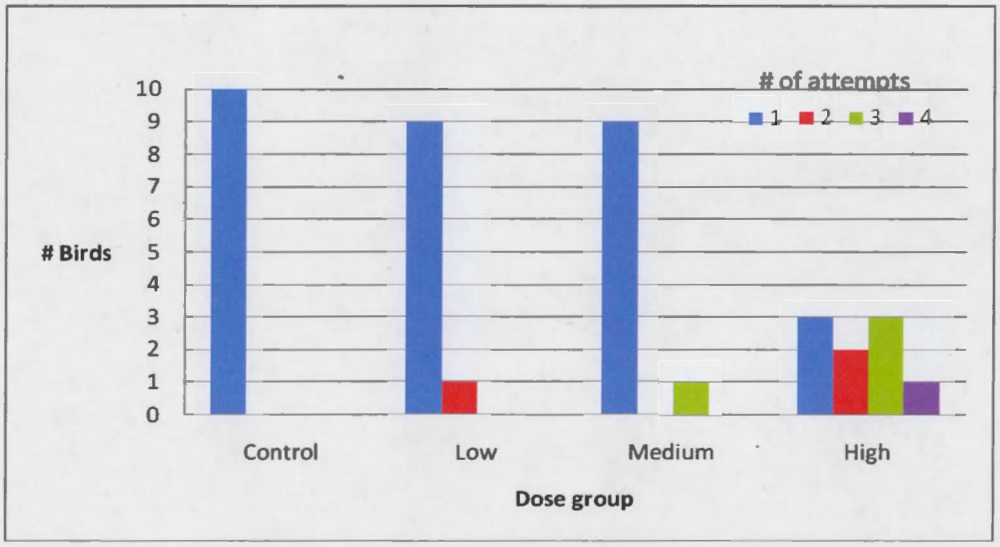


Figure 3.1 Male mallard agitation levels at 14 days post oil dosing (number of venepuncture attempts). There is a significant difference between the control dose group and the high oil dose. (Kruskal-Wallis; one way ANOVA; (P = <0.001).

When the male dose groups were re-evaluated at 334 days post-treatment, there remained a visible and persistent level of increased anxiety especially in the high oil dose males, however it was no longer significantly different (Figure 3.2).

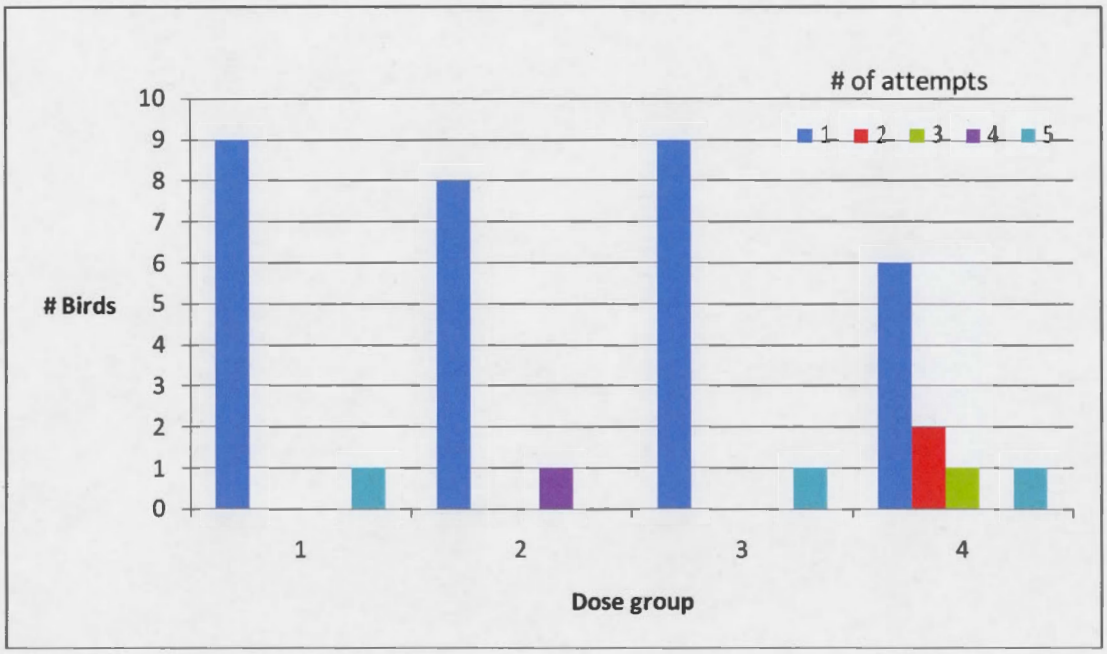


Figure 3.2 Male mallard agitation levels at 334 days post oil dosing (number of venepuncture attempts).

The female mallards were also showing agitation issues, throughout the treatment period which was reflected in notations in the day logs. Comments in the day log noted were exclusively directed to the high dose females and included audible heart beats while handling the birds, multiple attempts at venepuncture, and frenetic movements. At the termination of this series, the scoring system used with the male dose series was applied to the female mallards, and this highlighted a persistent increase in agitation in the high dose females (Figure 3.3).

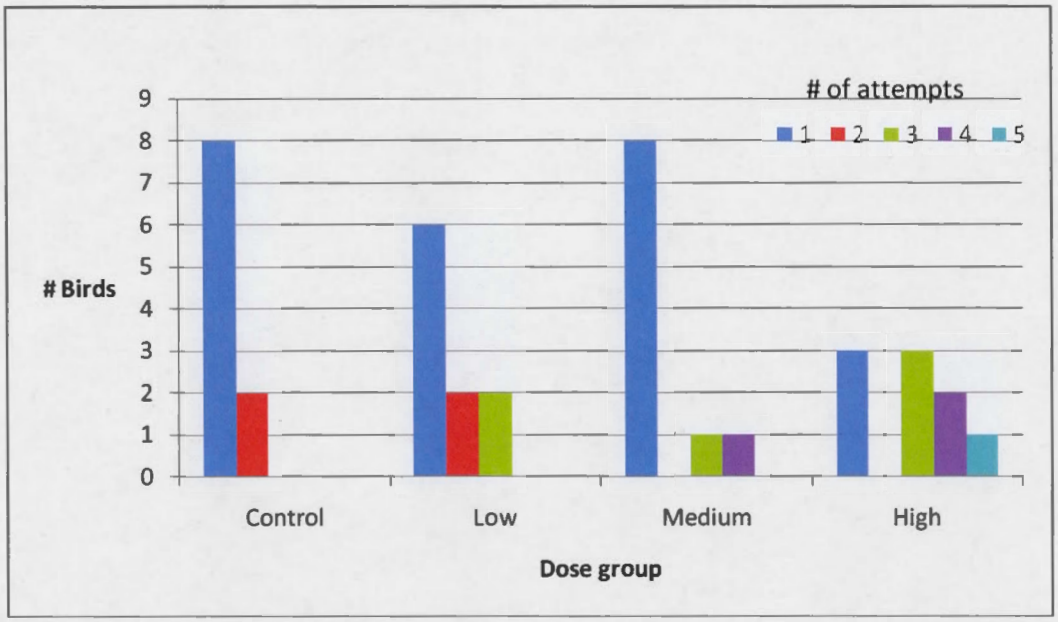


Figure 3.3 The female mallard agitation levels at 414 days post oil dosing (number of venepuncture attempts).

There proved to be a significant difference in the high oil dose females and the control female group (Figure 3.3) with regard to agitation levels; Kruskal-Wallis one-way ANOVA ($P = 0.043$).

3.1.2. Pair bonding

Pair bonding in mallards is initiated in late fall and is essentially complete at the end of February or early March of the following year. Disruption to pair bonding was the initial observation which led to the awareness that oil dosing in the research could have serious biological impacts on reproduction. It was found that there was a significant difference in female pair bonding between the control birds and the medium and high oil dose hens

(Figure 3.4). For the low dose females, there appears to be no significant impact on bonding behaviour from the crude oil ingested nine months earlier. The medium and high dose females, on the other hand, appear to have persistent failures to bond correctly as a result of the oil ingestion.

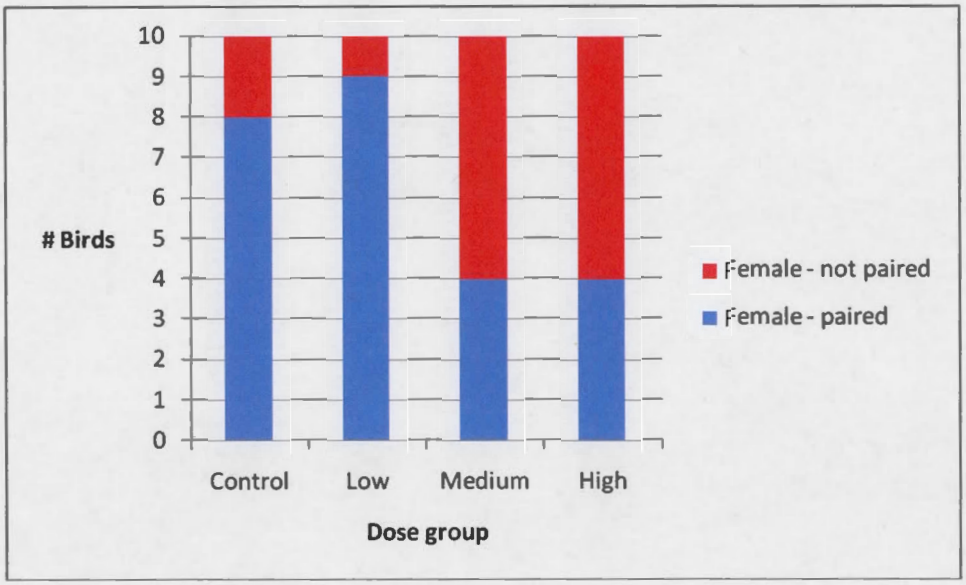


Figure 3.4 Pair bonded vs. non-pair bonded female mallards in May 2002 show significant differences between the expected pair bonding levels of the control hens compared to the pair bonding levels of the medium and high dose birds (chi-squared = 18.94; *df*=3; *P* = <0.001)

There was also a significant difference in male pair bonding between the control and oil dosed groups (Figure 3.5). Eight out of ten control pairs bonded, but surprisingly, only three out of the remaining nine low-dose male birds formed pairs.

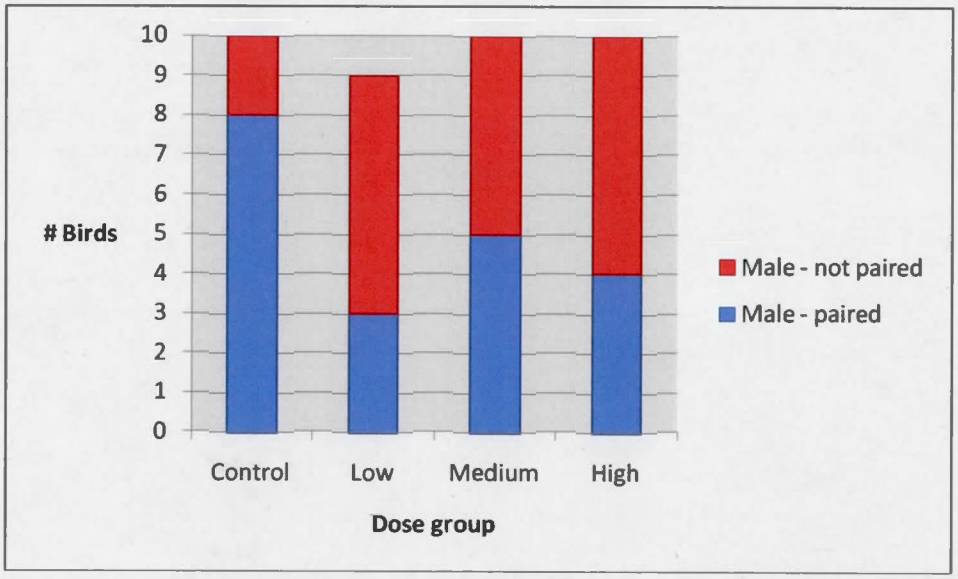


Figure 2.5 Pair bonded vs. non-pair bonded male mallards in May, 2002 show significant differences between the expected pair bonding levels of the control males and the pair bonding levels of the low, medium and high dose birds (chi-squared *df* = 3: *P* = < 0,002).

3.1.3. Nest construction

In 2002, the female mallards in the oil dose series were initially housed in the indoor aviaries. The four dose groups were all separated prior to the onset of expected egg lay and housed in one of the four indoor aviaries. Each aviary was stocked with ten nest boxes as the paired hens and their chosen mates were moved to them. Unpaired hens were added to their dose groups aviaries, along with extra male mallards. The nests were explored and organized by the hens in the control, low and medium oil dose groups. This meant the straw was compacted in the nest box and a nest bowl formed. The day log from the indoor aviaries note however, that the high dose nest boxes were stripped of their

straw on a regular basis. The straw provided was removed from the nest boxes and spread over the aviary floors, leaving the nest boxes denuded. The eggs laid in this time period compromised the first lay period.

Once the outdoor breeding pens were available, the paired birds were assigned to pens, while the unpaired hens were assigned a companion male mallard to share a pen. Their nests were closely monitored during the second lay period. Since the eggs were left with the hens, this would allow the hens to lay a true clutch. Where this clutch was attempted, all of the nests in the control, low and medium oil dosed birds had straw molded bowl lined with down (Figure 3.6). In cases where some attempt was made to form a nest bowl, this may be simply limited to a structure or compaction of the nest straw (Table 3.1). Not all resulted in egg production and in the case of the high dose groups, most eggs laid in all but one case, were either dumped in the nest box or on the pen floor.

Table 3.1. Nest boxes in the individual breeding pens where the hens had attempted to maintain a straw based structure.

Dose group	Nest bowl structure
Control	8
Low	8
Medium	10
High	1*

* the majority of the straw was ejected from all but one nest box.

The hens organized the provided nesting material (straw) into a base with a bowl depression (Figure 3.6). That depression was lined with down feathers plucked from the hen's breast region. The eggs were laid in this area, and covered with the down if the hen left the nest to drink, forage or defecate.

Figure 3.6. Control dose female nest box with a straw bowl lined with down plucked from the hen's breast area. (Note: All adult birds at the Delta Waterfowl facility were nasal tagged with colour-coded plastic markers. These posed no problem to the birds and were well tolerated by all birds.)

The high oil dose hens did lay eggs, however, their nest construction was disorganized or absent (Figure 3.7). In all but one of the high oil dose hens, the eggs that were laid to potential clutch size, had no parental care provided. The only nest that was constructed as expected for a mallard, was that of a pair-bonded hen and her mate. For all other high oil dose pairs, there was no nest structure and the few remaining bits of straw showed no

organization. No down was present and the eggs were laid and left haphazardly. These hens even damaged their own eggs. Many other high dose hens simply laid eggs around their pens, leaving them abandoned.

Figure 3.7 High dose female nest box showing six eggs laid on a thin bed of straw. Note that one egg was broken by the hen.

Successful nest building in both attempts to produce a viable clutch was another critical behaviour that was affected by the birds' treatment with oil.

3.2. Eggs

3.2.1. Egg production

All the female and male oil dose groups of a given dose level were initially housed in the same indoor aviary at the time of egg lay. Thus, egg production in the communally accessible nesting boxes did not allow us to evaluate the actual production on an individual basis. The egg production also represents the hens laying without the stimulation of a nest of eggs to provide feedback via the brood patch for lay to finish and incubation to begin. The female control and oil dosed groups were transferred to the outdoor breeding pens once they were available. This allowed for tracking of clutch production in the pair-bonded and assigned paired birds. Some of the mallard hens did produce a second clutch however, the disruption in moving the birds to the outside pens could also have caused changes to the reproductive cycle. The egg production from the indoor aviaries for both the female control group in 2002, and from the clean hens paired or assigned to the male control or oil dosed groups in 2003, can be used to determine the average egg production expected per hen within each of the oil dose groups (Table 3.2 and 3.3). The expected production is 13.8 eggs per hen in this flock. There was no true clutch formed as the eggs were removed daily. The data from the male oil dose series do not represent significant changes in egg production between the groups.

Table 3.2. Female mallard dose series total egg production in the indoor aviaries in 2002.

Dose group	Number of hens	# eggs in first lay	# eggs per hen
Control	10	141	14.1
Low	10	109	10.9
Medium	10	189	18.9
High	9	164	18.2

Table 3.3. Egg production from clean female mallards housed with the male mallard dose series in 2003.

Dose group	Total # eggs	# eggs per drake	# eggs per hen
Control	216	21.6	14.4
Low	166	18.4	11.1
Medium	220	22	14.7
High	201	20.1	13.4

These data represent the total egg production. The male data (Table 3.3) represent eggs from an equal number of hens (15 hens per dose group). Also included in Table 3.3 is the number of eggs per drake. The data from the male dose series do not represent significant changes in egg production between the groups.

Disruptive events during the reproductive period do result in reduced reproductive success. These influences can be either increased or decreased temperatures from the normal annual range (Hall, 1987). Handling and human presence also result in decreased egg lay (Bluhm *et al.*, 1983a,b). To eliminate potential bias, the female egg data (within

the same dose group) were collated from that collected while the birds were still in the indoor aviaries (Table 3.2, 3.3).

3.2.2. Egg fertility

Fertility was also addressed in this research. All eggs collected from the indoor pens were artificially incubated. With the female dose groups, artificial incubation continued allowing the fertile and viable eggs to hatch. The incubation of the male dose group eggs incubation was halted at day 18 by placing these eggs into refrigeration at 6°C. All eggs that failed to hatch were opened and examined for fertility, embryological development, and infection (Tables 3.4, 3.5).

3.2.3. Female dose group egg data

There are significant differences in the female oil dose groups' egg production between the fertile and non-fertile eggs (Figure 3.4). It appears that fertility is positively influenced by the ingestion of Brent Crude oil in the low dose group but not in the medium and high dose groups when compared to the control group. The data (Table 3.4) also indicate the high dose female oil dose groups show a trend towards lower hatchability.

The dead in shell embryos were examined and any developmental issues noted (Table 3.4). There were few gross abnormalities recorded and those embryos were fixed in

buffered formalin for further evaluation. One minor event occurred during the incubation with a power failure which resulted in a minor drop in the incubator temperature. This may have increased the mortality of the eggs in the incubator at that time.

The deformities in the eggs of the female control and oil dose groups were tabulated and no statistical differences were noted. However, it is interesting to note that there were obvious deformities that may yield greater information as to its developmental origin after further pathological examination.

There was little or no issue with infection in artificially incubated eggs during the research series involving the female dose groups (Table 3.4).

Table 3.4 Tabulated data for egg fertility data collected from male oil dose groups. Unless otherwise noted, the data show the percentage of eggs with the number in (). Data sets with statistically significant differences are denoted with the same letter.

Dose Group (# hens)	Total # eggs	Fertile eggs [a]	Infertile eggs [a]	Embryo with normal development - day 18	Embryo with abnormal development - day 18	Embryo died in shell - apparent normal development	Infected egg - no embryo observed	Infected egg - embryo observed	Fertile - no development after 24 hours
Control (15)	216	70.37 (152)	23.61 (51)	65.74 (142)	1.39 (3)	2.31 (5)	6.02 (13)	0.92 (2)	0 [b]
Low (15)	166	34.94 (58)	49.40 (82)	29.52 (49)	2.41 (4)	1.81 (3)	15.66 (26)	1.20 (2)	0
Medium (15)	220	75.91 (167)	16.82 (37)	66.82 (147)	1.82 (4)	5.00 (11)	7.27 (16)	1.36 (3)	1.20 (2)
High (15)	201	48.26 (97)	40.80 (82)	32.34 (65)	0	8.46 (17)	10.95 (22)	1.00 (2)	13.40 (13) [b]

[a] Comparison between the fertile eggs vs. the non-fertile eggs in the male oil dose groups vs. the control male group indicates the dose affects fertility. Power of performed test with alpha = 0.050: 1.000 chi-square= 86.878 with 3 degrees of freedom. (P = <0.001).

[b] Fertilization with no development of the embryo at 24 hours in the high oil dose group eggs showed statistical significance when compared to the control dose group data; Fisher's Exact test (P = <0.0001).

3.2.4. Male dose group egg data

The fertility of eggs produced by 'clean' females with the oil dosed males also indicates a relationship between the fertility and the dose group (Table 3.5). It is not observed as a consistent change in a dose-dependent manner from these data. In the low oil dose group, the data may reflect a contributing factor involving the loss of one male early in the lay season. The medium dose group appears to mirror that of the control group ratio of fertile to non-fertile eggs. The egg production results are comparable with research into the captive performance of male mallards in captivity (Stunden *et al.*, 1999) where 80% of the self-chosen adult mallard pairs produced fertile eggs. Fertility, however, does appear to be influenced by oil dose group in the male low and high oil dose groups.

The eggs from the male dose groups were all artificially incubated until day 18 when they were then refrigerated. All these eggs were later opened and examined. The stage of embryo development was assessed in all fertile eggs. Data collected included the days of development, obvious abnormalities, and the presence of infection within the egg (Table 3.4). Obvious deformed embryos were very rare, however, one anomaly that showed up that is significant. The high dose group eggs showed fifteen eggs that had been fertilized, however, all development was halted at the twenty four hour developmental stage (Caldwell and Snart, 1974). This anomaly was observed in two eggs only in the medium dose group.

The embryos in the male control and oil dosed groups were aged to determine the developmental age of each embryo (Caldwell and Snart, 1974). No major abnormalities

were observed in these eggs. The male control and oil dose groups however, showed an overall higher rate of infection within the artificially incubated eggs when compared to that found in the female oil dose groups data (Table 3.4).

Table 3.5. Tabulated data for egg fertility data collected from male oil dose groups. Unless otherwise noted, the data show the percentage of eggs with the number in (). Data sets with statistically significant differences are denoted with the same letter.

Dose Group (# hens)	Total # eggs	Fertile eggs [a]	Infertile eggs [a]	Embryo with normal development - day 18	Embryo with abnormal development - day 18	Embryo died in shell - apparent normal development	Infected egg - no embryo observed	Infected egg - embryo observed	Fertile - no development after 24 hours
Control (15)	216	70.37 (152)	23.61 (51)	65.74 (142)	1.39 (3)	2.31 (5)	6.02 (13)	0.92 (2)	0 [b]
Low (15)	166	34.94 (58)	49.40 (82)	29.52 (49)	2.41 (4)	1.81 (3)	15.66 (26)	1.20 (2)	0
Medium (15)	220	75.91 (167)	16.82 (37)	66.82 (147)	1.82 (4)	5.00 (11)	7.27 (16)	1.36 (3)	1.20 (2)
High (15)	201	48.26 (97)	40.80 (82)	32.34 (65)	0	8.46 (17)	10.95 (22)	1.00 (2)	13.40 (13) [b]

[a] Comparison between the fertile eggs vs. the non-fertile eggs in the male oil dose groups vs. the control male group indicates the dose affects fertility. Power of performed test with alpha = 0.050: 1.000 chi-square= 86.878 with 3 degrees of freedom. ($P = <0.001$).

[b] Fertilization with no development of the embryo at 24 hours in the high oil dose group eggs showed statistical significance when compared to the control dose group data; Fisher's Exact test ($P = <0.0001$).

3.3. Prolactin

All reproductive behaviours are initiated from seasonally programmed events and activities including age, day length, and nutrition. Internally, hormonally driven cascades are required to support the activities designed to ensure a successful reproductive outcome. In this research, one hormone was investigated in birds treated with different doses of oil relative to controls. Prolactin is linked to incubation, brooding behaviour and parental care. Samples from the baselines collected from all birds in this study 7 days prior to oil dosing (T = -7 days), post oil-dosing and early in the reproductive period and one last sample from 414 days post oil-dosing in the female dose groups were all tested for prolactin levels. Except for the last sample collected from the female mallards, there were no differences seen in prolactin levels between controls and different oil doses (data not shown). These data indicate that prolactin levels are responsive to the date and the gender of the mallards, as would be expected, and show no statistical difference between the control and oil dose groups.

Data for the prolactin levels in the female mallards at the time of termination of this experiment showed an unexpected result (Figure 3.15). For the September time period, prolactin levels should be at the baseline levels expected for non-breeding or post-reproductive birds. However, the high oil dose females showed an anomaly with their prolactin levels being higher than expected when compared to the control, low

and medium oil dose birds. This result however, sheds no further information other than an increased prolactin which may be stable, or either increasing or decreasing.

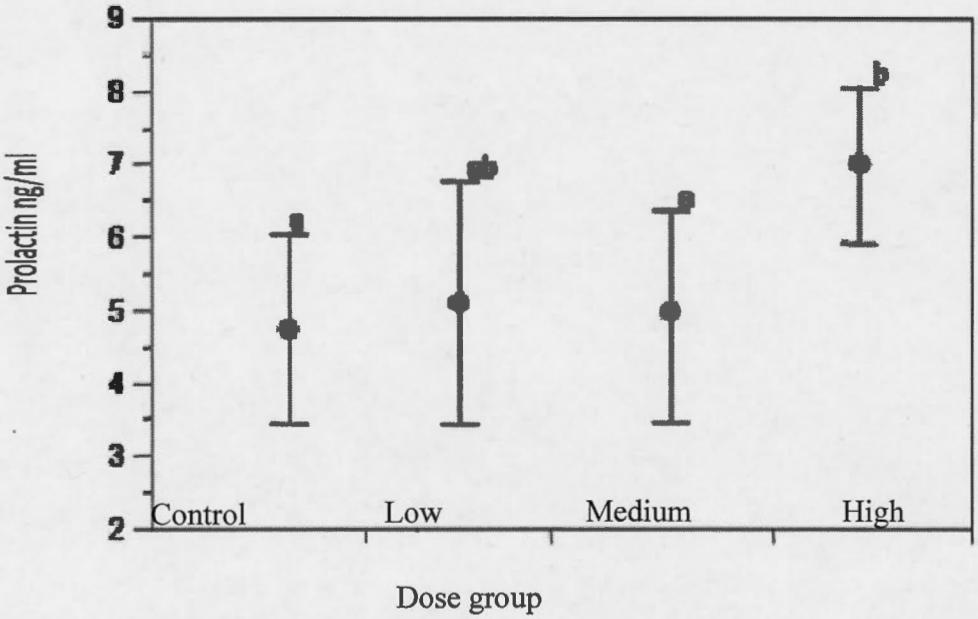


Figure 3.8 Prolactin levels from female oil dose groups at 414 days post oil dosing.

The coordinates in the graphs that do not share a common letter are significantly different. The bars indicate the SD. ANOVA p=0.0213. Multiple comparisons by Tukey-Kramer p<0.05.

Prolactin levels for the high oil dose female group results were significantly different (P< 0.05) from the control and medium dose groups. The low oil dose group had

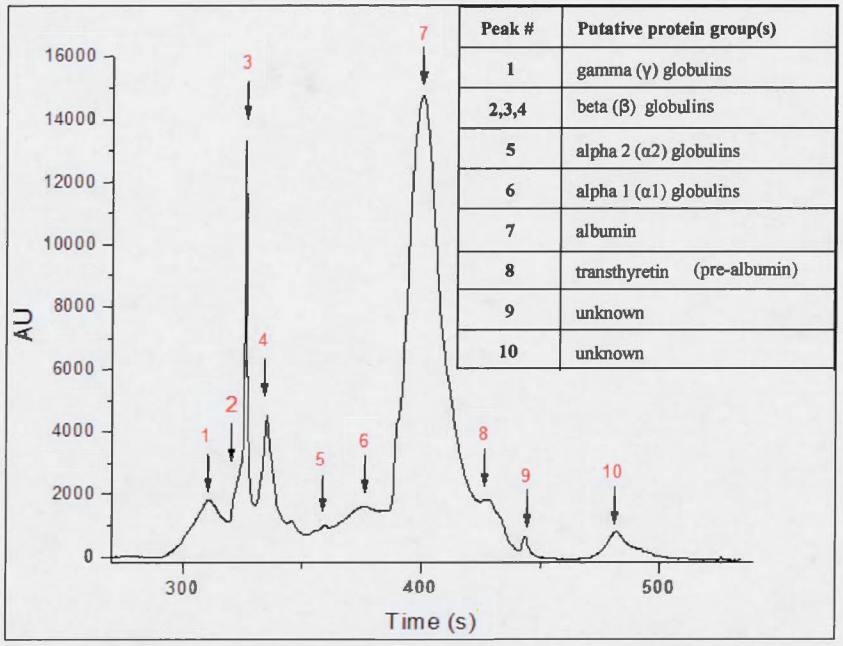
some overlap with the high dose group, but the minimum and mean levels were similar to those of the control and medium groups.

3.4. Protein profiles

Serum protein electrophoresis using capillary electrophoresis (CE) provides general diagnostic information and offers insight into the changes in the major protein groups within the individual. This research focused on the changes evident in mallards over time and dose impact, exploring changes in the specified protein groups linked to gender, dose level and biological outcome.

Each peak is given as a percentage of the total of the ten putative protein peaks as indicated in Figure 3.9. The data are tabulated with the ranges from each group to provide insight when evaluating cases in an oil spill. Statistically significant results are shown in bold typeset. Graphs are used where a specific protein is different according to ANOVA, across the dose groups for that sampling time. The results are graphed and letters are used to indicate results that are statistically different. The bars on the graphs show the standard deviations of the mean in the case of normally distributed data. Where the data were not normally distributed, non-parametric and descriptive statistics were used with medians and 5% to 95% confidence intervals.

Details of the statistical analysis and treatment effects tested for are presented in Appendix B.



Peak #	Putative protein group(s)
1	gamma (γ) globulins
2,3,4	beta (β) globulins
5	alpha 2 (α2) globulins
6	alpha 1 (α1) globulins
7	albumin
8	transthyretin (pre-albumin)
9	unknown
10	unknown

Figure 3.9 The protein groups identified by capillary electrophoresis (CE) and numbered to indicate the individual groups evaluated. The peaks are measured in absorbance units (AU) over elution time in seconds (s). The insert guide shows the peak number and the putative protein group.

3.4.1. The acute phase: days one to day five post oil dosing.

For blood samples collected in the first two days after oil dosing, the results show significant differences between male and female ducks for eight of the ten proteins quantified (Appendix B). In the case of gamma globulin (CE peak #1; Figure 3.9) (Figure 3.10), the values were greater in all females groups compared with their respective male groups. In the case of females, significantly higher values occurred in the medium and high dosed groups compared with controls. Male birds however, did not have any oil-related gamma globulin effects.

The alpha 2 globulin peak (CE peak #5; Figure 3.9) shows no gender based differences, however, the low and high oil dose female groups are statistically significantly higher that the control dose group (Figure 3.11).

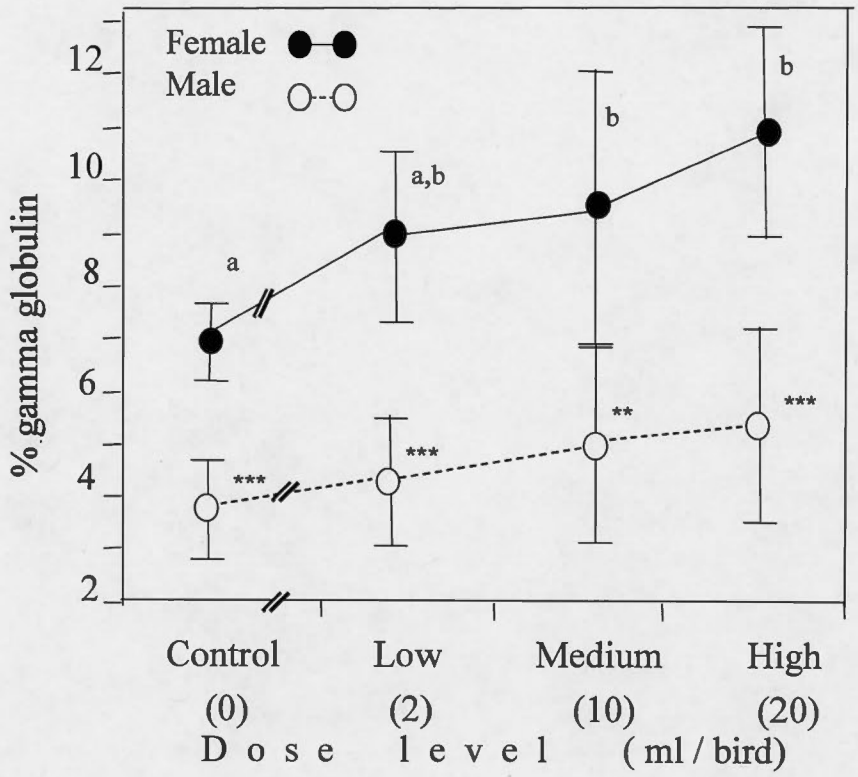


Figure 3.10 Gamma globulin (CE peak #1) levels on Days 1 and 2 (pooled, days are a NS effect) after oil dosing in both males and female mallards.The points in the graphs that do not share a common letter are significantly different. Main ANOVA effects are; day ($F_{1, 55} = 3.88$; NS), sex ($F_{1, 55} = 136.56$; $p < 0.0001$), dose ($F_{3, 55} = 8.39$; $p = 0.0001$) and day*sex interaction ($F_{1, 55} = 4.58$; $p = 0.0369$). The specific comparisons based on Least-Square-Means adjusted t-test are; day 1 vs. day 2 for males ($p = 0.0052$); day 1 vs. day 2 for females (NS); female vs. male day 1 (NS); female vs. male day 2 (NS); Specific comparisons in females are Control vs. High dose groups ($p < 0.0001$); Control vs. Medium dose groups ($p = 0.0265$). Significant between-sex differences at the 5% level for a given dose are indicated by * (* $p<0.0001$; ** $p<0.01$).**

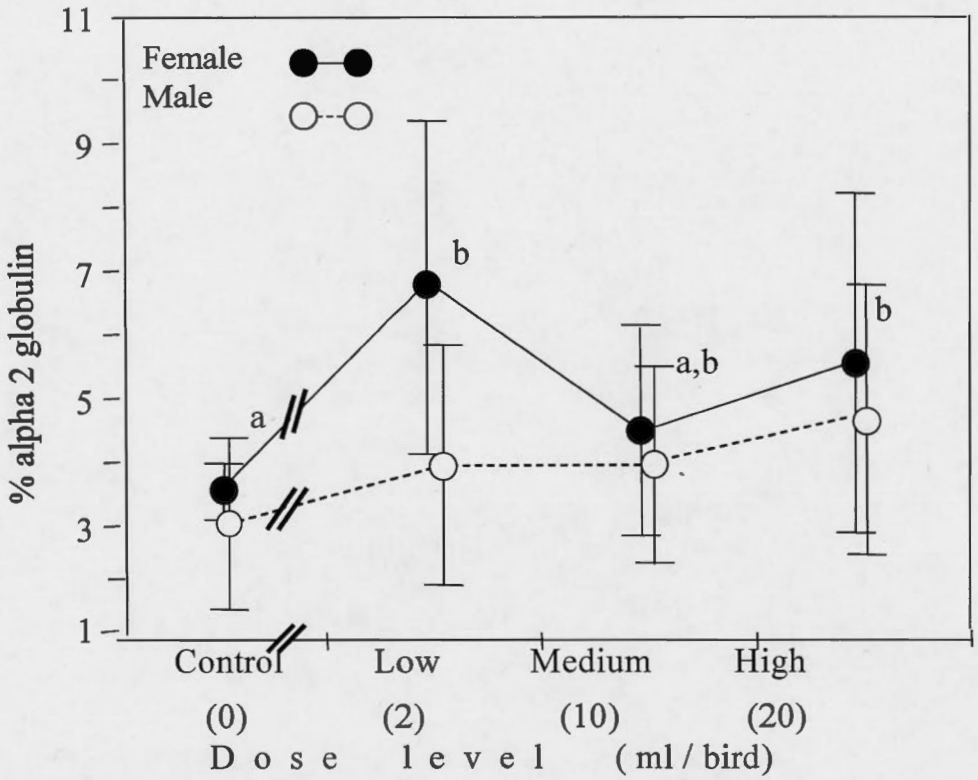


Figure 3.11 Alpha 2 globulins (CE peak #5) on Days 1 and 2 after oil dosing in both males and female mallards.The coordinates in the graphs that do not share a common letter are significantly different. Main ANOVA effects are; for day $F_{1,55} = 12.17, p = 0.0010$; sex $F_{1,55} = 15.41$, $p = 0.0002$; dose $F_{3,55} = 4.09, p = 0.0108$. The specific comparisons based on Least-Square-Means adjusted t-test shows significant differences between dose groups C vs. H $p = 0.0288$ and C vs. L $p = 0.0171$.

Albumin, a negative acute phase protein, shows a drop over all dose groups (Figure 3.12). There are no significant differences in the female oil dose groups between days one and two, however, the male oil dose groups do show a difference across these days.

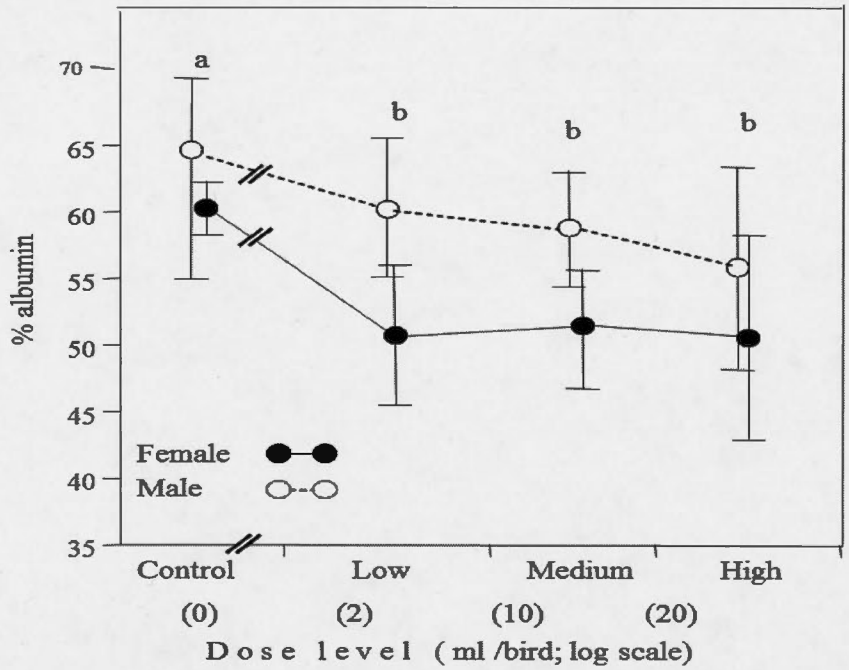


Figure 3.12 Albumin (CE peak #7) levels on Days 1 and 2 after oil dosing in both males and female mallards.The coordinates in the graphs that do not share a common letter are significantly different. The main ANOVA effects are; day $F_{1,55} = 4.51$, $p = 0.0382$; sex $F_{1,55} = 29.14$, $p < 0.0001$; dose $F_{3,55} = 8.02$, $p = 0.0002$ and for day*sex $F_{1,55} = 9.39$, $p = 0.0034$. The t-test showed differences for day 1 vs. day 2 for males $p = 0.0005$; day 1 vs. Day 2 for females, showed NS; females vs. males at day 1, NS; and females vs. males at day 2, $p < 0.0001$. Specific comparisons based on the Least-Square-Means adjusted t-test showed C vs. L, $p = 0.0120$; C vs. M, $p = 0.0153$; and C vs. H, $p < 0.0001$. The data points are shown staggered to make it easier to see the gender differences.

Transthyretin, in mammalian studies, is a negative acute phase protein, however, in this research gives a positive response (Figure 3.13) Also the gender differences are significantly different when comparing the dose groups. The responses within each gender are different across the dose groups.

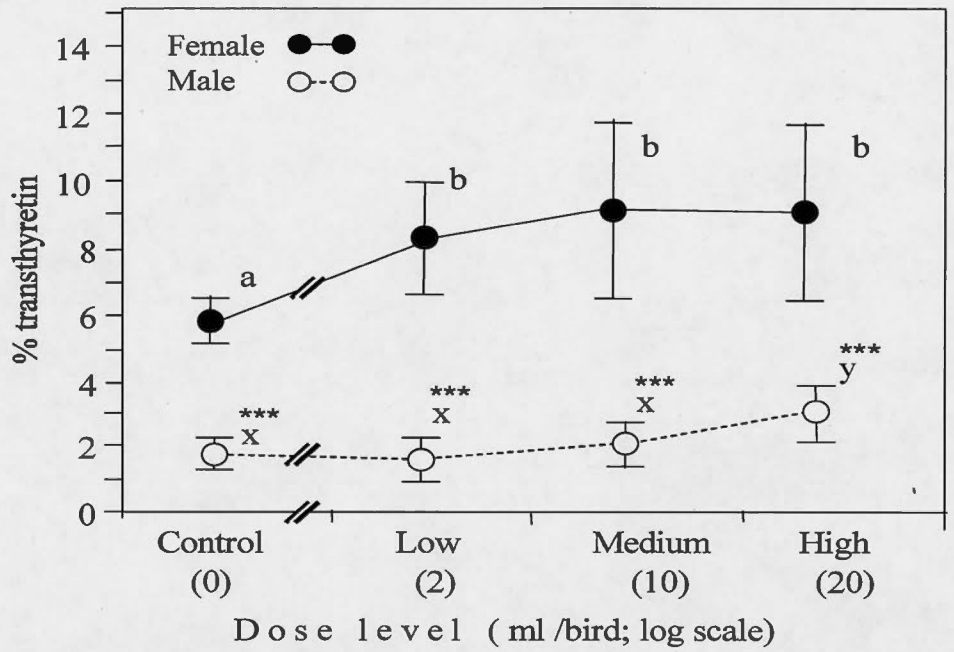


Figure 3.13 Transthyretin (CE peak #8) on Days 1 and 2 after oil dosing in both males and female mallards.The coordinates in the graphs that do not share a common letter are significantly different. Main ANOVA effects are for; day $F_{1, 55} = 7.57$, $p = 0.0080$; sex $F_{1, 55} = 29.14$, $p < 0.0001$; dose $F_{3, 55} = 9.24$, $p < 0.0001$; dose*sex $F_{1, 55} = 3.23$, $p = 0.0294$. The t-tests showed; female vs. male, control, $p < 0.0001$; female vs. male, low dose, $p < 0.0001$; female vs. male, medium dose, $p < 0.0001$; female vs. male, high dose, $p < 0.0001$, with between-sex differences at a given dose are indicated by * (* $p<0.0001$). The Least-Square-Means adjusted t-test for females, indicated; C vs. L, $p = 0.0096$; C vs. M, $p = 0.0001$; and C vs. H, $p < 0.0001$. The Least-Square-Means adjusted t-test for males; C vs. H, $p = 0.0068$, L vs. H, $p = 0.0009$; and M vs. H, $p = 0.0315$.**

Protein peak #9 (Figure 3.14) illustrates that the gender differences in this protein are significant. The results for the male and female control birds have very different ranges. Within the female dose groups, there is only a very slight non-significant response to the oil dosing, whereas, the males upper oil dose groups (medium and

high) demonstrate a significant potential range in response at an individual level to the oil dose during this phase.

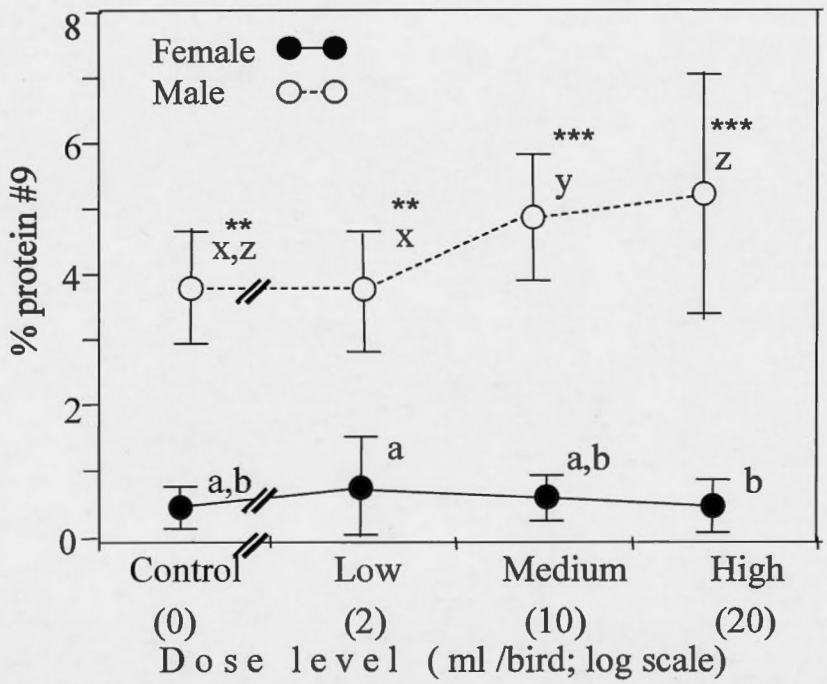


Figure 3.14 Protein peak #9 on Days 1 and 2 after oil dosing in both males and female mallards.The coordinates in the graphs that do not share a common letter are significantly different. Main ANOVA effects are; for sex $F_{1, 55}$ = 460.97, p <0.0001; day*sex $F_{1, 55}$ = 8.22, p <0.0059; and dose*sex $F_{3, 55}$ = 3.60; p =0.0190. The t-tests indicate; day 1 vs. day 2 for males, p =0.0099; female vs. male at day 1, p <0.0001; and for females vs. males at day 2, p <0.0001. Between-sex differences at a given dose are indicated by * (* p<0.0001; ** p<0.01).Within a given sex, specific comparisons based on Least-Square-Means adjusted t-test indicated for females; L vs. H, p = 0.0454; and for males; C vs. M, p = 0.0403; L vs. M, p = 0.0316; L vs. H, p = 0.0218.**

Protein peak #10 (Figure 3.15) also demonstrate significant gender differences with a dose response that is mirrored between the male and female groups. The low and high oil dose groups in both males and females are significantly different from one another.

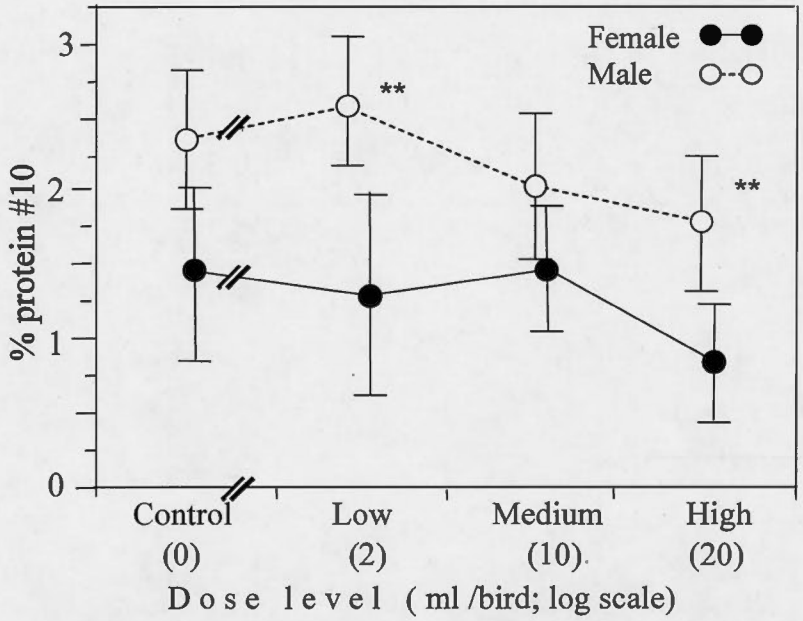


Figure 3.15 Peak # 10 on days 1 and 2 after oil dosing in both males and female mallards.The coordinates in the graphs that do not share a common letter are significantly different. Main ANOVA effects are; for dose $F_{3,55} = 7.56$, $p = 0.0003$; sex $F_{1,55} = 47.64$, $p < 0.0001$; dose*day $F_{3,55} = 3.84$, $p = 0.0145$. Between-sex differences at a given dose are indicated by * ($p<0.01$). The t-test showed day 1 vs. day 2, for the medium dose group, $p = 0.0014$. Within a given sex, the Least-Square-Means adjusted t-test for day 1; C vs. H, $p = 0.0239$ and for day 2; C vs. L, $p = 0.0355$; C vs. H, $p = 0.0043$; L vs. M, $p = 0.0092$; L vs. H, $p < 0.0001$; M vs. H, $p = 0.0386$.**

All the results from the day one and two time period post oil dosing for the female dose groups are tabulated (Table 3.6) to begin the process of developing a triage tool to aid oil spill rehabilitators. The tabulated data provide insight into a number of protein shifts in value range and changes that add to the potential for the diagnostic use of proteins during the acute phase. These include the changes documented with the individual proteins; gamma globulin, alpha 2 globulin, albumin and transthyretin.

Table 3.6. Female dose group protein ranges at days 1 and 2 post dosing. Bold typeset indicates significant differences. The numbers are shown as a percentage of the total of the peaks quantified.

T - 1, 2 days	Control		Low		Medium		High	
Protein peak	Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper
γ globulins	**5.52**	**7.73**	**7.14**	**12.3**	**5.66**	**14**	**8.66**	**12.1**
β globulins (2)	2.15	6.24	1.76	5	2.74	8.1	3.16	4.89
β globulins (3)	**2.08**	**5.27**	2.46	5.31	2.08	4.2	**1.76**	**3.21**
β globulins (4)	**5.27**	**7.84**	**5.97**	**10.1**	**4.84**	**9.87**	**2.96**	**9.34**
α 2 globulins	**3**	**4.19**	**3.3**	**10.4**	2.83	7.56	2.35	9.95
α 1 globulins	5.78	8.32	5.88	9.13	3.93	10.6	6.52	11.6
albumin	**59.2**	**63.9**	**45.3**	**60.2**	**44.5**	**57.2**	**38.5**	**62.3**
trans-thyretin	**4.71**	**6.74**	**5.98**	**11.4**	**5.84**	**12.7**	**6.34**	**14.4**
unknown #9	0.218	0.38	0.2	2.51	0.249	1.17	0.046	1.51
unknown #10	0.863	2.63	0.858	2.5	1.04	2.31	0.26	1.53

Table 3.6 details the pooled results for the female mallards sampled at days one and two. Values for each protein group that are significantly different or where there is a trend that adds information to the shift in homeostasis, are shown in bold. The gamma globulin result show that the medium and high oil dose groups have higher levels than the control group; ANOVA; (p= 0.001; Dunnett's test p< 0.05). High dose group females had significantly lower protein levels in peak #3 beta globulin when compared to control females; ANOVA; (p= 0.0025); Dunnett's test $p < 0.05$. Protein peak alpha 2 globulins showed that low dose females had significantly higher levels that those of the controls; ANOVA; (p= 0.025); Dunnett's test $p < 0.05$. Albumin shows that all dosed group levels were lower than the control group (ANOVA; (p = 0.0015); Dunnett's test $p < 0.05$). Transthyretin in the high and medium dose groups both have higher levels compared to the controls (ANOVA; (p<0.010) Dunnett's test $p < 0.05$).

Further analysis for the data from days 1 and 2 post oil dosing in the female dose groups showed that the protein peak #4 beta globulins was significantly lower in females that failed to form pair bonds in the following reproductive season (Student's t-test $p = 0.03$).

All the results from the day one and two time period post oil dosing for the male dose groups are tabulated (Table 3.7) to provide a diagnostic tool to aid oil spill rehabilitators begin the decision making process in a triage setting. The statistical analysis for these individual proteins; gamma globulin, alpha 2 globulin, albumin, transthyretin, and peaks #9 and 10, add to the ranges expected in male mallards at this time period for the acute phase response.

Table 3.7. Male dose group protein ranges at days 1 and 2 post dosing. Bold typeset indicates significant differences. The numbers are shown as a percentage of the total of the peaks quantified.

T - 1,2 days	Control		Low		Medium		High	
Protein peak	Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper
γ globulins	2.66	5.3	2.57	6.73	2.08	7.94	2.9	7.92
β globulins	0.531	1.67	0.344	1.75	0.8	1.85	0.542	2.53
β globulins	4.47	8.88	4.65	7.38	3.92	6.14	3.22	10.5
β globulins	7.68	15.4	7.96	13.7	7.66	11.9	7.36	15.9
α 2 globulins	1.48	6.24	1.6	7.35	1.75	6.35	2.22	8.03
α 1 globulins	2.75	8.5	2.83	14	3.05	9.06	2.82	9.53
albumin	**54.4**	**71**	**53.6**	**69.9**	**52.1**	**71.3**	**47.7**	**65.5**
trans-thyretin	**1.12**	**2.71**	**0.58**	**2.573**	**0.892**	**3.21**	**1.34**	**4.48**
unknown #9	**2.51**	**5.4**	2.78	5.56	3.88	6.82	**2.48**	**7.95**
unknown #10	**1.54**	**2.91**	1.73	3.17	1.08	2.5	**1.01**	**2.53**

Table 3.7 gives the results for the pooled protein results from days 1 and 2 in the male mallards. In the male mallards, transthyretin indicate a tendency towards being higher in those males that failed to form pair bonds in the subsequent reproductive season; Student's t-test; (p= 0.089). The high dose group also had significantly higher levels of the proteins transthyretin and the protein #9 than those of the control group; ANOVA; p<0.0007 and p<0.019 respectively; Dunnett's test $p < 0.05$. Peak #10 on the other hand was lower in the high dose birds when compared to the control birds; ANOVA; p= 0.004; Dunnett's test $p< 0.05$.

In examining the differences between the pooled results from days 1 and 2 in the male and female control groups (Tables 3.6 and 3.7), significant differences are noted. The gamma globulin, #2, #3, and #4 beta globulins, alpha 2 globulin, alpha 1 globulin, transthyretin and #9 peaks all show significantly higher values in the control male groups compared to the control female groups (Student's t-test p ranging from <0.0001 to 0.04) indicating normal gender based ranges.

Protein peak #10 shows the reverse, with the control female peak (Table 3.6) significantly greater than that of the control male (Table 3.7). Albumin levels show no statistically significant differences between males and females of the control groups. Further statistical examination exploring the effects of dose and sex as well as

the interactions between sex-dose on serum protein peaks at days one and two post treatment is detailed in Tables 3.8 and 3.9.

Further analysis showed that albumin in samples from male dose groups at day one and two post treatment was lower in males that failed to form pair bonds; Student's t-test; (p= 0.010) .

Table 3.8. Summary of probabilities and the tests used in exploring the differences between male and female data points at days 1 and 2 post oil dosing. Welch's test was used in cases of unequal variance.

Peak #	Probability	Statistical Test	Comment
γ globulins	P<0.0001	Student's T-test	Male values greater than females
β globulins - 2	P<0.0001	Student's T-test	Male values greater than females
β globulins - 3	P= 0.0004	Student's T-test	Male values greater than females
β globulins - 4	P= 0.0003	Student's T-test	Male values greater than females
α2 globulins	P= 0.0002	Student's T-test	Male values greater than females
α1 globulins	P= 0.044	Welch's Test	Male values greater than females
transthyretin	P<0.0001	Student's T-test	Male values greater than females
unknown	P<0.0001	Welch's Test	Male values greater than females
unknown	P=0.0033	Student's T-test	Male values lower than females

Table 3.9. Testing for the effects of dose and sex as well as the interactions between sex-dose on serum protein peaks at days 1 and 2 post oil dosing.

Peak	Effect of dose - probability	Effect of sex - probability	Dose - sex interaction - probability	Statistical test
γ globulins	P= 0.0001	P= 0.0001	N.S.	ANOVA 2 factor
β globulins - 2	N.S.	P= 0.0001	N.S.	ANOVA 2 factor
β globulins - 3	N.S.	P= 0.0001	N.S.	ANOVA 2 factor
β globulins - 4	N.S.	P= 0.0001	N.S.	ANOVA 2 factor
α2 globulins	P= 0.016	P= 0.002	N.S.	ANOVA 2 factor
α1 globulins	N.S.	P= 0.004	N.S.	ANOVA 2 factor
albumin	P= 0.0008	P= 0.0001	N.S.	ANOVA 2 factor
transthyretin	P< 0.0004	P= 0.0001	P= 0.03	ANOVA 2 factor
# 9	P= 0.0001	P= 0.0008	N.S.	ANOVA 2 factor

The control group female mallards show a significant difference in the #2 beta globulin peak at day 3 compared to those at day 4 (Figure 3.16) which were not seen in the low and medium dose groups for the same time period.

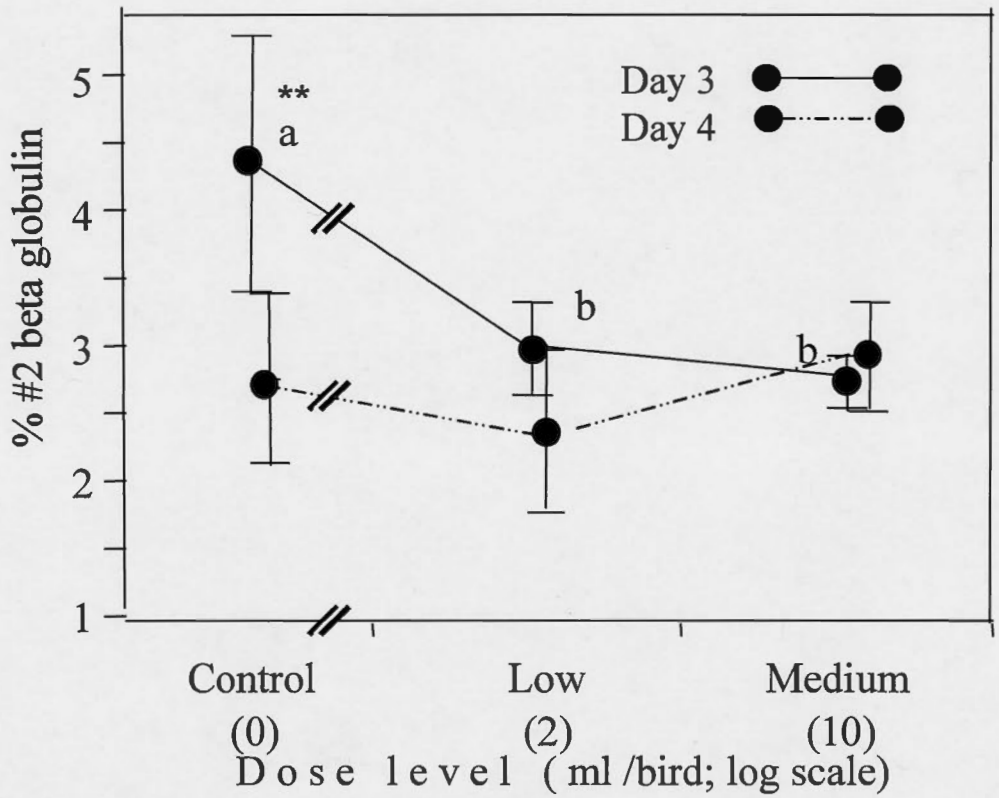


Figure 3.16 Protein peak #2 beta-globulin levels on days 3 and 4 after oil dosing in female mallards.The coordinates in the graph for Day 3 that do not share a common letter are significantly different. There are no significant differences seen in Day 4. ANOVA $F_{6,25} = 14.55$; $p < 0.0001$; Effect of dose $F_{2,25} = 3.85$; $p = 0.0357$; Effect of sampling day $F_{1,25} = 10.95$; $p = 0.0028$. Differences in sampling day are denoted by * (** p =0.0028). Multiple t-test based on LSM; $p < 0.05$. Data for the highest dose level are missing and therefore this dose level was not included in the analysis.

The control group female mallards show a significant difference in the #3 beta globulin peak at day 3 compared to those at day 4 (Figure 3.17). These differences are not seen in the low and medium dose groups for the same time period.

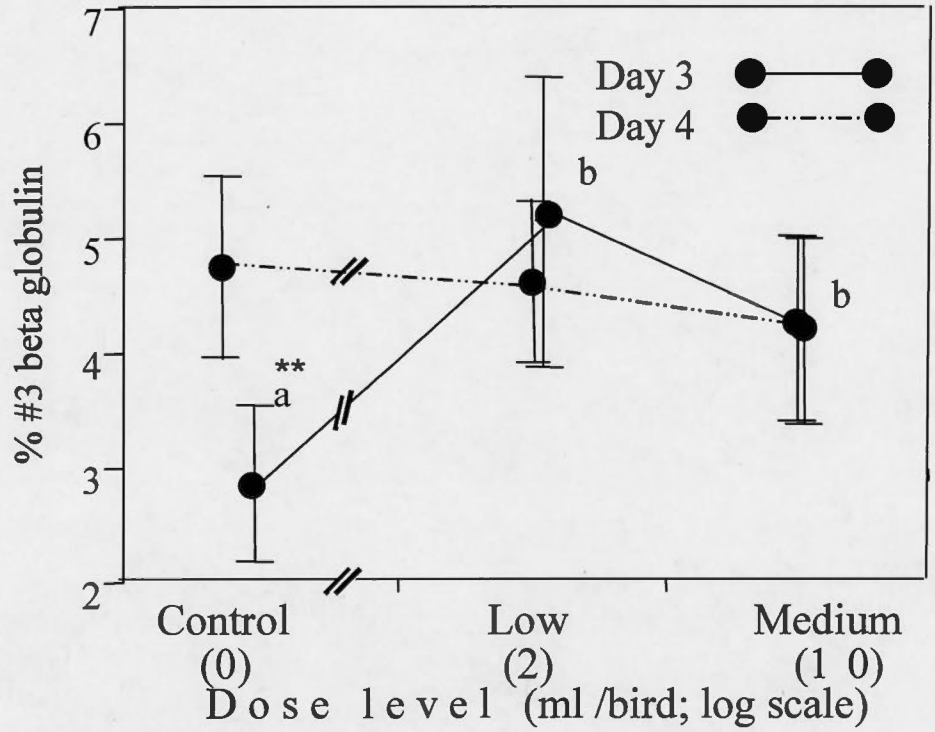


Figure 3.17 Protein peak #3 beta-globulin levels on days 3 and 4 after oil dosing in female mallards.The coordinates in the graph for Day 3 that do not share a common letter are significantly different. There are no significant differences seen in Day 4. ANOVA $F_{6,24} = 7.70$; $p < 0.0001$; Effect of dose $F_{2,24} = 4.81$; $p = 0.0282$; Effect of sampling day $F_{1,24} = 5.53$; $p = 0.0106$; Multiple t-tests based on the Least Square Means; $p < 0.05$. Differences in sampling day are denoted by * (** p =0.0106). The peaks are offset to allow ease of viewing.

The last protein peak in the beta globulin group, #4, shows a positive response in the low dose group, however there is no other statistically significant difference due to the day effect (Figure 3.18).

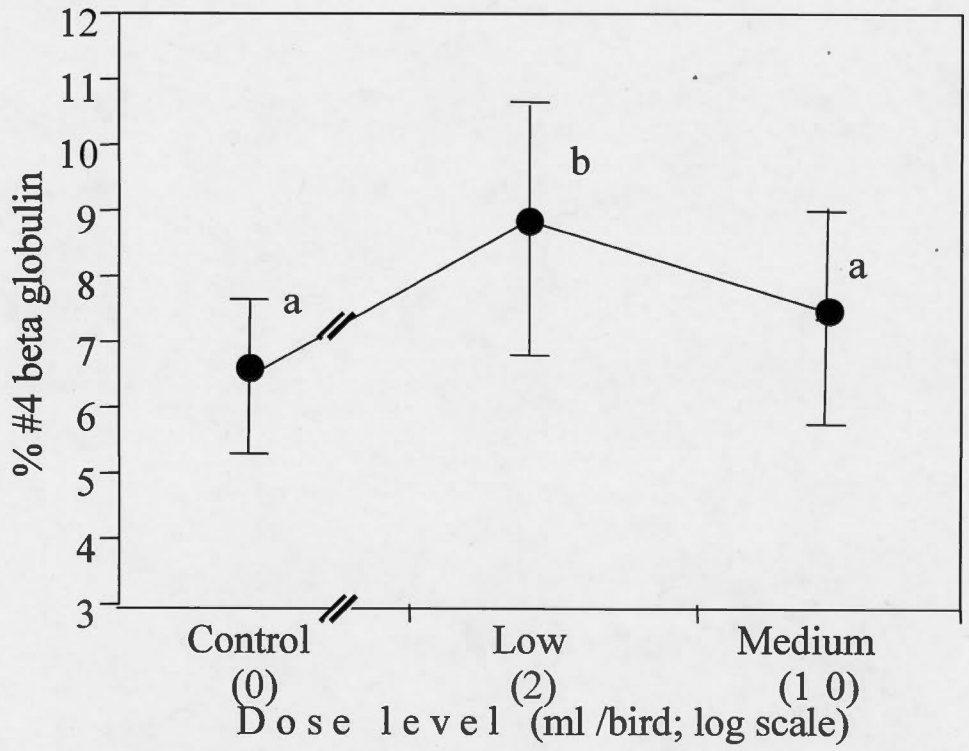


Figure 3.18 Protein peak #4 beta-globulin levels on days 3 and 4 (pooled) after oil dosing in female mallards.The coordinates in the graph that do not share a common letter are significantly different. ANOVA $F_{6,24} = 4.36$; $p < 0.042$; Effect of dose $F_{2,24} = 3.74$; $p = 0.0378$; Multiple t-tests are based on the Least Square Means; $p < 0.05$.

Table 3.10. Testing for the effects of dose and sex as well as the interactions between sex-dose on serum protein peaks at days 1 and 2 post oil dosing.

Peak	Effect of dose - probability	Effect of sex - probability	Dose - sex interaction - probability	Statistical test
γ globulins	P= 0.0001	P= 0.0001	N.S.	ANOVA 2 factor
β globulins - 2	N.S.	P= 0.0001	N.S.	ANOVA 2 factor
β globulins - 3	N.S.	P= 0.0001	N.S.	ANOVA 2 factor
β globulins - 4	N.S.	P= 0.0001	N.S.	ANOVA 2 factor
α2 globulins	P= 0.016	P= 0.002	N.S.	ANOVA 2 factor
α1 globulins	N.S.	P= 0.004	N.S.	ANOVA 2 factor
albumin	P= 0.0008	P= 0.0001	N.S.	ANOVA 2 factor
transthyretin	P< 0.0004	P= 0.0001	P= 0.03	ANOVA 2 factor
# 9	P= 0.0001	P= 0.0008	N.S.	ANOVA 2 factor

The pooled data is tabulated (Table 3.11) to give the ranges seen in female mallards at days three and four in the acute phase period. The high dose data add to the picture, allowing rehabilitation staff to identify birds impacted by a higher oil dose based on the ranges seen for the proteins evaluated. The tabulated ranges for the proteins do show statistically significant results within the beta globulins which are not noted as significant at days one and two after oil dosing. Albumin (decreased) and transthyretin (increased) are still showing significant changes over this period.

Table 3.11. Female dose group protein ranges at days 3, 4 post dosing. Bold typeset indicates significant differences. The numbers are shown as a percentage of the total of the peaks quantified.

T - 3,4 days	Control		Low		Medium		High	
Protein peak	**Lower**	**Upper**	**Lower**	**Upper**	**Lower**	**Upper**	**Lower**	**Upper**
γ globulins	5.05	9.17	5.28	9.42	6.53	9.81	5.41	10.7
β globulins - 2	**2.12**	**5.33**	**1.41**	**3.43**	**2.42**	**3.21**	**4.16**	**7.96**
β globulins - 3	**2.27**	**5.3**	**3.65**	**8.7**	**3.38**	**5.29**	**2.26**	**2.8**
β globulins - 4	**4.62**	**8.32**	**6**	**11.4**	**3.89**	**10.5**	**6.99**	**12.4**
α2 globulins	1.97	4.39	1.47	7.98	1.64	7.09	4.06	8.63
α1 globulins	3.46	10.1	1.26	9.25	5.03	11.4	6.61	9.58
albumin	**54.7**	**64.6**	**52.2**	**63.7**	**49**	**63.7**	**41.2**	**57.2**
transthyretin	**3.78**	**7.95**	**4.53**	**8.44**	**3.94**	**7.88**	**5.99**	**10.3**
unknown #9	0.392	0.58	0.239	1.52	0.359	1.09	0.145	0.284
unknown #10	1.11	2.6	0.911	2.25	0.955	3.09	1.08	1.61

The female mallards' #2 and #3 beta globulin protein peaks and albumin (Table 3.11) all showed significant differences associated with dose level (Wilcoxon Test; p= 0.004, p= 0.002 and p= 0.034, respectively). The #4 beta globulin protein peak also showed significant differences associated with dose level; ANOVA; (p= 0.039). This protein, #4 beta globulin, also showed a significant increase in the low dose group compared to the control group (Dunnett's test, p<0.05). Transthyretin also showed a significant difference associated with dose level (ANOVA, p= 0.016) and a significant increase in the high dose group compared to that control group (Dunnett's test, p=0.05). Pair bonding issues were not shown as significant at this period.

3.4.2. The transitional phase; from six to fifteen days post oil dosing

By day 6 of a response to a stressor, the organism is returning to its normal status. The tabulated data (Table 3.12) do show this as values return to the ranges seen in the control birds. The range in the gamma globulins still show a trend to an increased upper range which is also seen in the tabulated results from days one and two (Table 3.7).

Table 3.12. Male oil dose group protein ranges at days 6 and 7 post dosing. Bold typeset indicates significant differences. The numbers are shown as a percentage of the total of the peaks quantified.

T - 6,7 days	Control		Low		Medium		High	
Protein peak	**Lower**	**Upper**	**Upper**	**High**	**Lower**	**Upper**	**Lower**	**Upper**
γ globulins	2.34	**5.44**	3.29	**6.63**	3.45	**6.8**	2.63	**7.57**
β globulins - 2	0.554	1.87	0.614	1.63	0.914	1.33	0.559	1.53
β globulins - 3	5.97	8.41	5.33	8.02	5.38	6.61	3.8	7.92
β globulins - 4	8.09	12.4	7.25	12.7	7.39	15.7	8.24	14.1
α2 globulins	1.82	5.98	1.85	6.4	2.06	6.63	2.35	6.25
α1 globulins	3.34	**8.72**	2.21	6.68	3.84	8.15	2.52	**11.9**
albumin	54.9	67.2	55.5	67.9	51.3	70.3	55.5	67.3
transthyretin	0.542	4.45	0.648	2.26	0.772	2.13	0.464	4.55
unknown	0.189	4.86	1.62	4.01	2.36	5.67	2.82	5.41
unknown	1.76	2.9	2.01	3.16	1.47	2.85	1.94	3.16

No significant differences were seen at days 6 and 7 post dosing in the male (Table 3.12). However, gamma globulin, showed an increasing upper range trend related to dose. Also the alpha 1 globulin peak shows a subtle increase in the high oil dose group compared to the control, low and medium dose groups.

3.4.3. The chronic phase; after sixteen days post oil dosing.

The chronic phase of a response is seen once the bird has survived the initial assault and yet homeostasis has potentially not returned to the pre-impact homeostasis values. It can be expected that this process should occur some 16 days after the impact that induced an acute phase response based on the generalized and specific protein response patterns discussed in the Literature review.

3.4.4. Days 19 and 21 after oil dosing in male mallards

Gamma globulin in the male mallards at days 19 and 21 do show a statistically significant increase over the dose groups (Figure 3.19) compared to the control group.

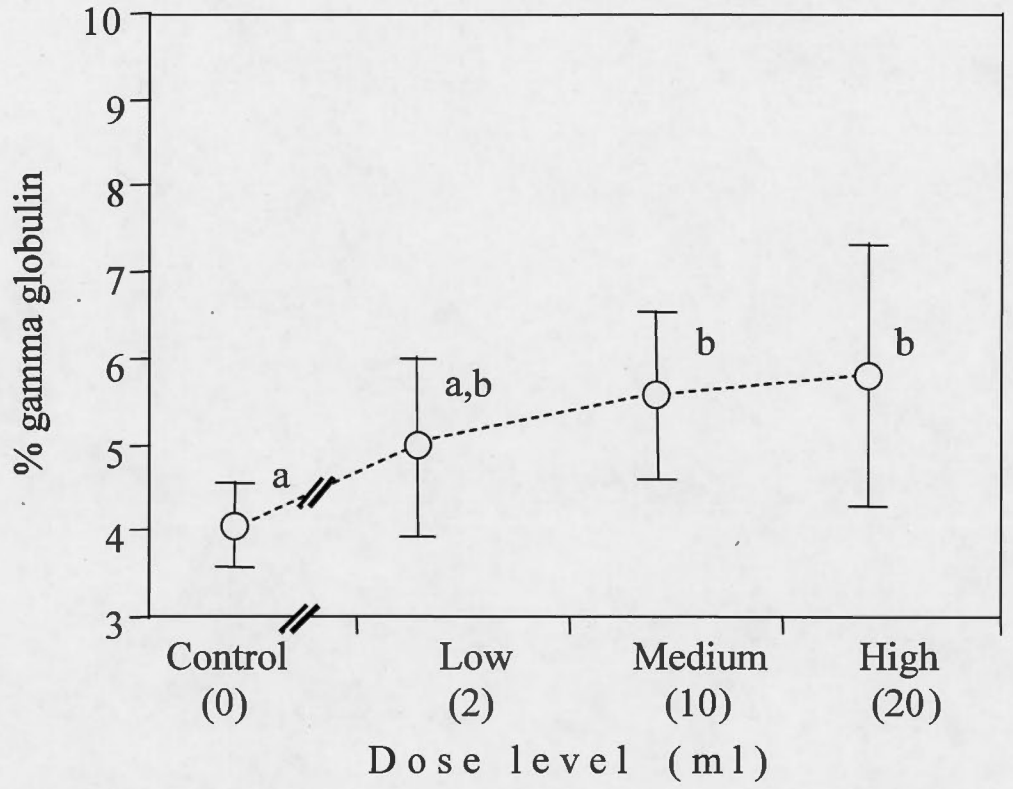


Figure 3.19 Gamma globulin protein peak levels on pooled data from days 19 and 21 after oil dosing in male mallards.The coordinates in the graphs that do not share a common letter are significantly different. (ANOVA $F_{7,29} = 6.01$; $p = 0.0105$; Multiple t-test based on LSM; $p < 0.05$).

The #2 beta globulins show a day effect that is significant in both the control group and the high dose birds (Figure 3.20). Also the increased amount of this protein in the high dose group on day 21 is statistically significant.

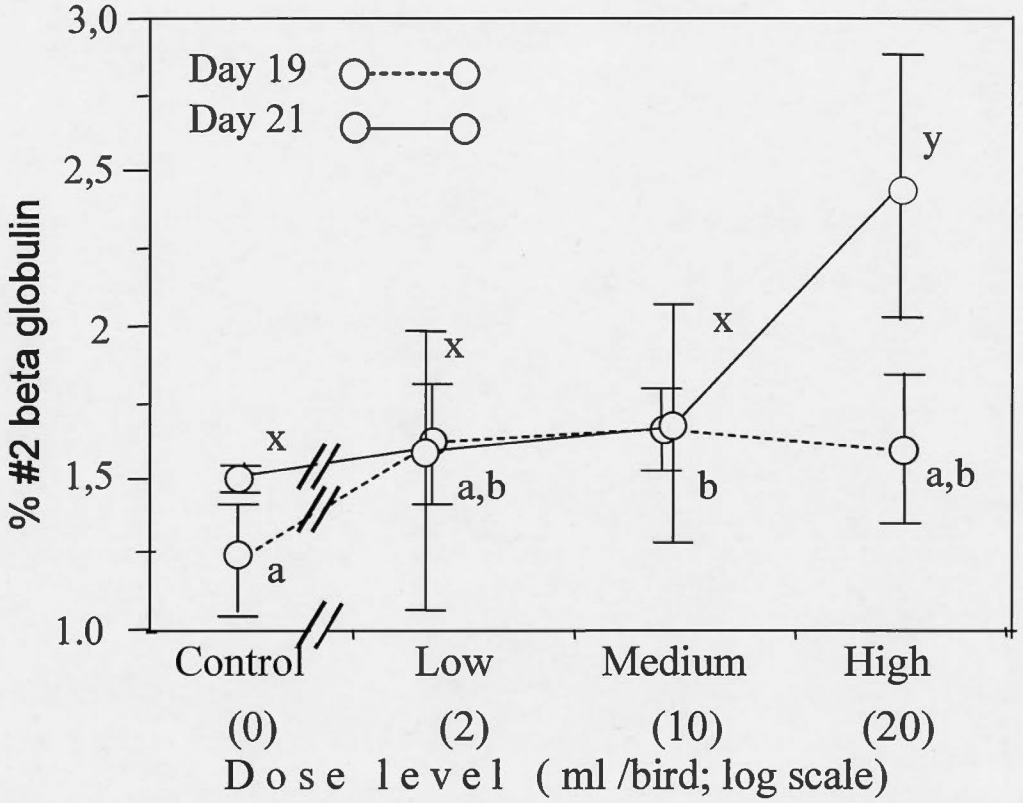


Figure 3.20. Protein peak #2 beta globulin levels from days 19 and 21 after oil dosing in male mallards.The coordinates in the graphs that do not share a common letter are significantly different. Main ANOVA effects are $F_{7,29} = 6.01$; $p = 0.0002$, with the effect of dose $F_{3,29} = 5.87$; $p = 0.0029$; the effect of sampling day $F_{1,29} = 7.72$; $p = 0.0095$; and the interaction dose x day $F_{3,29} = 3.86$; $p = 0.0194$. The Multiple t-test based on the Least Square Means; $p < 0.05$.

The #4 beta globulin (Figure 3.21) at days 19 and 21 in male mallards shows no effect of day, however, the decrease in this protein is statistically significant related to dose.

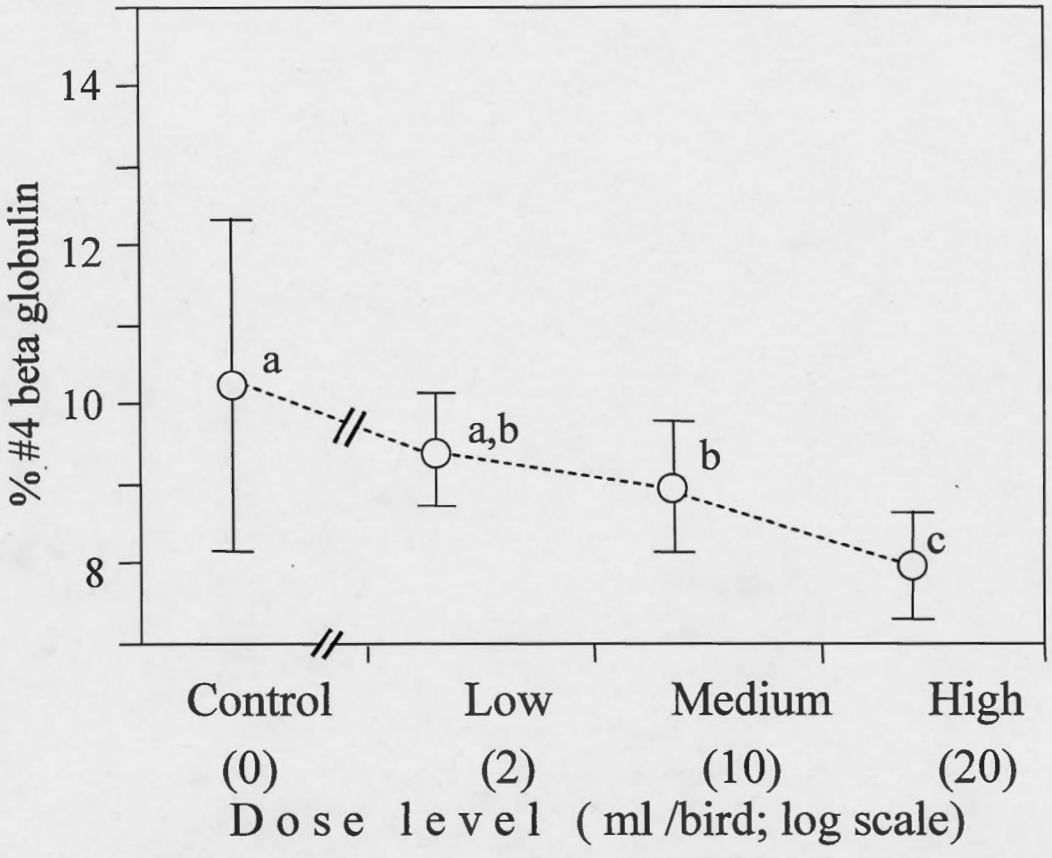


Figure 3.21 Protein peak #4 beta globulin levels on the pooled data from days 19 and 21 after oil dosing in male mallards.The coordinates in the graphs that do not share a common letter are significantly different. ANOVA $F_{7,29} = 4.25$; $p = 0.0024$. Effect of dose $F_{3,29} = 9.21$; $p = 0.0002$. Multiple t-test based on Least Square Means; $p < 0.05$.

The alpha 2 globulins (Figure 3.22) at this time do not show a date response (day 19 vs day 21), but do show an effect of the dose in the high oil dose group. The control group, low and medium oil dose groups are all statistically the same.

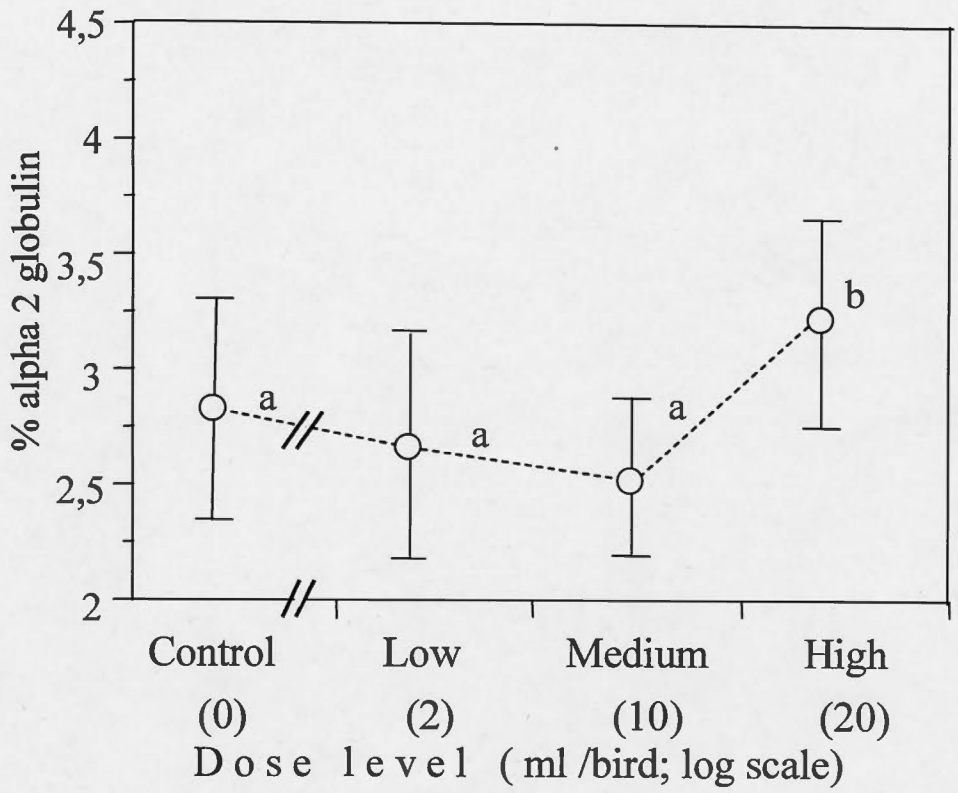


Figure 3.22. Protein peak alpha 2 globulins levels on pooled data from days 19 and 21 after oil dosing in male mallards.The coordinates in the graphs that do not share a common letter are significantly different. ANOVA $F_{7,28} = 4.69$; $p = 0.0014$; Effect of dose $F_{3,28} = 8.95$; $p = 0.0003$; Multiple t-test based on Least Square Means; $p < 0.05$.

Transthyretin results (Figure 3.23) indicate there is both a dose and sampling day response that is statistically significant. The increase over the oil dose groups in the transthyretin protein mirrors the results from the acute phase response.

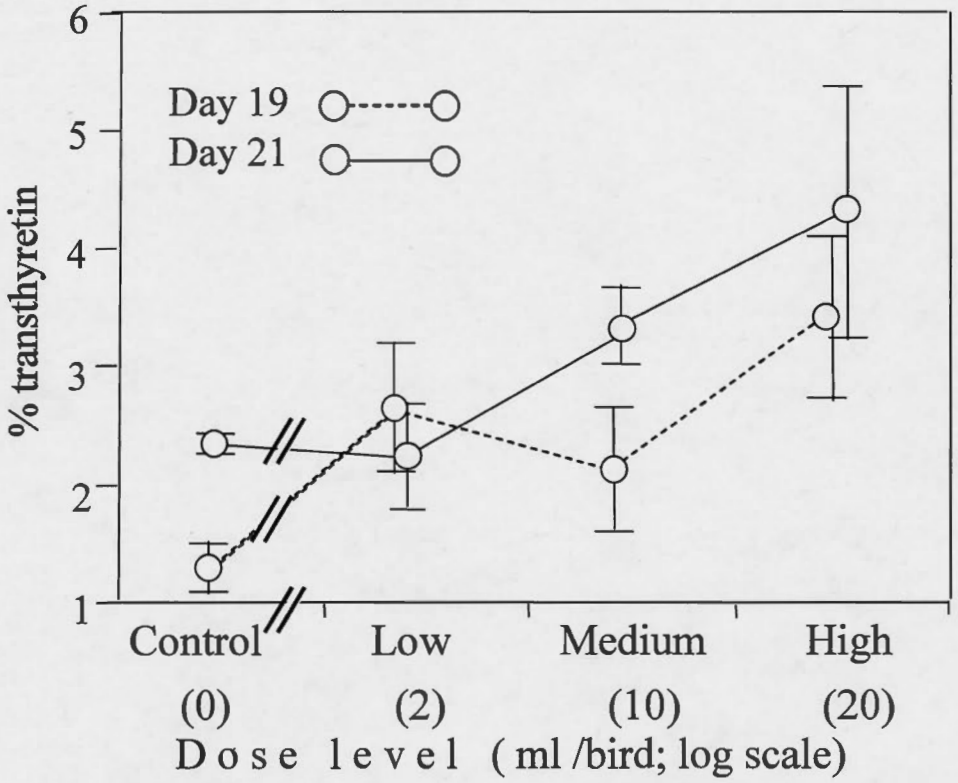


Figure 3.23. Protein peak transthyretin levels on days 19 and 21 after oil dosing in male mallards.The coordinates in the graphs that do not share a common letter are significantly different. The main ANOVA effects are; $F_{7,28} = 12.97$, $p < 0.0001$; effect of dose $F_{3,28} = 17.47$, $p < 0.0001$; effect of sampling day $F_{1,28} = 14.63$, $p = 0.0007$; interaction dose x day $F_{3,28} = 4.53$; $p = 0.0103$. Specific comparisons using a Multiple t-test based on the Least Square Means; effect of date; $p < 0.05$.

To aid the rehabilitation team to evaluate the status of mallards in care, the results are tabulated for this time period (Table 3.13). Linking the results of alpha 1 globulin, albumin, gamma globulin and #2 beta globulin proteins to a biological outcome, pair bonding, also aids with triage.

Table 3.13. Male dose group protein ranges at days 19 and 21 post dosing. Bold typeset indicates significant differences. The numbers are shown as a percentage of the total of the peaks quantified.

T - 19, 21 days	Control		Low		Medium		High	
Protein peak	**Lower**	**Upper**	**Lower**	**Upper**	**Lower**	**Upper**	**Lower**	**Upper**
γ globulins	**3.14**	**4.58**	**3.29**	**7.13**	**4.44**	**7.96**	**3.92**	**9.3**
β globulins - 2	**1.05**	**1.54**	**1.32**	**2.22**	**1.41**	**2.28**	**1.22**	**2.98**
β globulins - 3	4.17	6.72	4.4	6.6	4.37	7.36	4.7	8.28
β globulins - 4	**8.05**	**14.4**	**8.53**	**10.7**	**7.92**	**10.1**	**7.21**	**9.02**
α2 globulins	**2.26**	**3.54**	**2.03**	**3.47**	**2.17**	**3.27**	**2.71**	**4.29**
α1 globulins	**4.49**	**6.38**	**4.61**	**13.1**	**5.59**	**8.87**	**5.86**	**8.59**
albumin	**56.6**	**67**	**51.6**	**65.1**	**55.4**	**63.5**	**53.2**	**61.9**
transthyretin	**1.13**	**4.5**	**1.75**	**3.28**	**1.48**	**3.76**	**2.65**	**5.84**
unknown	2.1	4.33	2.36	4.6	2.39	5.91	3.08	5.16
unknown	2.36	4.6	1.55	3.34	2.49	3.85	1.7	3.21

The male oil dose group data from days 19 and 21 (Table 3.13) post dosing indicated further differences evolving with time. Gamma globulin and the #2 beta globulin protein peak showed significant differences associated with dose level (Wilcoxon, p= 0.005 and p= 0.0067, respectively). The non-pair bonding males in both these protein peaks also had higher levels (Wilcoxon, p= 0.021 and p= 0.0042, respectively). The #4 beta globulin protein showed a significant decrease across the dose groups (Wilcoxon, p= 0.0015). The alpha 2 globulin and transthyretin both showed a significant dose response (Wilcoxon, p= 0.018 and p= 0.0026, respectively). Also alpha 1 globulin and albumin, as with the gamma globulin and #2 beta globulin proteins, showed a response with both the dose level (Wilcoxon; p= 0.0039 and p= 0.023, respectively) and was lower in birds that failed to pair bond (Wilcoxon; p= 0.014 and p=0.021, respectively).

3.4.5. Day 72 after oil dosing in female mallards

The chronic phase is well established. It is still possible to see a dose response in the #2 beta globulin, #3 beta globulin and albumin protein groups. The #2 beta globulins (Figure 3.24) show a wide range in individual response in the oil dose groups, with some of the oil dose group birds responses higher than that of the control group.

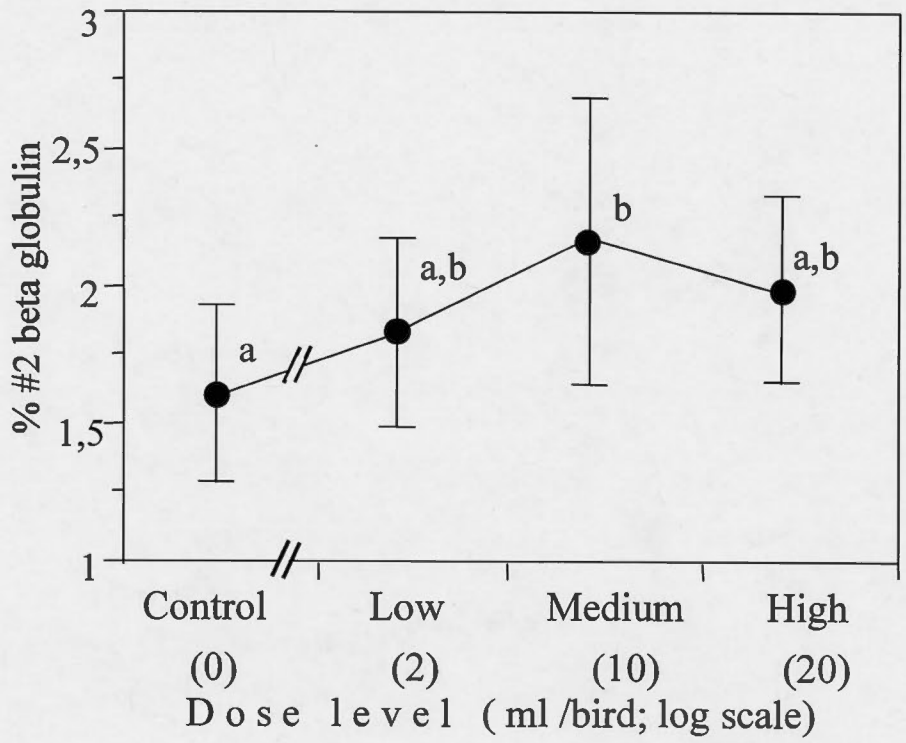


Figure 3.24. Protein peak #2 beta-globulin levels on day 72 after oil dosing in female mallards.The coordinates in the graphs that do not share a common letter are significantly different. ANOVA $F_{3, 34} = 3.4619$; $p = 0.0268$; specific means comparisons by Tukey-Kramer $p < 0.05$.

The #3 beta globulin group (Figure 3.25) shows no dose relationship in the low and medium oil dose groups compared to the control group. The result for the high dose group is not significantly different from the control group result.

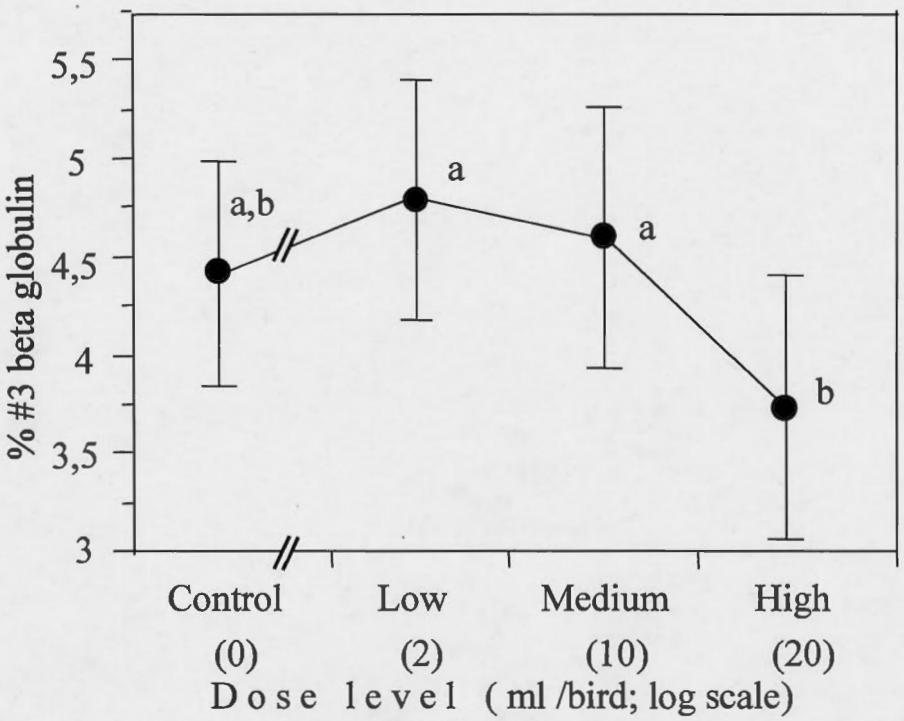


Figure 3.25 Protein peak #3 beta-globulin levels on day 72 after oil dosing in female mallards.The coordinates in the graphs that do not share a common letter are significantly different. ANOVA $F_{3,33} = 5.2310$; $p = 0.0046$; specific means comparisons by Tukey-Kramer $p < 0.05$.

Albumin, the negative acute phase protein still shows some dose responses (Figure 3.26), specifically in the medium oil dose group. The medium dose group are still statistically lower that the control dose group. The low and high oil dose groups both have results within the control range.

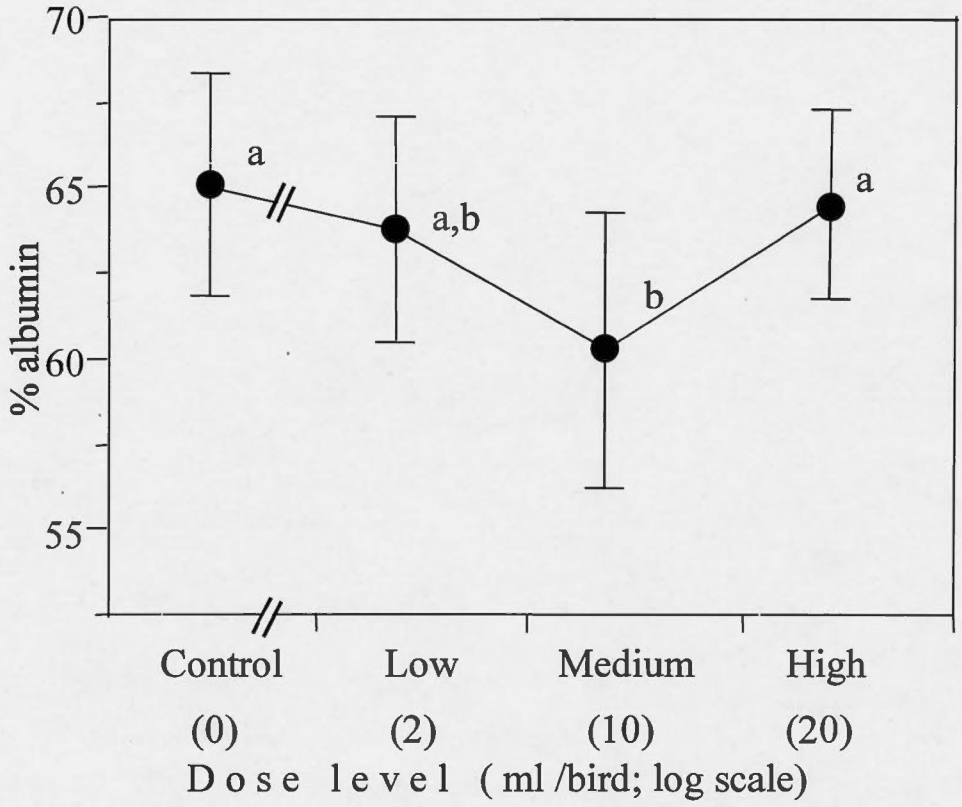


Figure 3.26. Albumin protein peak levels on day 72 after oil dosing in female mallards.The coordinates in the graphs that do not share a common letter are significantly different. (ANOVA $F_{3, 34} = 3.9403$; $p = 0.0163$; specific means comparisons by Tukey-Kramer $p < 0.05$).

The tabulated data (Table 3.14) for this time period show statistically that there are impacts within the #2 and #3 beta globulins and the albumin. The data do not show a visible difference in the ranges as collated, however, there are chronic changes associated with the dose groups still seen at this time period after dosing.

Table 3.14 Female dose group protein ranges at day 72 post dosing. Bold typeset indicates significant differences. The numbers are shown as a percentage of the total of the peaks quantified

T - 72 days	Control		Low		Medium		High	
Protein peak	**Lower**	**Upper**	**Lower**	**Upper**	**Lower**	**Upper**	**Lower**	**Upper**
γ globulins	3.76	9.25	4	9.21	4.16	8.08	4.27	8.09
β globulins - #2	**1.2**	**2.39**	**1.63**	**2.52**	**1.42**	**2.89**	**1.56**	**2.65**
β globulins - #3	**3.38**	**8.95**	**3.71**	**5.69**	**3.42**	**5.63**	**3.22**	**5.29**
β globulins - #4	5.17	7.94	4.25	8.64	4.79	7.6	4.92	7.47
α2 globulins	1.88	4.77	2.02	4.86	1.66	5.15	1.24	4.8
α1 globulins	4.72	7.2	3.94	9.76	5.47	11.1	4.31	11.7
albumin	**57.3**	**68.5**	**58.3**	**67.3**	**54.1**	**66.6**	**59.5**	**67.7**
transthyretin	2.98	5.11	2.67	8	3.37	9.31	2.98	5.51
unknown	0.35	1.19	0.293	1.56	0.15	1.91	0.201	1.43
unknown	2.07	3.14	2.15	3.19	2.4	3.93	2.46	3.27

The protein peaks #2 and #3 beta globulins and albumin (Table 3.14) all have significant differences associated with dose levels (Wilcoxon; p= 0.014, p= 0.022 and p= 0.040, respectively).

3.4.6. Day 278 after oil dosing in female mallards

The persistent increase in protein peak #2 beta-globulin (Figure 3.27) is linked to the dose group and is significantly different.

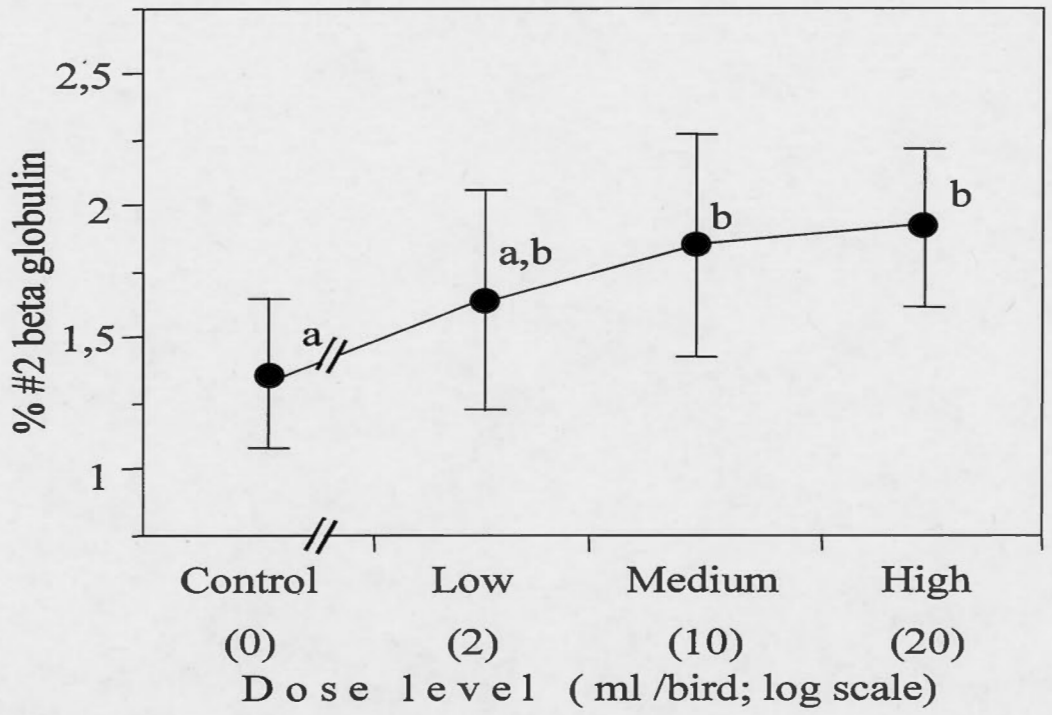


Figure 3.27. Protein peak #2 beta-globulin levels on day 278 after oil dosing in female mallards.The coordinates in the graphs that do not share a common letter are significantly different. (ANOVA $F_{3, 29} = 4.067$; $p = 0.0158$; specific means comparisons by Tukey-Kramer $p < 0.05$).

There are several trends noted at 278 days post dosing (Table 3.15) in the female dose groups. The #3 beta globulin protein peak, plus the peaks #9 and #10 appear to decrease in a dose dependent manner, especially at the upper levels of the range. However, the proteins alpha 1 globulin and transthyretin are increased in a dose dependent manner in the upper dose groups.

Table 3.15. Female dose group protein ranges at day 278 post dosing. Bold typeset indicates significant differences. The numbers are shown as a percentage of the total of the peaks quantified.

T - 278 days	Control		Low		Medium		High	
Protein peak	**Lower**	**Upper**	**Lower**	**Upper**	**Lower**	**Upper**	**Lower**	**Upper**
γ globulins	4.14	9.25	4.58	8.85	4.7	8.81	5.16	8.96
β globulins - 2	**1.08**	**5.27**	**1.12**	**2.28**	**1.21**	**2.53**	**1.49**	**2.36**
β globulins - 3	**2.2**	**5.09**	**3.18**	**5.09**	**2.54**	**4.49**	**2.5**	**3.72**
β globulins - 4	3.17	6.23	4.95	6.43	3.28	10.5	3.75	6.53
α2 globulins	1.54	4.52	1.1	3.02	0.77	5.29	0.785	5.52
α1 globulins	**5.28**	**7.88**	**4.54**	**8.27**	**5.23**	**9.42**	**2**	**8.65**
albumin	44.4	69.9	52.8	65.3	42.9	72.7	51.8	61.3
transthyretin	**1.7**	**9.15**	**2.4**	**10.5**	**3.17**	**8.39**	**4.77**	**14.8**
unknown 9	**1.53**	**16.4**	**2.25**	**13.3**	**2.01**	**13.4**	**2.72**	**14**
unknown 10	**1.32**	**7.84**	**1.2**	**3.36**	**0.876**	**3.16**	**0.347**	**3.64**

3.4.7. Day 334 after oil dosing in male mallards

The reduction in #3 beta globulin (Figure 3.28) group in the male mallards 334 days after dosing is significant, especially in the high oil dose group. The low dose and medium dose groups also share this trend, however, the medium oil dose group indicates wide variability within this group.

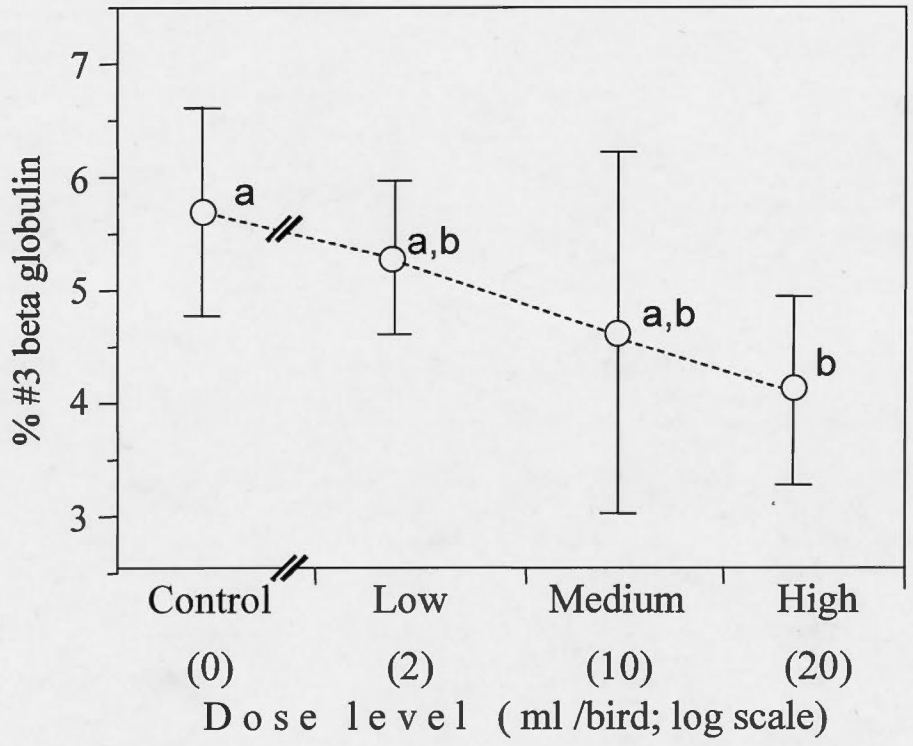


Figure 3.28. Protein peak #3 beta-globulin levels on day 334 after oil dosing in male mallards.The coordinates in the graphs that do not share a common letter are significantly different. ANOVA $F_{3,31} = 3.818$; $p = 0.0194$; Tukey-Kramer $p < 0.05$.

Transthyretin (Figure 3.29) at 334 days after oil dosing still shows a dose response relationship that although the data were not normally distributed, non-parametric and descriptive statistics are significant.

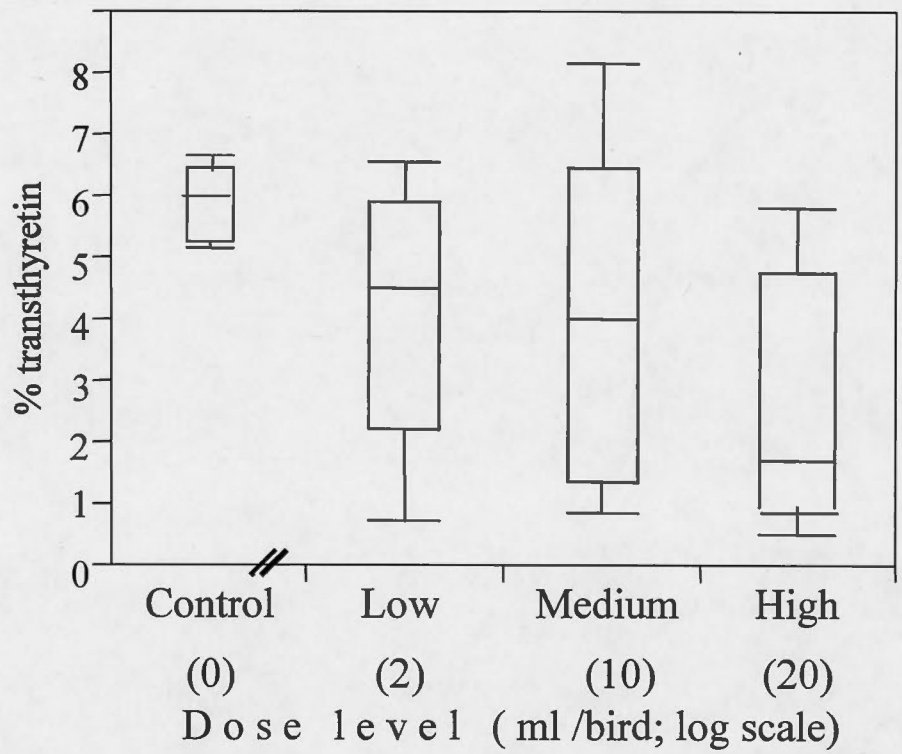


Figure 3.29. Transthyretin protein peak levels on day 334 after oil dosing in male mallards.Wilcoxon test, p = 0.0410. Note: data were not normally distributed, and therefore non-parametric test and descriptive statistics were used. Box plots show the medians and percentiles (10, 25, 75 and 90%).

Further changes are noted in the % range table for all the protein groups quantified (Table 3.16) which indicate a chronic shift in protein homeostasis. Transthyretin is still useful in exploring the ability of the male mallard to pair bond, whether in the oil dose groups or the control group. The protein peak #9 adds to this information concerning the male mallards ability to pair bond and is raised in any birds that failed to form pair bonds.

Table 3.16. Male dose group protein ranges at day 334 post dosing. Bold typeset indicates significant differences. The numbers are shown as a percentage of the total of the peaks quantified.

T - 334 days	Control		Low		Medium		High	
Protein peak	**Lower**	**Upper**	**Lower**	**Upper**	**Lower**	**Upper**	**Lower**	**Upper**
γ globulins	3.85	8.92	4.53	8.54	3.35	7.15	2.47	12.2
β globulins - 2	1.07	2.42	1.24	2.41	0.679	2.74	0.592	2.49
β globulins - 3	**4.36**	**7.22**	**4.17**	**6.51**	**2.72**	**7.24**	**2.93**	**5.64**
β globulins - 4	**5.59**	**10.5**	**6.2**	**12.1**	**4.15**	**13.8**	**5.96**	**10.1**
α2 globulins	2.59	3.61	1.94	7.63	2.27	3.45	2.27	6.52
α1 globulins	4.09	8.64	4.98	7.77	4.18	9.64	4.61	13
albumin	57.6	62.8	44.5	67.9	56.9	68.5	53.6	69.5
transthyretin	**1.33**	**6.68**	**0.747**	**6.56**	**0.883**	**8.18**	**0.543**	**5.85**
unknown #9	**0.207**	**2.83**	**0.396**	**5.76**	**0.582**	**3.24**	**0.276**	**4.01**
unknown #10	1.64	3.63	1.87	3.54	2.01	3.14	2.18	4.02

The data from the male dose groups, collected at 334 days post dosing (Table 3.16), shows significant differences in relation to the pair bonding. The #4 beta globulin protein shows a tendency for non-pair bonded males to have higher levels (Wilcoxon; p= 0.069). Transthyretin, is significantly lower in males that failed to pair-bond (Wilcoxon; p= 0.017), while the peak #9 is significantly higher in non pair-bonded males; Wilcoxon; (P= 0.017). In comparing the levels of proteins at the different dose levels, the #3 beta globulin protein and transthyretin both showed differences between the control and high dose groups. The #3 beta globulin protein showed that the high dose group have higher levels than the control levels (Dunnett's; p<0.05) and transthyretin high dose levels were lower that in the controls (Wilcoxon; p= 0.041).

3.4.8. Day 414 after oil dosing in female mallards

The final samples collected from the female dose groups (day 414 post oil dosing) show several trends. Specific statistically significant changes can be seen in the gamma globulin protein (Figure 3.30), #3 beta globulin protein (Figure 3.31) and the #9 protein (Figure 3.32). Trends are also noted in protein levels (Table 3.17) with a rise in the upper level of the range for the protein peaks #10 and decreasing levels in albumin being the most obvious.

The gamma globulins are significantly increased in response to the oil dose group at 414 days after oil dosing. The low and medium oil dose birds show a similar result, however, the high oil dose group shows an even greater response that was statistically significant.

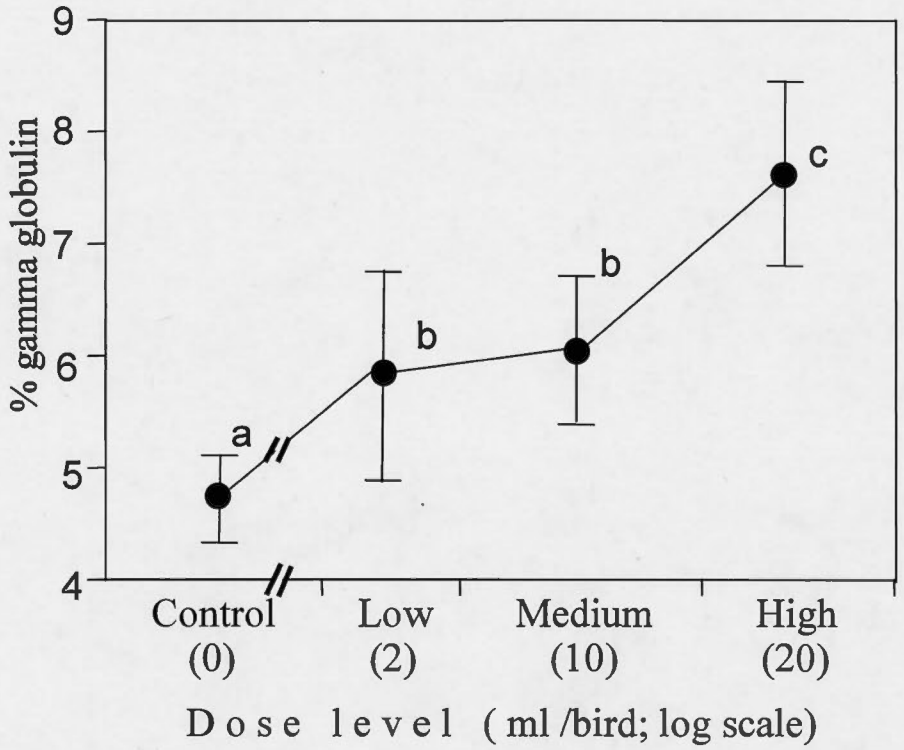


Figure 3.30. Gamma globulin levels at 414 days after oil dosing in female mallards.

The coordinates in the graphs that do not share a common letter are significantly different. ANOVA; $F_{3,25} = 16.013$; $p < 0.0001$. Tukey Kramer $p < 0.05$.

The #3 peak in the beta globulins does indicate that there is a statistically significant decrease in the level of this protein in response to dose in the medium and high oil dose female mallards at 414 days after oil dosing.

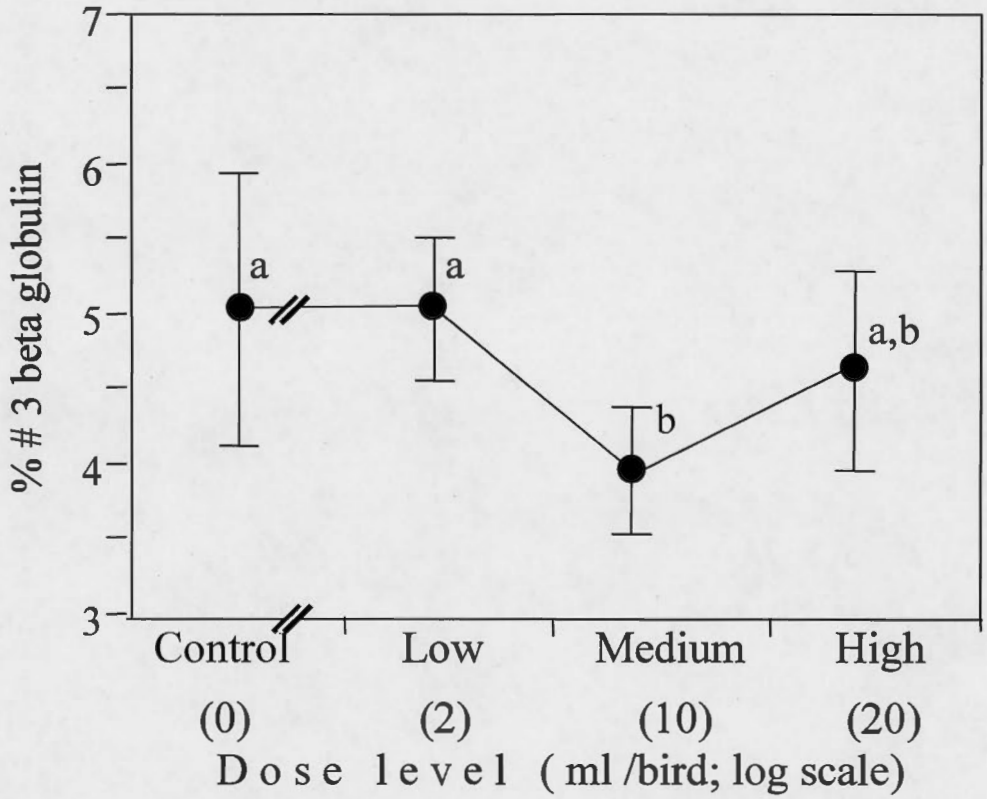


Figure 3.31. Protein peak #3 in the beta-globulins at day 414 after oil doing in female mallards.The coordinates in the graphs that do not share a common letter are significantly different. ANOVA $F_{3,25} = 4.6164$; $p = 0.0106$. Tukey Kramer $p < 0.05$

At this time, protein peak #9 (Figure 3.32) also showed a dose response relationship, that while not normally distributed, the non-parametric test and descriptive statistics aid in representing these data. There are birds in the oil dose groups that have maintained levels within the control birds' range, however some birds have a significant change in this protein in response to the oil dose from 414 days earlier.

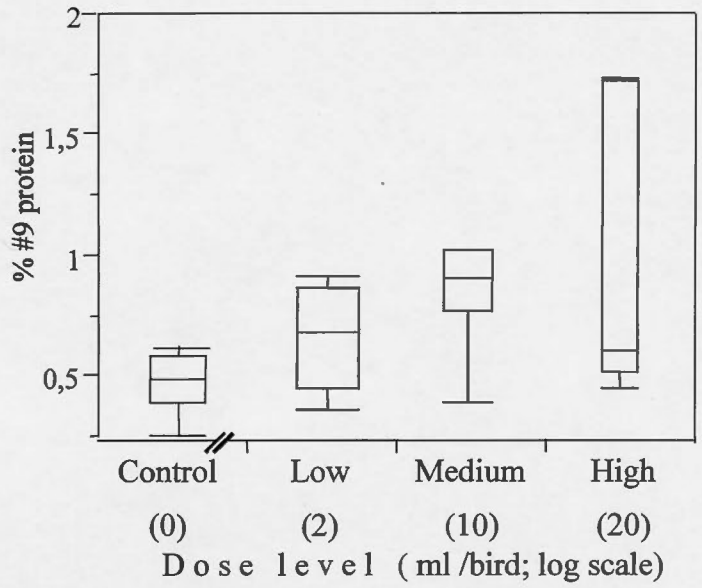


Figure 3.32. Unknown protein peak #9 in female mallards 414 days after oil dosing. (Wilcoxon p = 0.0359). Note: data were not normally distributed, and therefore non-parametric test and descriptive statistics were used. Box plots show the medians and percentiles (10, 25, 75 and 90%).

The tabulated data for the female mallards at 414 days after oil dosing show the ranges for the three proteins that show a statistical difference in the oil dose groups when compared to the control group (Table 3.17).

Table 3.17 Female dose group protein ranges at day 414 post dosing. Bold typeset indicates significant differences. The numbers are shown as a percentage of the total of the peaks quantified

T - 414 days	Control		Low		Medium		High	
Protein peak	**Lower**	**Upper**	**Lower**	**Upper**	**Lower**	**Upper**	**Lower**	**Upper**
γ globulins	**4.25**	**5.36**	**4.7**	**7.39**	**4.95**	**6.7**	**5.94**	**8.68**
β globulins - 2	1.56	2.71	1.52	2.5	1.85	2.41	1.58	3.07
β globulins - 3	**3.89**	**6.6**	**4.03**	**5.59**	**3.46**	**4.81**	**3.59**	**5.64**
β globulins - 4	6.49	10.1	6.1	11.1	5.57	10.5	6.5	9.68
α2 globulins	1.98	4.37	1.82	3.39	1.52	3.42	1.94	2.74
α1 globulins	4.64	10.5	6.08	11.4	6.47	11.8	8.06	10.8
albumin	58.4	65.2	56.9	63.8	55.4	66.2	54.9	60.6
transthyretin	2.82	6.61	2.44	6.31	2.96	7.06	3.08	7.01
unknown # 9	**0.256**	**0.626**	**0.365**	**0.918**	**0.39**	**1.98**	**0.446**	**2.43**
unknown # 10	2.74	3.43	2.44	3.43	1.19	3.69	2.29	4.06

3.5. Routine blood parameters and gross autopsy results

All blood samples collected were evaluated for their pcv. No birds appeared to have values considered outside the normal range throughout this experiment. Autopsy values for liver, testes and ovarian weight showed no oil dose response.

CHAPITRE IV

DISCUSSION

4.1. Behaviour

4.1.1. Agitation

The behavioural landscape that makes the individual bird function successfully within a species is critical to many aspects of survival. Any activity that places the individual outside the norm for its species leaves that individual vulnerable to many forces; poor nutritional intake because of poor foraging ability, loss of reproductive opportunity, and increased susceptibility to predation. The first intimation of an issue arising from the ingestion of oil in this research is linked to behavioural changes first noted in the weeks after dosing.

The collection of blood samples was routine and each step was designed to minimize the stress on the birds. Transporting the birds from the outside breeding pens to the laboratory for venepunctures was done by rapidly catching the bird and placing it, individually, in a darkened transport box. Each bird was weighed in the transport box and the venepuncture routine meant the bird was handled for less than 3 minutes. Typically one venepuncture, occasionally a second, was needed to obtain the required blood sample. In the weeks after the oil treatment, however, the dosed birds exhibited a behaviour (referred to here as 'agitation' behaviour) whereby up to five venepuncture attempts were necessary (Figures 3.1, 3.2, and 3.3). This agitation behaviour was primarily noticed in the birds in the higher oil dose levels. In contrast to this very specific behaviour in the high oil dose birds, heightened agitation by the

flock was noted on days where there were abnormal activities external to the research or even very hot days, and under these conditions, venepuncture attempts all had a similar degree of difficulty.

The issues arising from birds in an oil spill response situation are different in that the birds are all in a highly stressful situation, impacted by many other factors. They arrive with either hypo- or hyper-thermia, emaciation, dehydration, multiple health issues linked to the ingestion of oil, and all the while, being handled by predators (humans) in an unfamiliar landscape. Behavioural changes noted in birds during the course of an oil spill are often linked to increased preening and decreased foraging (Burger, 1997; Burger and Tsipoura, 1998). These behavioural changes are linked to the very real issue of feather maintenance and the increasing impact oil has on the organism which discourages it from feeding. Discussions regarding behavioural issues with members of the international oil spill rehabilitation personnel, including the staff based at the rehabilitation center at Fort George, Louisiana, during the Deepwater Horizon spill did not yield any specific information regarding behavioural changes in oiled birds. However, there are many factors that impacting a bird on admission; physiological damage linked the ingestion of the oil, emaciation and nutritional stress and clinically significant dehydration. These are the ones focused on in the triage and subsequent stabilization process. In one case, during the Deepwater Horizon spill response, at the sea turtle rehabilitation unit, it was noted that several

turtles were isolated because their behaviour was disruptive to other turtles in the main holding tanks. This, however, has been the only mention of specific issues in relation to behaviours that place an animal outside of the expected norm.

Within this research the mechanism for these behavioural changes was not specifically examined. However, in the human literature, agitation is associated with many factors, including regulatory issues within the dopaminergic, serotonergic, noradrenergic and GABAergic pathways (Lindenmayer, 2000; Engleborghs, *et al.*, 2008). The observational data in this research link a bird that over responds to restraint or other foreign stimuli to the dose level of crude oil. At this stage, the pathway for this response is conjecture and may be very difficult to evaluate in an oil spill rehabilitation center. However, normal or expected captive handling responses need to be documented to aid in exploring the responses of oiled birds that may support behavioural changes linked to an underlying neurological impact.

The use of post release survival studies can illuminate the potential for specific species impacted by petrochemicals to cope and survive. Several studies using banding and radio-tracking data have indicated that long term survival for some species is similar to non-oiled individuals (Anderson *et al.*, 1996; Underhill *et al.*, 1999; Golightly *et al.*, 2002). However, in the literature and discussions with researchers exploring post release survival, data indicate that where there are

mortality events, they are at their peak in the three to four weeks after release (personal communication Adam Grogan, RSPCA Wildlife Division)(Sharpe, 1996; Goldsworthy *et al.*, 2000). This may be the result of inappropriate behaviours leading to the mortality events.

These studies highlight the need to understand the toxic impact of each major oil, and the tolerance levels of different species to not only, the toxicity of oil, but also the ability of species to cope with the stress of captive care. This research offers an observational clue to aid in identifying those individuals impacted by the toxic components of the petrochemical involved. Behavioural changes are not routinely recognized or noted in triage. These changes may be quite subtle and species specific, but, given the outcome from this research, it may be a very useful indication that further investigation in needed in determining the toxicological impact on the individual bird

4.1.2. Pair bonding

Pair bonding is essential for normal reproductive cycles (Weller, 1965). Mallards pair bond earlier than other *Anas* species in the Northern Hemisphere (Johnsgard, 1960; Bluhm, 1988; Rohwer and Anderson, 1988) with almost 100% of female mallards expected to be paired by spring (Baldessare and Bolen, 1994). Even mate loss prior to spring migration is rapidly responded to with 97% of the widowed female mallards forming a new pair bond (Lercel *et al.*, 1999). This highlights the importance that pair bonding plays in the annual reproductive cycle of the mallard. Male mallards guard the female as she lays (Cunningham, 2003), and begins incubation. Once incubation is underway, the male then leaves and joins primarily male-male groups as they begin their moult into eclipse plumage. Oil ingestion has the potential to impair birds reproductive cycles (Holmes *et al.*, 1978; Cavanaugh and Holmes, 1982; Rattner *et al.*, 1984; Cavanaugh *et al.*, 1983; Cavanaugh and Holmes, 1987; Butler *et al.*, 1988; Holmes and Cavanaugh, 1990). These effects are either that the reproductive cycle is lost or delayed in oil impacted birds. It must also be noted that in these cases, the oiling event through the research design or a real world event, coincided with the reproductive season.

The next major observation period came seven months after oil treatment as the whole research flock was observed in the communal indoor aviary. The female mallards from the first cycle of dosing were housed over winter (2001 – 2002) with

an excess number of males. The following year (2002 – 2003), the dosed males were housed with an excess number of females. To ensure maximum reproduction, it is critical to give the female mallards opportunities to choose their own mates (Lebret, 1961; McKinney, 1992). Although it is probable that 100% of wild mallard females will pair bond (Baldessarre and Bolen, 1994), the pairing in captive birds is different with 80% of the females expected to pair bond in the Delta Waterfowl flock. These results are comparable with research into the performance of male mallards in captivity (Stunden *et al.*, 1999) where 80% of the females that initiated self-chosen pair bonds produced fertile eggs. Every opportunity in this research was given for the hens to maximize their reproductive effort.

Bluhm (1985) indicated that canvasback hens (*Aythya valisineria*) would forego breeding if their pair bond was disrupted, leading the author to conclude that pair formation was a crucial reproductive step for this species. In mallards, however, this is not thought to be such an issue (Humburg *et al.*, 1978; Ohde *et al.*, 1983; Lercel *et al.*, 1999). However, these researchers explored the role of re-establishing a new pair bond once the original one was dissolved by death or the loss of one member of the pair. Research to date does not address the issue surrounding the absence of a pair bond in the first place, as was the case in the present research.

Research data indicate pair bonding is a necessary part of the reproductive cycle and that decreased pair bonding rates will potentially result in reduced recruitment into the population. Pair bonding in the research birds was reduced in the medium and high dose oil dosed females, and in all three oil dosed male groups. From the data (Figures 3.4 and 3.5) pair formation occurs in 80% of the control birds in both research series forming pair bonds, compared to 57% in the female oil dosed groups and 41% in the male oil dosed groups. There is a gender response difference between the male and female oil dosed groups, with the threshold for females mallards being between 2 and 10cc's of oil while the male threshold is <2cc of crude oil. This translated into 3 out of the low oil dose male group forming pair bonds, while the female low oil dose group saw 9 out of the 10 birds forming pairs.

One issue that is hard to answer in the male dose groups is whether the un-paired females did not form pair bonds because the males were unable to offer the cues needed, or the females themselves evaluated the males and found them unsuitable, choosing to forgo the reproductive cycle. The outcome however, is that the reduced pair-bonding seen in these captive birds, may well translate into pair-bonding issues in wild populations, disrupting a very essential process. In the case of this research, these results are also of significance as this essential step is often very difficult to evaluate in the field. If the expected numbers of paired birds fail to arrive in the breeding grounds, is this because the population itself has decreased or is there a

problem with the reproductive process itself? The conditions maintained in this research facility plus the protocols used to observe the dose groups allowed these data to be collected, and their significance noted. The results in this significant step in reproduction are worrisome and given that pair-bonding is a crucial step, which if it fails, can ultimately result in a population level impact.

4.1.3. Nest construction

Notes on the hens in the female dose groups, while still in the indoor aviaries indicated that the nest boxes were treated differently by the high dose hens. The straw placed inside to provide the foundation for a nest, was often ejected. This was then further validated once the hens were all moved to the outside breeding pens and able to attempt a second clutch. The high dose hens almost invariably followed the same actions, all of the straw was tossed out of the nest boxes, with only one hen managing to form a nest and incubate the eggs to hatch. The control, low and medium oil dose groups did not show similar behaviours, and any true clutch formed (≥5 eggs), was generally incubated in a normal nest, until hatch. The majority of the nest boxes were entered, even if there was no egg lay, and the straw structured or compacted. To date, there is no mention of this issue in the literature or in conversations with field researchers.

The hormonal control for nest construction may be linked to estradiol levels which peak during the nest construction period in mallard hens (Bluhm *et al.*, 1983a). It is also raised for 2 to 5 weeks prior to egg production in canvasback ducks (Bluhm *et al.*, 1983b). In both species it then drops with the onset of egg lay. In this research, estradiol levels were not explored as the timing of the needed sampling may have impacted this biological function, however, it does offer a possible pathway for further exploration to understand this phenomenon.

4.1.4. Egg production

Since the dose groups were divided into one of four indoor aviaries prior to egg laying, all the eggs were identified with their parental dose group as they were collected. They were held at room temperature for one week prior to placing in the incubator as per the Delta Waterfowl recommended procedures. All the eggs from 2002 and 2003 laid in the indoor aviaries were artificially incubated and reared. Because of this procedure the normal signaling for incubation to begin by the hen, the presence of an incubation patch stimulated by her clutch was absent (Caldwell and Cornell, 1975). There was no signal, other than the biological boundaries, to limit egg production. Once the hens were moved to the outside pens where suitable nest boxes were provided and brooding behaviour was possible, a second attempt to produce a clutch initiated. The dose group differences noted while the birds were inside,

became even more evident. Within all dose groups, several hens produced eggs. Almost all nests that were initiated and maintained had the normal nest components; moulded nest and down-lined bowl. The exception was the nests in the female mallard high oil dose groups. The straw was mostly removed and if eggs were laid, they were not cared for and often cracked or broken. There was only one hen in the high dose group that managed to re-nest, lay a clutch and incubate most of her eggs to hatching.

Wild mallards lay from one to thirteen eggs in a clutch. If the number is higher, it can be assumed that another hen has contributed to the nest (Palmer, 1976; Pehrsson, 1991; Alisaskas and Ankney, 1992). Continuous lay in mallards has shown a range of 5 to 18 eggs with the mean 12.1 eggs per hen (Arnold *et al.*, 2002). Prince *et al.* (1970) showed egg production in the Delta flocks (base population, F1 and F2 generations) ranged from 9.5 to 12.1 eggs per hen over three clutches. This research project resulted in 13.8 eggs per hen laid, which is certainly within the expected production range. The slight increase seen in this research projects egg production may be related to the use of increased day length early in the reproductive cycle, something that had not been done in earlier work.

Other studies exploring petroleum and oviposition have, in general, noted reduced egg production in mallards as well as other species (Holmes *et al.*, 1978; Wootton *et al.*, 1978; Coon and Dieter, 1981; Cavanaugh *et al.*, 1983; Fry *et al.*, 1986; Albers, 2006). The primary difference between these references and my research involves the timing of the oil ingestion. Mallards in this research were given doses of oil equivalent to those acquired through a spill scenario, nine months earlier. Most research into the issues surrounding egg production and oil have meant that the birds were ingesting oil concurrent with, or immediately prior to oviposition.

Total egg production for each hen of the female dose groups (Table 3.3) shows an increased egg production in the medium and high dose groups. A primary hormone linked to egg production is luteinizing hormone. Mallard studies (Goldsmith and Williams, 1980; Bluhm *et al.*, 1983a) show luteinizing hormone increasing sharply prior to initiation of lay, fluctuating during the lay period, and declining with completion of the clutch. Progesterone also follows a similar pattern in mallards (Bluhm *et al.*, 1983a). Once incubation begins, both luteinizing hormone and progesterone levels fall and prolactin increases (Goldsmith and Williams, 1980; *Bluhm et al.*, 1983a). The egg production per hen in the medium and high oil dose groups is increased in relation to the control hens (34% and 29% respectively). This represents an increased cost to the hen, placing her outside the expected upper range for mallard egg production (Arnold *et al.*, 2002). This extended egg lay may indicate

that the triggers to halt egg production are not functioning as per the control group. The underlying issue may be that the luteinizing hormone levels have remained increased or that prolactin levels have not risen such that brooding behaviour is initiated and gonadal recrudescence begun (Bartke *et al.*, 1980; Sharp *et al.*, 1998).

Fertility in the female dose groups is interesting in that the low dose group laid fewer eggs with a greater percentage of these eggs being fertile. The overall fertility however, is not changed across the groups. Nest success also means that live young are not only hatched but fledge (Dzus and Clark, 1998). This experiment also looked at potential hatch success for the female dose groups. The trend to lowered hatch success shows that even if a hen produces greater numbers of eggs, poor hatchability may undo that advantage. That is if all of the other behaviours required to achieve success are also met.

The oil dosed group males were overwintered and given the opportunity to pair with healthy hens representing a range of ages from one to six years old. Egg production per hen reached expected levels of production, independent of the pair bonding ability of the males. Fertility was also not impacted in all groups. However, there were embryonic development changes were noted in the high dose group. The protocol for the eggs in this section of this research meant incubation was halted at day eighteen and the eggs were cooled to kill the embryos. On examination of the egg contents, it

was apparent that the developmental process was very successful for a majority of the embryos, with the majority of them developed to day 18. The high dose male group however, showed problems including a higher incidence of mortality prior to day eighteen incubation (17.5%) and of even more concern, a significant number of eggs (15%) that were fertilized but failed to develop past the twenty-four hour period of development.

Fertility has been shown to be negatively impacted by oil ingestion (Holmes *et al.*, 1978). Other areas where reproductive success was negatively impacted was in the reduced success rate for hatching (Holmes *et al.*, 1978; Cavanaugh *et al.*, 1982; Giese *et al.*, 2000). Cavanaugh *et al.* (1982) noted a drop in hatchability in eggs from contaminated mallard hens when compared to control birds of 71% to 53%. Much of the research examining the aspect of reproduction was carried out where oil was fed either continuously to the birds, immediately prior to egg lay, or observed in birds that were heavily impacted by an oil spill, rehabilitated and released.

Though relatively few embryos were noted with obvious abnormalities, they were interesting. One hen in the medium oil dose group produced an embryo with anencephaly. There were also a number of embryos from all oil dosed groups with apparent hydrocephalus. These are being held for pathological examination. The most obvious deformities were all observed in the female dose groups. The male research

group showed few obvious deformities, however the mortalities in the embryos indicate a problem with their development. The most intriguing outcome for the male dose group embryos was the fertilization of the egg with no development past twenty-four hours. In the wild, or in research where eggs were not examined, this phenomena would be overlooked. Yet it speaks to a potential teratogenic effect. The other factor which makes this an intriguing problem is that the oil dosing was done during the period of testicular refractoriness. There are no studies to date that have noted this phenomenon.

In reviewing the literature, there are few sources citing deformities caused by petroleum products. The primary issue appears to be the impact of polycyclic aromatic hydrocarbons on eggs and the subsequent embryological abnormalities (Albers 1983; Hoffman and Gay 1981; Fry, 1995).

One other impact in the male dose groups occurred with the incidence of infection in the eggs. As there were infections noted in eggs from all dose groups, it is suspected there was an underlying issue, perhaps arising from an incident over the winter when the water system failed and the birds were without fresh water on a daily basis for 2 weeks. The numbers of eggs in this group were not spread evenly across all groups, perhaps reflecting a loss of cloacal integrity compounded by an underlying physiological response induced by the oil ingestion.

4.2. Prolactin

The neurochemical array involved in so many of the processes required to both live and reproduce are intimately linked through areas within the brain (Silver and Ball, 1998). Induction and suppression pathways of neurotransmitters and hormones are slowly being elucidated and more is understood about the complexity involved (Kang *et al.*, 2007). Since many of the observations and outcomes which involve reproductive behaviour documented in this research indicated an underlying and as yet undefined mechanism, analysis of the prolactin levels provided valuable information on this facet. The samples from the baseline collections (T= -7 days), immediately after oil dosing, in April of the following year and at the final collection of bloods when the research was terminated were analyzed. The choice of these samples was based on availability, rather than those that would illuminate prolactin's role during egg lay and incubation and the temporal changes associated with the reproductive cycle. This is also true for other hormones that may further illuminate and direct research. Bluhm *et al.*, (1983a,b) noted that estradiol in both canvasback ducks (*Aythya valisineria*) and mallards, rose prior to egg lay, then decreased with the first eggs laid. Progesterone peaked and then dropped prior to egg lay, then peaked again during incubation in these birds. Prolactin however, increased over time in all groups, even stressed and non-laying birds, although the increase was not as high as in the laying birds. Eggs appear to be the primary factor linked to the presence of increased prolactin. This was demonstrated when the brood patch was anesthetized,

prolactin levels dropped in incubating mallards (Hall and Goldsmith, 1983). Yet, the last sample, day 414 post oil dose treatment, in the female mallard series, showed a higher than normal or predicted result (Figure 3.8). This is especially intriguing also because of the timing of this sample. Prolactin generally increases through egg lay to reach a peak at the onset of incubation. It then remains high throughout the incubation period. Once hatching has occurred, prolactin declines through the weeks of parental investment in the ducklings, to a baseline at the onset of autumnal recrudescence (Bluhm, 1988). This blood sample collected in September should reflect the baseline levels that occur in the rest of the flock. However, the results from the high oil dose females are increased, reflecting an unexpected result in the normal cycle of prolactin. Due to the limited sampling period and that there were no reproductive behaviours noted or expected at this time, it is difficult to explain this result. The other issue relates to the single time period sample which cannot provide detail of an increasing or decreasing level for prolactin.

There are few references to the impact that environmental contaminants play on prolactin levels in avian species in the laboratory or in the wild. Organohalogen contaminants have been linked to decreased prolactin levels in glaucous gulls (*Larus hyperboreus*) (Verreault *et al.*, 2008). One *in vitro* study found that the pesticide toxaphene increases prolactin mRNA levels (Graham *et al.*, 2003). Petroleum products are noted to impact prolactin negatively in domestic and wild mallards

(Harvey *et al.*, 1981; Cavanaugh *et al.*, 1983). However, these research results are often gathered with the oil dosing coinciding with the reproductive cycle itself. The results in this research are seen in the high dose female birds more than 400 days after an environmentally relevant dose of oil was administered to the birds. The combination of the observational data (increased egg production and poor nest construction) and the increased level of prolactin following oil dosing may help underline a breakdown in cues for expected normal activities arising from a neurochemical base. In a field setting this could then translate into reduced successful reproductive effort with reduced recruitment. Wolfaardt (2007) reported that 26% of penguins oiled and rehabilitated failed to breed in subsequent years. This loss was considered a permanent shift in these birds ability to reproduce. No data is available on the amount of oiling except that they were oiled and captured for 'de-oiling'. For whatever reason, a potential shift in biochemical cascades required for successful reproduction, can have an impact at the individual level. Where the population is a limited one, this can translate into population level impacts.

4.3. Protein electrophoresis

4.3.1. Introduction

The main goal in this research is to add a simple, yet effective test to gauge the toxicological damage suffered by the bird. The early use of the electropherogram was based on an understanding of the normal or expected ranges for each of the major protein groups; albumin, alpha 1 and alpha 2 globulins, beta globulin and gamma globulin. With research and increasingly sophisticated technology, these major protein groups have been explored to view the many individual proteins contained in these major groups. This base of knowledge is not readily available for non-mammalian species, especially those with little agricultural importance. It is also unknown whether the responses and individual proteins are ubiquitous or if there are analogues. The results described here provide insight into the changes elicited by a toxic assault in the mallard.

The individual protein graphs show results for those days where there is a significant difference due to dose group and gender. Statistical analyses performed on the % abundance tables for the protein peaks, show that there are several areas of impact during the evolution of the acute phase response into a chronic long term state. .

4.3.2. The acute phase: days one to day five post oil dosing

In the acute phase, the first changes occur in the twenty-four to forty-eight hour period after treatment (Figures 3.10, 3.11, 3.12, 3.13, 3.14, 3.15 and Tables 3.6 and 3.7). Both male and female mallard results indicate that not only did birds in the dosed groups respond to the oil dosing, some of these responses also acted as predictors of a much more long term impact; the change in pair bonding potential. There are also gender related differences in their response. In the evolution of a spill event, this early result is more likely to reflect the toxic impact of the oil itself. Once the bird is oiled and has resisted attempts to capture it for several days after the oiling event, the picture does change. Dehydration, starvation and secondary effects by the oil such as ulceration of the gastro-intestinal tract and anemia begin to add their assault on the body's responses with homeostasis being challenged (Tseng, 1999). These may obscure the protein changes in the days post impact that can be attributed to the toxic impact of the oil. For example, albumin, the most abundant protein is affected by a number of conditions including malnutrition. It has historically been used as a nutritional marker (Johnson, 1999; Beck *et al.*, 2002; Covinsky *et al.*, 2002). However, some human studies have shown it to be relatively insensitive to nutritional issues (Johnson, 1999). This may mean that once the birds are in the rehabilitation setting and the stabilization and rehydration issues have been addressed, albumin may still remain a useful protein, indicating changes corresponding to the toxic impact.

The protein peak #1, gamma globulin protein group is comprised of the immunoglobulins which function as antibodies (Davison *et al*, 2008). They are comprised of three main classes of avian specific immunoglobulins, IgY, IgM, and IgA. The data from the first days after oil dosing show that gamma globulins are increased in all the oil dose groups (Figure 3.10). Generally, changes in the gamma globulins are in response to acute or chronic inflammation, infection, chronic hepatitis and immune mediated disorders. There is also a significant gender difference, with the male control range lower that the female control.

The alpha 2 globulins (Peak #3, Figure 3.9; Figure 3.11) are often increased in kidney pathology. The results in this research have demonstrated an increased response in the low oil dose female mallards over the medium and high oil dose groups. The male oil dose groups show a slight increase through the oil dose groups which is not significant. Cray and Tatum (1998) note that this protein generally represents 4 to 8% of the total proteins in an electropherogram. It is therefore unlikely that the research results represent a shift from the normal range.

Albumin, a negative acute phase protein, does graphically demonstrate that each of the oil dose groups are undergoing a significant physiological stress. The male and female control results show no statistical difference, however, the response is gender driven for the oil dose groups. This major protein is also involved in maintaining the

acid-base balance and as a transport carrier for vitamins, mineral, hormones and fatty acids.

Transthyretin, a mammalian negative acute phase protein, shows a positive response in this avian research through this period. There is no literature to support this unexpected result in avian species. The response is also very significantly different between the male and female mallards, from the control ranges and then the responses between genders to the oil dose levels. This proteins major roles include the transport of thyroxin and retinoic acid (Richardson, 2002). Protein peaks #9 and #10 both show significant dose and gender responses during the acute phase period. Further research will be needed to identify these two peaks.

The beta globulins (reported in this research as the #2,3,4 beta globulins) do show a response to the toxic stressor during the acute phase period which is expected, as many of the avian acute phase proteins are found in the beta globulin region (Cray and Tatum, 1998).

4.3.3. The transitional phase; from six to fifteen days post oil dosing

Through this period the body is working towards returning to its normal state. There are no significant differences seen in this period.

4.3.4. The chronic phase; after sixteen days post oil dosing

Once the birds are moving into a chronic phase of the response after three weeks post dosing, the male mallards still show that pair bonding issues are observable with several protein peaks still low when compared to the control values (Table 3.13).

The other major result seen with several of these proteins, is that they remain dose responsive through the many months covered by this research. Gamma globulins do remain increased through the experimental period. This may mean that the body remains in a heightened state of readiness to confront any systemic assault.

Other changes are also persistent in the beta globulins, especially the peak #2 and #3. This group of proteins does include the acute phase proteins fibronectin and haptoblobin (Cray, 1997). Fibronectin acts as a substrate for cell migration in the embryo (Yamada *et al.*, 1992) and in feather morphogenesis (Haake and Sawyer, 1982). Haptoglobin in mammalian systems binds to hemoglobin to ensure the retention of iron and to limit the oxidative damage potential of the hemoglobin (Wassell, 2000). Its role is thought to be similar in avian species however, it does not occur in all avian species (Wicher and Fries, 2006). To explain the chronic changes in the beta globulins seen in the mallards will require further investigation.

Transthyretin also remains statistically different, initially as a positive response, followed by a significant negative response in the male mallards (Figure 3.28) at 334 days after oil dosing. The role of transthyretin in the transport of thyroxine and retinol is well established (Herbert *et al.*, 1986; Chang *et al.*, 1999). Cookson *et al.*, (1988) reported increased transthyretin in white storks (*Ciconia ciconia)* during the moult period. Some of the mallards in this study were blood sampled during periods where moult was underway, however, this is balanced by the control groups which were in the same condition as all the oil dosed groups. One other area where transthyretin has been explored is in the falcons infected with aspergillosis. Transthyretin has responded positively to this fungal infection, however, these results are still being prepared for publication (C. Cray personal communication).

4.3.5. Proteins – the bottom line

The changes that are documented over the course of this experiment do offer insight into the loss of protein homeostasis consistent with animals that have suffered damage within primary pathways that are linked to liver function, the primary site of protein manufacture. They also highlight the need to ensure gender is known or determined as soon as possible in the oil spill response for avian admissions. This research does support that mallards do exhibit a chronic change in the proteins evaluated, and thus homeostasis is shifted, measurable and outcomes may be linked to these changes that will impact the longevity and reproductive ability of these birds.

CONCLUSION

5.1. CONCLUSION

This research was undertaken to evaluate the use of the electropherogram (acute and chronic phase proteins) as an additional tool in the assessment of oiled birds entering a rehabilitation centre. Protein electrophoresis can evaluate the level of toxic impact from petrochemicals. It offers an understanding of the physiological response of the bird, based on the quantity of ingested oil, and links it to potential long term outcomes as seen in this study.

The primary question was whether a particular bird can overcome the impact of the toxic assault from the petrochemical, or would euthanasia be the most effective response. On admission to a rehabilitation center, birds are first examined to determine their health status and amount of oiling. If there are secondary issues such as fractures or wounds, euthanasia is often the preferred course of treatment. The next step involves drawing a blood sample to evaluate the bird's level of hydration, anaemia and potential infection. The stabilization procedure begins for those birds considered capable of tolerating and benefiting from the rehabilitation process, and where there are suitable facilities to undertake this commitment.

Acute phase protein determination, specifically the levels of the gamma globulin, beta globulins, albumin and transthyretin proteins all provide valuable information about the health status of the bird during the period of potential admission to a facility. This

process also requires that the sex of the bird is known to ensure the acute phase protein values are indeed linked to the appropriate gender.

There is a transition phase between the acute and chronic response phases, when protein values indicate a healing process and an attempt to return to normal physiological values. However, most oiled birds will remain in captive settings until they reach the chronic phase, and here changes to the proteins may again begin to arise which are still linked to the level of oil ingestion. Evaluation of the bird continues throughout the rehabilitation period, with blood samples being taken at regular intervals, which allows for the protein monitoring to continue. From discussions with oil spill rehabilitators and researchers, the highest mortalities are seen in the first few weeks after release. This is documented through band return notifications and radio-tracking of birds from rehabilitation centers (Sharpe, 1996; Goldsworthy *et al.*, 2000; Newman *et al.*, 2003).

This outcome may be related to the behavioural issues observed in this research. The level of agitation, as quantified by the number of venepuncture attempts, is a numerical representation of the over-reaction of the mallards to handling. If these birds consistently over-respond to a stimulus, it may represent a problem once they return to the wild. Foraging and predator avoidance are essential activities which are necessary for survival. It is possible that the over-responsiveness to environmental

stimuli such as the presence of predators or other birds that has resulted from oil toxicity, can result in maladaptive behaviour and therefore mortality from predation, starvation, or mal-functioning of the immune system. If the bird does survive to enter a breeding season, its likelihood of success is potentially diminished by impaired ability to form the necessary pair bonds, perform maternal behaviours, or produce viable off-spring.

An interesting aspect of this research is the timing of the dosing. Dosing was done after the birds had finished their annual reproductive cycle and were changing into eclipse plumage. Doses at environmentally low levels (Hartung, 1964) at a time of low hormonal activity still elicited impacts observable through the following year.

For the birds in this study, there are apparently three targets of impact for the oil as it enters the body via the digestive tract: gonads, liver, and hypothalamus. The incidence of fertilized eggs that failed to develop past the first 24 hours of incubation in the high dose male group may indicate damage within the testes. In females, the egg hatchability dropped with the upper dose group. All groups produced grossly abnormal embryos and eggs that failed to develop as expected; however, there appears to be a tendency to increased problems in the upper dose groups.

The changes in protein profiles are most likely the result of liver damage. The persistence of changes associated with dose level for the period of the study is disturbing and may potentially be observable by histopathology. This was not covered by this study, although samples have been collected and could be processed at a future date. Serum and plasma samples have also been stored and will be to further evaluate shifts in biochemical homeostasis.

The hypothalamus is the control center for many of the neurochemical cascades that drive life processes. Pair bonding, essential to the continuation of the species, did not occur in many of the birds in the oil dose groups. The underlying mechanisms leading to this event are not understood; however, research focused on several pathways including opioid, dopamine and norepinephrine pathways (Ottinger and Baskt, 1995) indicates their involvement in many of the early reproductive cycle behaviours. Agitation is another phenomenon with underlying neurochemical pathways. In humans, the pathways implicated also include the dopaminergic, serotonergic, noradrenergic and GABAergic systems (Lindenmayer, 2000).

The protein changes induced during an acute phase event (Gruys *et al*, 1994), have multiple and overlapping pathways (Suffredini *et al*, 1999). By following the protein changes seen in this research, from the acute to a chronic phase, it is possible that even relatively minor oiling events elicit a long term perturbation reflected in dose

level differences. These changes may also reflect an impact on the neuro-transmitters responsible for initiating an acute phase response, possibly within the dopaminergic pathway.

The underlying issue is one of humane response, what is the best course to take in an oil spill situation? Where possible, using protein electrophoresis as an assessment tool may aid in identifying those animals that have been profoundly impacted by the oil toxicity and have a very limited chance at survival and successful reproduction.
In addition, the chronic changes seen in the birds in this study, with dose related protein changes still evident throughout the research period, could be of use in monitoring birds outside the rehabilitation setting in the aftermath of an oil spill event. Birds ingesting oil via their food should also show these protein changes although they may not show exterior signs of oil spill exposure, and the impact of food contamination should be able to be estimated. Of particular interest would be those species dependent on the aquatic food chain including species that are vulnerable to oil spills, e.g. penguins, gannets, pelicans and gulls, and those less likely to be exposed to oil, such as shorebirds.

In summary, protein electropherograms are a valuable new assessment tool in the rehabilitation efforts for birds impacted by an oil spill event.

5.2. CONCLUSION

Cette recherche a été entreprise pour évaluer l'utilisation de l'électrophorégramme de protéines (phase aiguë et chronique) comme un outil supplémentaire dans l'évaluation des oiseaux mazoutés qui entrent dans un centre de réhabilitation. L'électrophorèse des protéines peut évaluer le niveau de l'effet toxique des composantes pétrochimiques. Elle propose une compréhension de la réponse physiologique de l'oiseau, sur la base de la quantité d'huile ingéré, et elle la relie à d'éventuels résultats à long terme comme on le voit dans cette étude.

La principale question était de savoir si un oiseau peut notamment surmonter l'impact de l'agression toxique à cause des composantes pétrochimiques, ou si l'euthanasie est la meilleure réponse. Lors de leurs admission à un centre de réhabilitation, les oiseaux sont d'abord examinés afin de déterminer leur état de santé et de la quantité d'huile impacté. S'il y a des problèmes secondaires, comme des fractures ou des blessures, l'euthanasie est souvent le cours de traitement recommandé. L'étape suivante consiste à prélever un échantillon de sang pour évaluer le niveau d'hydratation de l'oiseau, l'anémie et infection potentielle. La procédure de stabilisation commence pour les oiseaux considérés comme étant capables de tolérer et de bénéficier des processus de réhabilitation, et où il y a des installations appropriées à prendre cet engagement.

La détermination des protéines de phase aiguë, en particulier les niveaux de la gammaglobuline, globulines bêta, l'albumine et la transthyrétine, fournissent toutes des informations précieuses sur l'état de santé de l'oiseau pendant la période d'admission potentielle dans un établissement. Ce processus exige également que le sexe de l'oiseau soit connu pour assurer que les valeurs de toxicité aiguë des protéines de phase soient en effet liées au sexe approprié.

Il y a une phase de transition entre les phases d'intervention aiguës et chroniques, lorsque les valeurs de protéines indiquent un processus de guérison et une tentative de retour à la normale des valeurs physiologiques. Cependant, la plupart des oiseaux mazoutés restera dans les milieux captifs jusqu'à ce qu'ils atteignent la phase chronique, et ici des modifications apportées aux protéines qui sont liés au niveau de l'ingestion d'huile peuvent de nouveau commencer à émerger. L'évaluation de l'oiseau continue tout au long de sa période de réhabilitation, avec des échantillons de sang qui sont prélevés à intervalles réguliers, ce qui permet un suivi continuel des protéines. Selon des discussions avec des réhabilitateurs expert en déversements d'huile et des chercheurs, les plus hauts taux de mortalité sont observés dans les premières semaines après la libération des oiseaux. Ceci est documenté grâce à des notifications de retour de bande et radiopistage des oiseaux provenant des centres de réhabilitation (Sharpe, 1996; Goldsworthy et al, 2000; Newman et al, 2003).

Ce résultat peut être lié à des problèmes de comportement observés dans cette recherche. Le niveau d'agitation, telle que quantifiée par le nombre de tentatives de ponction veineuse, est une représentation numérique de la sur-réaction des canards colverts à la manipulation. Si ces oiseaux sur-réagir à un constamment stimulus, cela peut représenter un problème une fois qu'ils retournent à l'état sauvage. La recherche de nourriture et l'évitement des prédateurs sont des activités essentielles qui sont nécessaires pour la survie. Il est possible que la sur-réactivité aux stimuli environnementaux tels que la présence de prédateurs ou d'autres oiseaux qui résultant de la toxicité du pétrole, peuvent entraîner un comportement inapproprié et donc la mortalité peut être due à la prédation, la famine, ou le mauvais fonctionnement du système immunitaire. Si l'oiseau survit pour entrer dans une saison de reproduction, sa probabilité de succès est potentiellement réduite par altération de la capacité à former des liens de couple nécessaires, effectuer des comportements maternels, ou produire une progéniture viable.

Un aspect intéressant de cette recherche est le moment du dosage. Le dosage a été fait après que les oiseaux aient fini leur cycle de reproduction annuel et qu'ils étaient entrain de muer vers un plumage d'éclipse. Les doses à niveaux environnementaux faibles (Hartung, 1964) durant une période de faible activité hormonale ont suscité les effets observables au cours de l'année suivante.

Pour les oiseaux dans cette étude, il y a apparemment trois cible pour l'impact de l'huile l'lorsqu'elle pénètre dans le corps par l'intermédiaire du tube digestif: les gonades, le foie, et l'hypothalamus. La proportion d'œufs fécondés qui ont échoué à se développer au-delà des 24 premières heures d'incubation dans le groupe des males à forte dose peut indiquer des dommages dans les testicules. Chez les femelles, le taux d'éclosion d'œufs a diminué avec le groupe recevant la dose supérieure. Tous les groupes ont produit des embryons grossièrement anormaux et des œufs qui ont échoué à se développer comme prévu, mais il semble y avoir une tendance à des problèmes accrus dans les groupes recevant les doses supérieures.

Les changements dans les profils protéiques sont probablement le résultat de dommage hépatique. La persistance des changements associés à la dose pour la période de l'étude est inquiétante et peut potentiellement être observable par l'histopathologie. Ceci n'a pas été couvert dans cette étude, bien que les échantillons ont été recueillis et pouvaient être traitées à une date ultérieure.

L'hypothalamus est le centre de contrôle pour la plupart des cascades neurochimiques qui animent les processus vitaux. La formation du couple, indispensable à la continuité de l'espèce, n'a pas eu lieu dans la plupart des oiseaux dans les groupes recevant les doses d'huile. Les mécanismes sous-jacents menant à cet événement ne sont pas compris; cependant, la recherche axée sur plusieurs voies, in durant les voies

opioïde, de la dopamine et de la noradrénaline (Ottinger et Baskt, 1995) indique leur implication dans la plupart des comportements précoces du cycle de reproduction. L'agitation est un autre phénomène avec des voies neurochimiques sous-jacentes. Chez l'humain, les voies impliquées incluent également les dopaminergiques, les sérotoninergiques, les systèmes noradrénergiques et GABAergique (Lindenmayer, 2000).

Les changements de protéines induits lors d'un événement de phase aiguë (Gruys et al, 1994), ont des voies multiples et qui se chevauchent (Suffredini et al, 1999). En suivant les modifications des protéines observées dans cette recherche, de la phase aiguë vers une phase chronique, il est même possible que les déversements d'hydrocarbures relativement mineurs puissent susciter une perturbation à long terme en fonction des niveaux de dose. Ces changements peuvent aussi refléter un impact sur les neurotransmetteurs responsables de l'initiation d'une réponse de phase aiguë, possiblement dans la voie dopaminergique.

Le problème sous-jacent est celui de la réponse humaine, quelle est la meilleure voie à prendre dans une situation de déversement de pétrole? Lorsque cela est possible, l'utilisation de l'électrophorèse des protéines comme un outil d'évaluation peut aider à identifier les animaux qui ont été profondément touchés par la toxicité du pétrole et qui ont une chance très limitée de survie et d'avoir un accouplement réussit.

En outre, les changements chroniques observés dans les oiseaux de cette étude, avec les changements de protéine liée à la dose évidente tout au long de la période de recherche, pourraient être utiles dans la surveillance des oiseaux en dehors du cadre de réhabilitation à la suite d'un déversement d'hydrocarbures. Les oiseaux qui ont ingéré du pétrole via leur nourriture devraient aussi montrer des changements de protéines, bien qu'ils ne démontrent pas de signes extérieurs de l'exposition à en déversement de pétrole, et l'impact de la contamination des aliments devrait pouvoir être estimé. Il serait particulier l'évènement intéressant d'étudiés des espèces qui dépendent de la chaîne alimentaire aquatique, y compris les espèces qui sont vulnérables aux déversements de pétrole, par exemple, les pingouins, les fous de Bassan, les pélicans et mouettes, et ceux qui sont moins susceptibles d'être exposés à l'huile, tels que les oiseaux de rivage.

En résumé, les électrophérogrammes de protéines sont un nouvel outil d'évaluation précieux dans les efforts de réhabilitation pour les oiseaux touchés par un déversement d'hydrocarbures.

APPENDICE A

OIL SPILL REHABILITATION RESPONSE FOR BIRDS

A. Introduction

Whether it is an individual bird, a small number of impacted animals or a major spill, the care of the birds is based on the same process. Caring for oil impacted birds is well documented with several dedicated organizations committed to providing scientifically based response and trained personnel (Table A.1).

Table A.1 Oil Spill Non-Governmental Organizations (NGO's) based in North America

Name	Address	Website
Tri-State Bird Rescue and Research	110 Possum Hollow Road, Newark, Delaware, 19711, USA	http://www.tristatebird.org/
IBRRC - International Bird Rescue	444 W. Ocean Boulevard, Suite 777 Long Beach, California 90802, USA	http://www.bird-rescue.org/
OWCN - Oiled Wildlife Care Network	UC Davis Wildlife Health Center TB 128 Old Davis Road Davis, CA 95616, USA	http://www.owcn.org/

By exploring a specific oil event, all the various areas of rehabilitation can be placed in context. The Deepwater Horizon oil spill, 65 km off the coast of Louisiana, USA in the Gulf of Mexico, began on the 20th April, 2010, with an explosion at the well-head, 1500, below sea level (Kerr *et al.*, 2010). This major event can act as a very recent example of the professional response to oiled birds using current techniques in care and rehabilitation and will be used in this appendix to illustrate the activities that contribute to the wildlife rehabilitation response mounted in an oil spill.

A.1 Capture

A.1.1 The reality of capturing oiled birds.

The first issue is that of actually capturing the oiled birds. This can be quite difficult as lightly oiled birds may still be able to fly. Also, pelagic species will retreat to the water for safety, or remain able to dive if capture is attempted from a boat. The length of time a bird is compromised prior to capture can add significantly to the problems faced in rehabilitation.

The expertise represented in the NGOs (Table A.1) currently working on wildlife impacted by oil often offers wildlife its best chance for recovery. This includes active involvement in the capture of lightly oiled and mobile birds. Teams with the expertise

in boat captures can often catch birds under difficult situations, including capture at night. The oiled birds resting on the water will allow a team to approach closely (personal communication, Jay Holcombe, IBBRC). It takes a great deal of stealth and team coordination to be effective and successful as any noise will spook the birds, making them flee. This activity also is regulated in most countries, covered by health and safety laws, plus wildlife and environmental legislation, all of which must be addressed prior to any field activities.

A.1.2 The Deepwater Horizon situation

During the Deepwater Horizon spill response, teams were assigned to the capture and transport of oiled birds to assigned rehabilitation centers. These were trained personnel with the appropriate Occupational Health and Safety Administration (OSHA) and British Petroleum (BP) permits. For example, while I was assisting in the field surveys in Louisiana, I was not permitted to even touch an oiled bird as I did not have the appropriate permits, despite my past experience in the field of wildlife rehabilitation and oil spill response.

A.2 Admission

A.2.1 Overview

The birds admitted to rehabilitation centers can arrive in various conditions; lightly oiled, yet very debilitated; moderately oiled with compromised health; or heavily oiled with very few obvious health issues. To add to the problem is the type of oil or petroleum product. Its toxicological and physical properties can then add a myriad of problems to the care and health impacts of the birds admitted to the center (Frink and Welte, 1990; Tseng, 1999; Berg, 2003). On top of the obvious issues posed by the oil, injuries and underlying illnesses can add another dimension. These issues can include gastro-intestinal erosion, eye ulceration and irritation, and respiratory effects, in addition to standard issues such as dehydration and emaciation.

A.2.2 Deepwater Horizon admission process

Tri-State Bird Rescue and Research, Delaware, USA, was contracted by British Petroleum (BP) to be responsible for the wildlife response component of the disaster management. The International Bird Rescue Research Center (IBRRC) and the Oiled Wildlife Care Network (OWCN) were also involved with other components of the effort, including media relations and chelonian and marine mammal rescue and care. Birds were captured and transported to centers along the Gulf of Mexico, primarily

southern Louisiana, Mississippi, Georgia and Florida. I was based at Fort George, Buras, Louisiana. On arrival at the center, the birds were given an initial exam. Injuries or obvious illness were grounds for euthanasia unless deemed minor and treatable within the context of the oil spill response process. Any oil product was wiped from the oral cavity, and eyes flushed with copious saline. The birds were leg banded, photographed and a feather sample collected for later fingerprinting of the oil to ensure it was linked to the presumed source (Fig. A.1 and A. 2). The chain of evidence paper trail is crucial for all admissions, even for all wildlife dead on arrival. All dead and euthanized animals were stored, frozen and later examined for evidence of oil impact. The photographic evidence, feather sample and paperwork for each bird are stored and may be used in legal actions taken against the responsible parties.

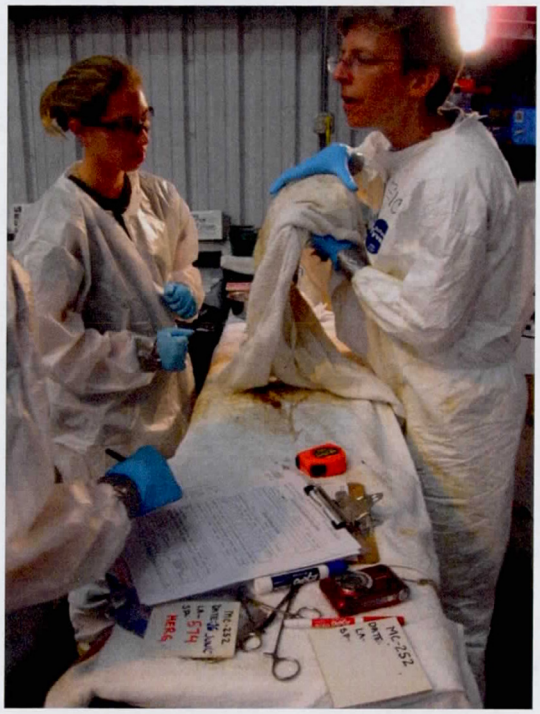


Figure A.1 Admission process at Fort George, LA. Dr. Erica Miller (right) from Tri-State Bird Rescue and assistant examine a brown pelican (*Pelecanus occidentalis*) being admitted to the rehabilitation facility. Note the protective clothing worn at all times when handling oiled birds in this event

.

Figure A.2 All body surfaces were checked and records kept about the extent of oiling on each admission, as can be seen on this brown pelican

A.2.3 Blood sampling and tests

A blood sample is collected from the birds for a baseline evaluation of the hydration state, hemoglobin and plasma solids which are presumed to be total protein although glucose and cholesterol can also elevate this value (Figure A.3). However, these admits are often so debilitated that the total solid value can be considered to be mainly protein.

Figure A.3 Blood sample being collected from the transverse medial metatarsal vein in a brown pelican.

One simple field test performed is the hematocrit. A small sample of whole blood is collected in a capillary tube. This tube is then centrifuged at 12,000 rpm for 5 minutes in an appropriate centrifuge. The capillary tube is then placed on a hematocrit reader (Figure A.4) where the base of the blood column is placed at the zero mark, the tube adjusted such that the top of the blood column lines up with the 100% mark. The hemoglobin is then recorded as a percentage value. In general, a reading below 30% indicated a potential anemia whereas a reading over 60% indicates a significant level of dehydration. Normal ranges are not known for many species, however, an avian hematocrit generally ranges between 35 to 55% depending on the species (Thrall *et al.*, 2004).

Once the blood samples were processed, those birds with severe anemia or dehydration were re-evaluated. If still deemed capable of overcoming these issues, further oral fluid therapy was given, otherwise the bird was euthanized.

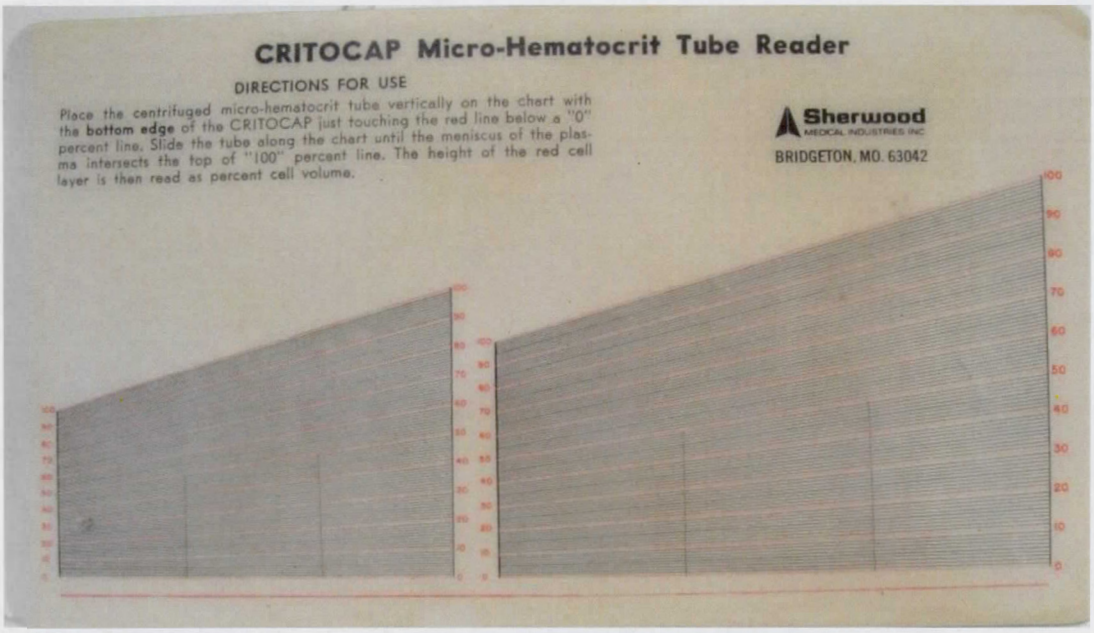


Figure A.4 Hematocrit reader used to evaluate the percentage of red blood cells in a blood volume.

The other simple test done at this time evaluates the total solids in the plasma fraction of the capillary tube sample. A refractometer similar to that illustrated in Figure A. 5 is used to measure the total solids by using the refraction of light through a liquid that contains chemicals (Figure A. 6). In this case, the predominant molecules are that of proteins, although cholesterol and sugars can elevate the reading. However, this quick test yields a great deal of information about the hydration level, with high levels of protein (>6g/100ml) indicating significant dehydration. Very low levels of protein (<2g/100 ml) can indicate starvation or kidney failure. Low protein levels, as in the case of starvation, will respond by increasing over the next days as the bird receives nutrients, while a failure to rebound may indicate further investigation is needed.

Figure A.5 Hand held refractometer used to quantify total solids which are generally equivalent to total protein levels.

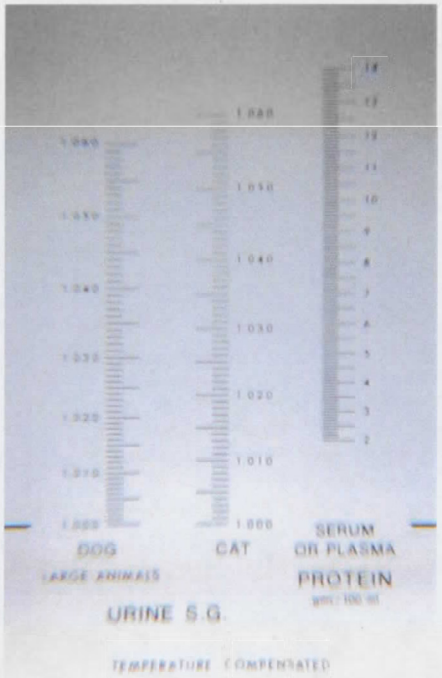


Figure A.6 Scale used in the refractometer. The putative protein scale on the right side is in gm/100ml.

A.2.4 Stabilization process

On admission, each bird is started on rehydration therapy. Warmed oral fluids, either Pedialyte® or Lactated ringers®, were administered into the gastro-intestinal tract via a tube, to start rehydrating the birds. Where there was evidence of oil ingestion, a dose of Sulcrate® (sucralfate) was also added to the oral fluids. Sulcrate® acts as a gastro-duodenal cytoprotective agent, protecting the gastro-intestinal tract from further impact of the oil and preventing absorption of any oil remaining in the gastro-intestinal tract.

A.3 Housing

Each bird is placed in a cage appropriate to its species, age and condition. For flock birds such as pelicans (brown (*P. occidentalis*) and white (*P. erythrorhynchos*)), a common admission in the Deepwater Horizon spill, they were housed in 8' square pens made of plywood sheets. The flooring of these 4' high pens was disposable sheeting material, changed daily to ensure that any oily fecal material and waste products could be removed rapidly, limiting further oil contact for the birds housed in the unit. The pen tops were an open mesh to help with observations and ventilation. Smaller birds and those in intensive care were housed in plastic cat and dog crates (Figures A.7 and A.8). Supplementary heating was provided for chilled or weak birds by using infra-red lamps clamped beside the cage. All caging was set up to allow ease of observation and handling for further treatments.

Figure A.7 A crate set up for smaller species needing intensive care. Food appropriate to the species is provided. The red colouration is from heating lamps.

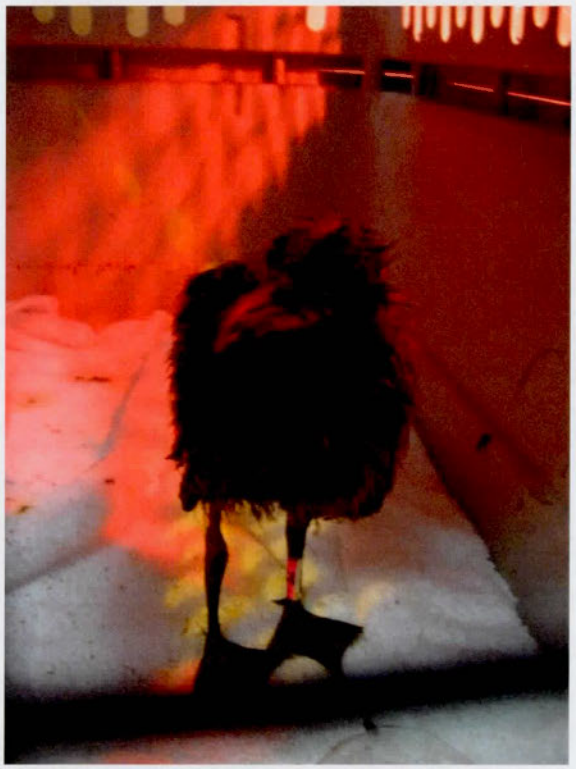


Figure A.8 A laughing gull (*Larus atricilla*) chick, covered in oil. Note the leg band for identification.

A.4 Feeding and monitoring intake.

Each pen also had a water source and trays of food appropriate to the species. The pelicans for instance, had whole fish available, while the laughing gull chick's fish were cut into small pieces. Food intake was monitored through regular weigh in's of each bird (Figure A.9), ensuring anorexic birds are noted and re-evaluated.

Figure A.9 Daily weights were recorded. Two members on that team are pictured here placing a pelican in the plastic container, tared and ready, on the scale.

A.5 Human health and safety

Ventilation in the temperatures in this region was critical and air was moved through the rehab center, an empty warehouse, with industrial fans. The temperature with humidity often registered between 40°C and 49°C posing a health hazard for the human caregivers and birds alike, especially as protective clothing is critical and required by OSHA (Fig.A.10). To help with venting the warm air under the Tyvek®

safety suits, slits were placed down the back (Figure A.11) however, no skin could be exposed to the product as contact dermatitis had been reported in people previously. All joins were sealed using duct tape (Figure A.12). Safety glasses were used when simply transporting birds (Figure A.10) instead of the full face shield used when in the washing lines.

Figure A.10 Full safety gear required for the handling of oiled birds.

Figure A.11 To help dissipate heat from around the body, slits were allowed in the backs of the Tyvek® suits.

Figure A.12 Joins between the gloves and suits were sealed using duct tape. The trick was to make a tab of the tape end to ensure easy removal.

Figure A.13 Safety glasses were required whenever handling any birds as seen here as a handler carries a brown pelican.

A.6 Stabilization

The period for stabilizing the birds is dependant on many factors; the health of the birds, the number of birds in care, the staffing with experience and expertise to handle the wash routine, the water supply and needed materials such as the Dawn® dish detergent, towels, and safety equipment for the team. Until a bird is bright, alert and responsive (BAR), which means it is stable, well hydrated, gaining weight, its

hemoglobin and serum proteins or total solids in the expected normal range and its behavior indicative of a bird feeling better, it cannot be washed. In pelicans for instance, these large birds are difficult to handle when in normal health. Over the first days post admission, they are often very easy to handle, putting up little fight and posing little risk to the handler. It is very obvious to experienced handlers when the bird begins to feel better and objects vigorously to being handled. Once the bird meets all the criteria needed, it is then deemed stable enough to undergo the wash process. In the case of the Deepwater Horizon spill, most birds required at least 5 days of care before the wash was considered.

A.7 The wash line.

A.7.1 Preparation

Once deemed ready, the birds scheduled for washing had their food withheld for some hours before being handled. This helps minimize the issue of regurgitation.

The oil from this spill formed a tarry coating over the birds. To begin the softening process, the birds were 'pre-basted' with methyloleate, a liquid that is soluble in organic liquids and considered a safe chemical. Once this liquid has been applied to all tarred areas, the birds were placed in a holding crate for 30 minutes. Then, the birds are ready to wash.

A.7.2 Washing birds

Water temperature is critical and maintained at 42°C throughout the wash process, ensuring a temperature that will help with the penetration of the wash liquids into the tar/methyloleate coating. At this temperature, the birds also do not experience hypothermia. Dawn® detergent is used at between 5 and 15% in the first wash, helping to hydrolyse the oil complex and hold it in the water column. The other process also used in this response incorporated a water spray unit with the Dawn® concentrate in the applicator container to spray detergent laden water into the feathers. As the tubs of detergent were soiled, the bird was moved to a clean tub, and the process continued until the water stayed clean (Figure A.14).

Figure A.14 The wash line at Fort George, LA.

Then the rinse is begun with the bird moved to a clean area of the wash line (Figure A.15). These team members are only required to wear safety glasses and not the full face shields as there is no danger of oily product impacting the handlers by this stage of the process.

Figure A.15 The rinse team at Fort George, LA

The structure of feathers is crucial to restoring waterproofing (Rijke, 1970), and the rinse cycle essential ensuring the final step. This step takes time as all the soap must be removed from the now clean feathers. The bird is rinsed from the beak and head down area to the feet, ensuring the feathers are restored to normal conditions. Once all the soap has been rinsed from the feathers, they are essentially washed dry. The test to know of the soap is removed, is that the water begins to 'bead up' on feathers as their hydrophobicity returns (Figure 16). However, the final drying step finishes this process (Figure 17) with the birds held in a very clean area with warmed dry circulating air. They are continually observed for signs of stress and hydration

problems. As can be seen, syringes (Figure 17) with prepared fluids are ready for use in any bird showing signs of distress.

Figure A.16 Water beads on the head feathers of a northern gannet (*Morus bassinus*).

Figure A.17 The drying room at Fort George, LA. Rehydration fluids are always kept available to support any bird in distress through this process. The arrow indicates the fluids held in readiness for any bird in distress.

This process can take as little as an hour, but also can take longer, with many people required. Birds showing any distress are returned to a cage to rest and the wash process continued as soon as the bird is ready. In general, the pelicans cope extremely well with the whole process. This is not so for many species, although it is an issue of monitoring each bird as an individual.

A.8 The final steps

Once the birds are cleaned, the next stage requires time and care. The birds are observed for a day or so in small caging units with limited access to water, ensuring they are truly waterproof as they preen and wash themselves, and have coped with the stress of the wash-line. The next step requires that the birds are placed in outside flight cages fitted with pools appropriate to the species, to finish their recovery. This may take days to weeks depending on several factors. The individual bird's health is monitored and 'weigh in's' scheduled regularly to ensure they are maintaining or gaining weight. The pools where they are housed are also crucial, with constantly flowing clean water to ensure they do not loose waterproofing from fecal or food contaminated water. If waterproofing has not been totally restored or is lost again, they will be rewashed. Birds that have been severely debilitated also need to regain aerobic strength prior to release. This means caging that allows for flight. Also, it is desirable to have species specific caging to minimize interspecific aggression and support natural behaviors.

A.9 Release

The release of these birds is also governed by many factors. First, each bird should be in good health, with blood parameters and body mass in the normal ranges for that species and fully water proofed with feathers in good condition. Weather plays a role and several days of stable weather forecast is needed to ensure a success reintegration into the wild.

The most critical factor is that they are released into a clean environment. For example, in the Deepwater Horizon spill, many pelicans were relocated to colonies along the Texas coastline and also the eastern coast line of Florida.

A.10 Summary

There are several factors involved in responding to an oil spill involving wildlife. They all need to be integrated into the response to ensure human health and safety concerns are addressed first. Then the wildlife, in this case, the birds must be assessed at each step of the process, ensuring only those deemed truly healthy and fit, will be released.

APPENDICE B

STATISTICAL EVALUATION OF SERUM PROTEIN DATA

Data were transformed (arc-sine$\sqrt{}$(percentage ÷ 100)) to achieve normality of the data or the residuals, and occasional outliers were removed as indicated in the summary tables (below). Unless otherwise indicated, analysis of variance (ANOVA) was performed using different models that are indicated in Tables B.1, B.2, B.3, B.4, B.5, B.6, B.7, and B.8. When male and female birds were included in the same blood series and the blood collection was necessarily conducted over a 2-day period (methodological limitation due to the large number of samples), the ANOVA model was designed to test for the effects of dose level, day of blood sampling and sex as the main effects, in addition to the interactions between these parameters. When blood was collected from either male or female mallards over a 2-day period, the ANOVA model tested for the effects of dose level, day of blood sampling and their interactions. In the case that blood samples were collected from either males or females on a single day, the effect of dose level was evaluated with a simple ANOVA. Only probabilities <0.05 are presented in the summary tables that follow.

Where the ANOVAs identified significant effects, specific means comparisons were achieved using the Tukey-Kramer test with $p < 0.05$ or by calculating Least-Squares-Means and using a T-test adjusted for multiple means comparisons. These results have been presented in graphic form in the results section of the thesis.

In the case that the data, the transformed data or residuals were not normally distributed even after removing outliers, the data were analyzed with the nonparametric Wilcoxon test for a general dose-related effect without specifically comparing pairs of dose levels. These results have been presented in graphical form in the results section of the thesis.

Table B.1 Summary of statistical analyses (ANOVA) for serum proteins in male and female mallards at sampling time – days 1 and 2.

Peak	Effects						
ID	dose	day	sex	dose*day	dose*sex	day*sex	d*d*s
1	0.0001[a]		<.0001			0.0369	
2							
3							
4		0.0478	<.0001				
5	0.0108	0.001	0.0002				
6		0.0082	<.0001			<.0001	
7	0.0002	0.0382	<.0001			0.0034	
8	<.0001	0.008	<.0001		0.0294		
9			<.0001		0.019	0.0059	
10	0.0003		<.0001	0.0145			

[a] Probability values.

Table B.2 Summary of statistical analyses (ANOVA) for serum proteins in female mallards at sampling time – days 3 and 4.

Peak	Effects		
ID	Dose	Day	Dose*Day
1			
2	0.0357[a]	0.0028	
3[b]	0.0282		0.0106
4	0.0378		
5		0.0403	
6			
7			
8		0.0115	
9			
10			

[a] Probability values.

[b] the outlier bird # P82 was removed from the data set for this peak

Data for the highest dose level are missing and therefore this dose level was not included in the analysis.

Table B.3 Summary of statistical analyses (ANOVA) for serum proteins in male mallards at sampling time – days 6 and 7.

Peak	Effects		
ID	dose	day	dose*day
1		0.0126[a]	
2[b]		<0.0001	<0.0002
3			
4			
5		0.0078	
6			
7		0.0095	
8	0.0398	<0.0001	
9			0.0367
10			

[a] Probability values.

[b] the outlier bird # B58 was removed from the data set for this peak

Table B.4 Summary of statistical analyses (ANOVA) for serum proteins in male mallards at sampling time – days 19 and 20.

Peak	Effects		
ID	dose	day	dose*day
1	0.0141[a]		
2			
3			
4	0.0029	0.0095	0.0194
5			
6[b]	0.0003		
7			
8[c]	<0.0001	0.0007	0.0103
9			0.0545
10			

[a] Probability values.

[b] the outlier bird # B65 was removed from the data set for this peak

[c] the outlier bird # G59 was removed from the data set for this peak

Table B.5 Summary of statistical analyses (ANOVA) for serum proteins at sampling time - day 72 – female mallards.

Peak	Effects
ID	dose
1	
2	0.0268[a]
3[b]	0.0046
4	
5	
6	
7	0.0163
8	
9	
10	

[a] Probability values.

[b] the outlier bird # Y80 was removed from the data set for this peak

A Wilcoxon test was performed on data for peak # 1.

Table B.6 Summary of statistical analyses (ANOVA) for serum proteins at sampling time - day 278 – female mallards.

Peak	Effects
ID	dose
1	
2[b]	0.0158[a]
3	
4	
5	
6	
7	
8	
9	
10	

[a] Probability values.

[b] the outlier bird # P76 was removed from the data set for this peak

Table B.7 Summary of statistical analyses (ANOVA) for serum proteins at sampling time - day 334 – male mallards.

Peak	Effects
ID	dose
1	
2	
3	0.0194[a]
4	
5	
6	
7	
8	0.041
9	
10	

[a] Probability values.

A Wilcoxon test was performed on data for peaks # 2 and # 8.

Table B.8 Summary of statistical analyses (ANOVA) for serum proteins at sampling time - day 414 – female mallards.

Peak	Effects
ID	dose
1	<0.0001[a]
2	
3	0.0106
4	
5	
6	
7	
8	
9	0.0359
10	

[a] Probability values.

A Wilcoxon test was performed on data for peak # 9.

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