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Effects of Early Life Exposure to the Organophosphate Flame Retardant, Triphenyl Phosphate on Stress and Stress Related Behaviour in Birds

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Figure 10. Mean stress response (change in in corticosterone levels) in Japanese quail chicks across treatments. No significant effects were observed across treatment groups ($p = 0.342$).

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LIST OF ABBREVIATIONS

AVT: Arginine vasopressin

BDE-99: 2,2', 4,4',5- Pentabromodiphenyl Ether

CRF: Corticotropin releasing factor

Cort: Corticosterone

DDT: Dichlorodiphenyltrichlororethane

DON: Deoxynivalenol

EDC: Endocrine Disrupting Chemicals

EE2: 17 α-ethinylestradiol

FR: Flame Retardant

GAS: General Adaptation Syndrome

HBDE: Hexabromocyclododecane

HPA Axis: Hypothalamic – Pituitary – Adrenal Axis

HPI Axis: Hypothalamic- Pituitary- Interenal Axis

OPFR: Organophosphate Flame Retardant

PCB: Polychlorinated biphenyl

TI: Tonic Immobility

TPHP: Triphenyl Phosphate

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ABSTRACT

Our environment is contaminated by hundreds of thousands of chemicals. While regulations have been established to control production and release of some of these chemicals, thousands of new chemicals are introduced to the market on an annual basis. Traditional toxicity tests have focused on outcomes such as mortality, impaired growth and reproduction. However, recent studies have been promoting behaviour as a measure of exposure to contaminants, given that it is more sensitive than traditional toxicological endpoints, can encompass multiple levels of biological organization, and is directly related to an organism's fitness. Among other effects, it alters behaviour in response to acute stressors, and helps regulate key life history decisions such as reproduction. Evidence in the literature has demonstrated that exposure to contaminants impacts the stress response and associated behaviours, however, these are few in number.

Triphenyl phosphate (TPHP) is an organophosphate flame retardant that has been classified as a priority chemical under the Government of Canada's Chemicals Management Plan. Preliminary studies have demonstrated that TPHP causes behavioural changes, physical deformities and alterations to the gene expression of the stress axis (Hypothalamus-pituitary-interrenal (HPI) axis) in zebrafish (*Danio rerio*). However, little is known about this compound's potentially toxic effects in other taxa. The objective of my thesis is to determine the effects of TPHP on stress and stress-related behaviour in Japanese Quail. Developing quail were exposed to TPHP *in ovo* and orally for the first week of life. Treatment groups consisted of safflower oil (control), predicted environmentally relevant levels of TPHP (5 ng/g) and higher levels of TPHP (50 ng/g and 100 ng/g). In addition to basic measures of mortality and deformities, individuals from all treatment groups were subjected to behavioural tests involving tonic immobility (an extreme fear response), contact with a novel object and tendency to explore a novel environment. Baseline and stress-induced corticosterone concentration were also measured. Exposure to TPHP was not associated with any dose-dependent effects on mortality endpoints (hatching success), deformities, tonic immobility endpoints (number of attempts required to initiate immobile state, average duration of immobile state), novel area exploration (time taken to enter the novel area and percentage of time spent in the familiar area), and corticosterone (baseline and stress corticosterone concentrations). However, chicks exposed to higher doses of TPHP (100 ng/g) administered

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significantly more pecks (18.0 ± 2.8) upon a novel object in comparison to the lower dose group (5 ng/g) and control (10.3 ± 0.9 and 9.5 ± 1.4 pecks respectively).

As mentioned above, while baseline and corticosterone concentrations were measured, the results were difficult to interpret due to the assay being difficult to validate, and only 44% of the birds were successfully blood sampled past the 3 minute mark. Baseline values varied between 0.42 ± 0.1 and 2.9 ± 0.95 ug/mL, while stress values varied between 2.3 ± 0.61 and 11 ± 1.9 ug/mL. The change from baseline to stress samples varied between 2.0 ± 0.4 and 7.65 ± 1.4 ug/mL. The average pre-stress corticosterone was lower than the post-stress corticosterone, however there was a positive relationship seen between sampling time and post-stress corticosterone concentrations. Lastly, no relationship between TPHP and corticosterone was observed, which should be interpreted with caution given the issues with the assay.

Overall, these data suggest that TPHP affects neophobic behaviour in Japanese Quail exposed to 100 ng/g of TPHP. This exceeds the 5 ng/g that was initially defined as an environmentally relevant dose when the study was designed. However, in light of recent evidence that TPHP is rapidly metabolized in birds, this implies our initial definition of environmental relevance may not be correct. Alterations to the stress response could prove to be extremely detrimental to any species, as it could lower the ability to cope with acute environmental changes, and evade predators. Increasing our understanding of behavioural effects of TPHP in birds will support risk assessment to effectively minimize any potential deleterious impacts on wildlife.

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RÉSUMÉ

Notre environnement est contaminé par des centaines de milliers de produits chimiques. Bien que des règlements ont été établies pour contrôler la production et la libération de certains de ces produits chimiques, des milliers de nouveaux produits chimiques sont introduits sur le marché chaque année. Les tests de toxicité traditionnels se sont concentrés sur des résultats tels que la mortalité, et le développement et la reproduction altérées. Cependant, des études récentes ont indiqué que le comportement pourrait être une bonne mesure de l'exposition aux contaminants, étant donné qu'il est plus sensible que les critères toxicologiques traditionnels, qu'il peut englober plusieurs niveaux d'organisation biologique et qu'il est capable d'évaluer l'effet sur un organisme. La réponse au stress a des liens directs avec la forme physique d'un organisme. Entre autres, elle modifie le comportement en réponse à des facteurs de stress aigus, et aide à réguler les décisions clés du cycle de vie comme la reproduction. Des preuves dans la littérature ont démontré que l'exposition aux contaminants a un impact sur la réponse au stress et les comportements associés, mais ces derniers sont peu nombreux.

Le phosphate de triphényle (TPHP) est un retardateur de flamme organophosphoré qui a été classé comme produit chimique prioritaire dans le cadre du Plan de gestion des produits chimiques du gouvernement du Canada. Des études préliminaires ont démontré que le TPHP provoque des changements de comportement, des déformations physiques et des altérations de l'expression génétique de l'axe du stress (axe Hypothalamus-pituitary-interrénale (HPI)) chez le poisson-zèbre (*Danio rerio*). Cependant, on sait peu de choses sur les effets potentiellement toxiques de ce composé dans d'autres taxons. L'objectif de ma thèse est de déterminer les effets du TPHP sur le stress et le comportement lié au stress chez la caille japonaise. Les cailles en développement ont été exposées au TPHP dans l'oeuf et oralement pendant la première semaine de leur vie. Les groupes de traitement étaient composés d'huile de carthame (témoin), de niveaux de TPHP pertinents pour l'environnement (5 ng / g) et de niveaux plus élevés de TPHP (50 ng / g et 100 ng / g). En plus des mesures de base de la mortalité et des déformations, les individus de tous les groupes de traitement ont été soumis à des tests de comportement impliquant l'immobilité tonique (une réponse de peur extrême), le contact avec un nouveau objet et la tendance à explorer un nouveau environnement. La concentration de base et la concentration de corticostérone induite par le stress ont également été mesurée. L'exposition au TPHP n'a été associée à aucun effet

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dépendant de la dose sur les critères d'effet de mortalité (succès d'éclosion), les déformations, les critères d'effet d'immobilité tonique (nombre de tentatives nécessaires pour intier l'état immobile, durée moyenne de l'état immobile), la nouvelle zone (temps nécessaire pour entrer dans la nouvelle zone et pourcentage de temps passé dans la zone familière) et la corticostérone (concentrations de base et de stress de corticostérone). Cependant, les poussins exposés à des doses plus élevées de TPHP (100 ng / g) ont significativement administré plus de coups de bec (18,0 ± 2,8) sur un nouveau objet par rapport au groupe exposé à une dose plus faible (5 ng / g) et au groupe témoin (10,3 ± 0,9 et 9,5 ± 1,4 picore, respectivement).

Comme mentionné ci-dessus, bien que les concentrations de base et de corticostérone aient été mesurées, les résultats ont été difficiles à interpréter car le test était difficile à valider, et seulement 44% des oiseaux ont été échantillonnés successivement (prélèvement sauguin) après 3 minutes. En outre, le test s'est avéré difficile à valider. Les valeurs de base variaient entre 0,42 ± 0,1 et 2,9 ± 0,95 ug/mL, tandis que les valeurs de stress variaient entre 2,3 ± 0,61 et 11 ± 1,9 ug/mL. La différence entre les échantillons de base et les échantillons de stress variait entre 2,0 ± 0,4 et 7,65 ± 1,4 ug/mL. La moyenene de corticostérone avant le stress était inférieure à la moyenne corticostérone après le stress, mais une relation positive a été observée entre le temps d'échantillonnage et les concentrations de corticostérone après le stress. Enfin, aucune relation entre le TPHP et la corticostérone n'a été observée, ce qui doit être interprétée avec prudence étant donné les problèmes posés avec le test.

Dans l'ensemble, ces données suggèrent que le TPHP affecte certains comportements liés au neophobie chez la caille japonaise exposée à 100 ng / g de TPHP. Cela dépasse les 5 ng / g qui ont été initialement définis comme une dose pertinente pour l'environnement lorsque l'étude a été conçue. Cependant, à la lumière des preuves récentes que le TPHP est rapidement métabolisé chez les oiseaux, cela implique que notre définition initiale de la pertinence environnementale peut ne pas être correcte. Les modifications apportées à la réponse au stress pourraient s'avérer extrêmement nuisibles à toute espèce, car elles pourraient diminuer leur capacité à faire face à des changements environnementaux aigus et aux prédateurs. Une meilleure compréhension des effets comportementaux du TPHP chez les oiseaux permettra de sountenir l'évaluation des risques afin de minimiser efficacement tout impact potentiellement délétère sur la faune.

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CONTRIBUTION OF AUTHORS

This thesis explore the effects of contaminants on avian behaviour. It consists of a literature review chapter and a data chapter. I did the research and writing for the literature review chapter, with editing help from my supervisor, Dr. Jessica Head.

The contents of the second chapter were a part of a team project, funded by the Canadian government's Chemical Management Plan (CMP) through Dr. Kim Fernie (Environment and Climate Change Canada). The overall goals of this project were to assess potential risks of the organophosphate flame retardant, Triphenyl Phosphate (TPHP) to birds, using Japanese quail as a model organism. The experimental design of the overall project was established by Dr. Jessica Head, Dr. Kim Fernie, and Dr. Mélanie Guigueno before I was recruited as a student. The planned research was implemented principally by me and Dr. Guigueno with support from François Ste Marie Chamberland (Honours student), Karine Khatchikian and Sabrina Evans (undergraduate students), and Diane Langan (animal facility technician). My main roles in the overall project included responsibility for egg injections (with MG and FSC) monitoring of hatching and chick care (with MG, FSC, KK, SE, DL), design of the housing corrals (with deeply appreciated input and assistance from Ian Ritchie), design and implementation of behavioural tests (with MG and FSC), incubator checks during the incubation periods (with MG and FSC), post-hatch weighing and dosing (with MG). The final 'take-down' of the experiment (euthanasia of the animals and dissections) were principally spear-headed by Dr. Guigueno, Dr. Fernie, and Glenn Barrett (ECCC technician).

In addition to our roles in the overall project, Dr. Guigueno, François Ste. Marie Chamberland and I all had responsibilities for design, implementation and reporting of specific components. I was responsible for the corticosterone and stress-related behavioural tests which formed the basis for Chapter 2 of my thesis. With mentorship from Dr. Guigueno, Dr. Head and other team members, I designed studies, gathered data and statistically analyzed corticosterone concentrations, neophobia, exploratory behaviour and tonic immobility tests. The other behavioural endpoints (aggression, dominance hierarchy) formed the basis of François Ste Marie Chamberland's Honours thesis. The results of some of my stress-related behavioural tests

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(presented in Chapter 2 of this thesis) and François Ste Marie Chamberland's aggression tests formed the basis for a paper recently accepted for publication in the Journal of Environmental Pollution, in which I share first co-authorship with Dr. Mélanie Guigueno (Hanas & Guigueno et al. 2020). My thesis chapter was used as the first draft of this paper. Dr. Guigueno incorporated Francois' behavioural data. The paper was revised and edited by Dr. Guigueno and Dr. Head prior to being submitted for publication. In addition to behaviour, other endpoints were also explored in the overall project. For example, Dr. Guigueno recently published a paper on mortality, deformities, metabolism, thyroid hormone signaling and growth (Guigueno et al. 2019). I am a co-author on this paper.

Published papers stemming from my MSc thesis work:

Hanas, A. K., Guigueno, M. F., Fernie, K. J., Letcher, R. J., Ste-Marie Chamberland, F., & Head, J. A. (2020). Assessment of the effects of early life exposure to triphenyl phosphate on fear, boldness, aggression, and activity in Japanese quail (Coturnix japonica) chicks. *Environmental Pollution*, *258*. https://doi.org/10.1016/j.envpol.2019.113695

Guigueno, M. F., Head, J. A., Letcher, R. J., Karouna-Renier, N., Peters, L., Hanas, A. M., & Fernie, K. J. (2019). Early life exposure to triphenyl phosphate: Effects on thyroid function, growth, and resting metabolic rate of Japanese quail (Coturnix japonica) chicks. *Environmental pollution*, *253*, 899-908.

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INTRODUCTION

Birds are charismatic fauna of their respective ecosystems and are readily monitored not only by researchers, but citizen scientists as well. They are a ubiquitous class of animals that occupy numerous trophic levels and habitats globally (Smits & Fernie, 2013), which enables multiple routes of exposure to environmental contaminants (ie: through the aquatic environment and bioaccumulation and biomagnification through the food chain as top predators). It is these characteristics that make them reliable sentinels of environmental change (Burger & Gochfeld, 2004).

One of the largest anthropogenic alterations our environment has experienced over the last century is the increased presence of chemicals such as pesticides, organic pollutants, metals, and emerging contaminants (e.g. flame retardants). The deleterious effects of contaminants were bought to the attention of the public in Rachel Carson's Silent Spring (1962), which sparked an environmental movement that prompted stricter government regulations on pollutants. However, despite the establishment of these laws, thousands of new chemicals are bought to the market annually. In addition to new chemicals, there is a backlog of old chemicals introduced decades ago, on which Canada's Chemicals Management Plan is tasked to perform risk assessments. Traditional toxicological endpoints included in risk assessments are median lethal concentration (LC_{50}), cancer, growth and development. However, these approach can be costly in terms of animal life and finance (Peterson et al., 2017a). There have been several studies in the literature exploring alternate methods. One of the endpoints considered is behavioural tests in the laboratory and field.

Behavioural research is considered to be less expensive and less invasive (especially when performed in the field) than traditional LD50 tests. As an assay of fitness, behaviour is essential for the survival of organisms (Peterson et al., 2017a). It has shown to be affected by contaminants, to be an indicator of multiple levels of biological organization, and can be used as an early warning tool (Hellou, 2010; Peterson et al., 2017a). This means that behavioural alterations may be observed prior to any phenotypical or genotypical alterations. A type of behaviour shown to be affected in the literature is stress- related behaviour (Love et al., 2003;

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Tartu et al., 2014). Stress related behaviour is regulated by the stress response, an integral process for an organism's survival. With the stress response, organisms are capable of making behavioural changes in reaction to acute stressors such as predation and food shortage (Cockrem, 2007).

My thesis addresses the effects of an emerging contaminant on stress and stress related behaviour in birds by developing methods to assess behavioural alterations upon exposure to contaminants. I use laboratory based behavioural studies to determine the effects of triphenyl phosphate (TPHP) on corticosterone and fear based behaviour. The thesis is composed of two chapters, as follows:

In Chapter 1, the objective of this chapter is to summarize the current knowledge related to exposure and effects of contaminants on bird behaviour, narrowing in on effects on bird stress and stress related behaviour. There are many contaminants known to be neurotoxic, thus their effect on behaviour is well known. Contaminants can alter multiple forms of behaviour such as mate selection, foraging, chick rearing and motor movements. There is also evidence that contaminant exposure impacts corticosterone levels.

In Chapter 2, I describe the results of a laboratory based experiment involving exposure of Japanese quail chicks to the organophosphate flame retardant TPHP. All three of these behavioural tests have been used previously in assessing the effects of chemical exposure on behaviour (Oliveri, Bailey, & Levin, 2015; Quinn, 2012; Swaddle et al., 2017). This study is among the first to utilize neophobia and exploratory behaviour to quantify the effects of chemical exposure in Japanese quail.

In conclusion, behaviour is rapidly becoming a widely accepted ecotoxicological endpoint. However, there is still much work to be done in order to use it to its fullest extent, for example, better understanding how heat stress and other environmental factors affect behaviour. Since behaviour is such a sensitive endpoint, it is important to understand how it is affected by conditions in our experimental designs. The objectives of my thesis were therefore to 1) develop methods to assess stress related behaviours in birds exposed to contaminants, 2) determine

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whether varying levels of TPHP affects corticosterone concentrations of Japanese quail chicks and lastly, 3) determine if TPHP affects stress related behaviour utilizing the aforementioned neophobia, novel area exploration and tonic immobility endpoints.

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CHAPTER 1: LITERATURE REVIEW

1.0 BEHAVIOURAL ECOTOXICOLOGY

Driven by a complex interaction between an organism's external environment (habitat, feeding opportunities, predation risk, etc) and internal biology (hormonal processes, neurobiology, psychophysical constraints, etc), behaviour is a powerful, highly adaptive method utilized by organisms to maximize their life fitness with the 'tools' they are given. Some behaviours are very specific, and necessary for an organism's survival (such as antipredatory, foraging, sociality and reproduction), and any alterations to them could prove to be devastating to an organism's fitness (G. R. Scott & Sloman, 2004).

Behavioural ecotoxicology is a branch of ecotoxicology that examines the effects of contaminants on an organism's behaviour. Behavioural changes can be the result of hormonal, physiological and neurological alterations driven by acute or chronic exposure to pollutants (Peakall, 1996; Walker, 2003; Zala & Penn, 2004). Taxa differ in their sensitivity to pollutants, and present different behavioural responses due to different behavioural repertoires and ecological niches. By assessing an organism's behaviour, we are not only able to determine whether or not they are at risk for increased exposure to chemicals, but also consider the whole organism response and any fitness consequences exposure could entail (Hellou, 2011). This in turn, could lead to valuable information for gauging population level effects.

Additionally, some researchers consider the examination of an organism's behaviour in response to chemical exposure, to be more sensitive than traditional toxicological testing methods (Hellou, 2011). Upon exposure to non-lethal doses of chemicals, behavioural alterations have been observed in an organism's mating behaviour, predator avoidance, activity levels and social interactions (Peterson et al., 2017). This is exemplified by lead, a persistent, heavy metal contaminant. At high concentrations, lead is fatal to a wide variety of species. However, at lower concentrations, it has been shown to alter the behaviour of an organism. For example, a study done by Gorissen, Snoeijs, Duyse & Eens (2005) found that exposure to non-lethal concentrations of lead significantly impacted the dawn singing behaviour of great tits (*Parus*

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major). Great tits with higher concentrations of lead had a smaller song repertoire in comparison to birds at the control sites. Furthermore, some chemicals do not directly cause mortality, however can cause long term detrimental population effects through modifications of behaviour. For example Scholz et al., (2000) found that the pesticide diazinon is not lethal to chinook salmon (*Oncorhynchus tshawyscha*), but it has devastating effects on salmon populatons. The juveniles do not develop proper olfactory imprinting, and thus are unable to execute 'homing techniques' as adults.

The value of behavioural ecotoxicology to the field of ecotoxicology has been well summarized in a review article by Peterson et al., (2017). As mentioned above, non-lethal doses of contaminants can cause effects that alter behaviours that are intrinsic to an organism's life history. For example, contaminants can impair an organism's reproductive behaviour. The alteration of reproductive behaviours can take many forms. For example, a study done by Weber (1993) exposed male-female pairs of fat head minnows (*Pimephales promelas*) to sublethal waterborne lead concentrations of 500 ug/l. He found that the pairs exposed to the lead treatment tended to engage in courtship, nest building and spawning behaviours significantly less frequently than the controls.

One of the chemical groups that are well known for disrupting reproductive behaviours are endocrine disrupting chemical (EDCs). As their name implies, EDCs are chemicals that interfere with the endocrine system, whether by design or inadvertent side effects (Clotfelter, Bell, & Levering, 2004). Exposure to these chemicals can affect an organism's ability to compete for mates, as exemplified in (Hoffmann & Kloas, 2012), who exposed African clawed frogs (*Xenopus laevis*) to 5 different concentrations of 17 α-ethinylestradiol (EE2), a common component of contraceptives. Male frogs exposed to EE2 experienced lower sexual arousal, evidenced by decreased production of 'advertisement calls' and increased production of rasping calls (calls that characterize a sexually unaroused state of a male). Furthermore, at increased concentrations of EE2, males began to develop higher concentrations of vitellogenin within their blood, an indication of feminization. The cumulative alterations in the male frog's behaviour led to females finding them less attractive and thus selected not to mate with them.

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Chemical exposure can also alter feeding behaviour. Organisms employ feeding behaviour that maximizes net energy gain, in order to maintain fitness (Abrams, 1991). This behavioural alteration can result in can result in altered prey preference (Weber, 1996). Weber exposed juvenile fathead minnows to lead, and found that individuals exposed to lead tended to target smaller, younger prey items in comparison to the controls.

Lastly, exposure to chemicals can cause alterations in social organization. When applicable, sociality in animals can be quite complex, and have integral fitness consequences (Scott, 1956). Interactions with conspecifics can determine an individual organism's place in the social hierarchy. Henry and Atchinson (1979, 1984) exposed blue gill sunfish (*Lepomis macrochirus*) to methyl parathion, copper, cadium and zinc. They found that heavy metal exposure tended to make fish more subordinate and hypoactive, while effects of methyl parathion exposure caused hyperactivity and increased displays of dominance (aggression).

2.0 AVIAN ECOTOXICOLOGY

Avian ecotoxicology has played an enormous role in our understanding of chemical effects on the environment. For decades, it has been largely accepted that birds have an increased sensitivity to 'shared risks' such as disease and environmental hazards (P. Rabinowitz, Scotch, & Conti, 2009). For example, from 1911 – 1985, British miners used canaries as early warning signals for carbon monoxide poisoning. The smaller stature and increased breathing rate of the birds caused them to succumb much quicker to the deleterious effects of the deadly gas, enabling miners to take action and evacuate the mine before they too, died from inhalation. Despite the canary's key role in worker safety, this area of research did not start to gain its roots until the late 1920s, when researchers started actively investigating the link between fowl and waterfowl mortality, and lead shot ingestion. The findings from these studies paved the way for lab based exposure studies on numerous other chemicals such as arsenic, strychnine and white phosphorous (Vogt, Cottam, Cahalane, & Leopold, 1939).

Upon the publication of Rachel Carson's Silent Spring (1962), the effects of contaminants on birds began to receive further public awareness. This ground-breaking book

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drew the attention of professionals and the public to the effects of the legal pesticide DDT on the reproduction of raptors and fish-eating birds. The shells of eggs laid by DDT exposed birds were thin, and extremely fragile. Consequently, the eggs would be crushed when a parent bird attempted to incubate them.

Today, we have a more holistic understanding of the role of birds as sentinels of the environment and human health. One of the characteristics that make birds good sentinels is that they occupy many different trophic levels (from primary to tertiary consumers), and therefore have differences in diet. These differences implicate birds in a wide variety of terrestrial, aquatic and marine ecosystems, and thus they are privy to different types of environmental and anthropogenic perturbations. This is especially the case for birds at higher trophic levels that have the potential to biomagnify high levels of environmental contaminants. For example, piscivorous birds (such as osprey and thick billed murre) are considered to be reliable sentinels of marine ecosystems, and are often used to monitor mercury contamination (Goodale et al., 2008). In contrast, birds whose prey is mostly composed of small mammals such as rodents (such as different species of owls and hawks), as used to monitor the presence of rodenticide in the environment. Lastly, insectivorous and omnivorous songbirds are also valuable indicators of terrestrial mercury concentration (Jackson et al., 2014).

In addition to being present in a number of different trophic levels and ecosystems, many birds species have widespread ranges. This includes sentinel species such as raptors (Espín et al., 2016), gulls (Burger & Gochfeld, 2004) and starlings (Eens et al., 2013). This allows for convenient comparison of environmental health between multiple different locations. A example of this is (Thomas et al., 2011), who examined and compared the effects of second generation anticoagulant rodenticides on great horned owls (*Bubo virginiarus*) across Canada.

Furthermore, birds make a good sentinel species simply due to the fact that they are an easy and accessible animal to monitor and sample. Many birds occur in large colonies, which offer a huge study population in one location (Smits & Fernie, 2013). Or, some solitary nesters can be coaxed to utilize a site with the use of nest boxes (Mo et al., 2018). Bird embryos can also be easily incubated within the lab, which has led to well characterized developmental models

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(Smits & Fernie, 2013). Bird embryos are an extremely useful tool in studying potential effects of a contaminant in the environment, due to certain chemicals (especially those lipophilic in nature) being transferred from the mother to the embryo. This process is called maternal transfer, and it occurs when a mother is harboring persistent chemicals within her tissues (Adkins-Regan, Ottinger, & Park, 1995). During development, the chemicals can then be in turn, transferred to the forming embryo, with the potential for important impacts on the embryo's phenotypic and/or genotypic expression (Adkins-Regan et al., 1995).

Furthermore, birds can be on the receiving end of indirect and direct behavioural effects from contaminants (Walker, 2003). Direct effects usually involve decline in populations bought on by pollutant exposure. The pollutant exposures can elicit changes in behavioural strategies utilized by birds in order to successfully obtain food, avoid predators and reproduce. Indirect effects are usually caused by the effects of pollutants on the bird's food choice (ie prey), and thus can lead to population declines in this regard.

3.0 AVIAN BEHAVIOURAL ECOTOXICOLOGY

As mentioned above, the increased sensitivity to chemicals, in addition to the wide trophic and environmental presence of birds, makes them an ideal taxon to monitor for effects elicited by contaminants. Bird behaviour in response to toxicants has been examined both within the lab and the field. While each approach has its strengths and weaknesses, combined, they give a holistic understanding of how birds are affected by chemicals. In order to effectively use birds as sentinel species, it is important to characterize sublethal effects of chemical exposure in order to prevent mortality and potential consequent ecosystem damage (Zala & Penn, 2004).

Listed below are several different chemicals that have been shown to exert adverse health effects at environmentally relevant concentrations during dosing studies. Birds have consistently been shown to have altered behaviour in response to exposure, cementing them as possible behavioural subjects to monitor chemicals in the environment.

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3.1 Mercury

Several studies have demonstrated that birds experience observable alterations in their behaviour in response to exposure to emerging and legacy chemicals. For example, the legacy chemical methylmercury is most well known for being associated with 'Minamata Disease', a neurological syndrome caused by chronic exposure to methylmercury (Takeuchi et al., 1957). First noted in Minamata, Japan in 1956, the disease was triggered by a chemical plant releasing copious amounts of methylmercury waste into surrounding waters, and thus being taken up by marine organisms such as fish and shellfish. The fish and shellfish were in turn consumed by Minamata inhabitants, thus causing the accumulation of methylmercury that would eventually cause severe neurological symptoms. Given its neurotoxic tendencies, researchers have worked over the past 5 decades to define sublethal exposure models using behaviour as an endpoint.

Arguably, one of the most important concepts within an organism's life history are trade–offs, defined as the management of risk when making day to day decisions managing foraging, predator evasion and reproduction (Komers, 1997). Cognitive effects of methylmercury exposure can impact a bird's management of trade-off decisions concerning predator evasion and foraging (Kobiela, Cristol, & Swaddle 2015) . They took a captive population of zebrafinches and exposed a subset of them to environmentally relevant concentrations of methylmercury. They placed birds in an experimental arena, and videorecorded them over three days of increasing perceived predation risk. They measured the bird's regulation of body mass, vigilance behaviour, and willingness to return to foraging after a disturbance. They found that the birds exposed to methylmercury lost significantly more mass and waited significantly longer to forage when exposed to high predation risk. The findings of this study suggest that methylmercury exposed birds may react more strongly to the effects of predation, and thereby increase their risk of starvation.

Spalding et al., (2000) examined the effects of methylmercury exposure on great egret (*Ardea alba*) fledglings. They found that egrets exposed to methylmercury had a significantly reduced appetite and weight compared to control birds. Another study done by Bouton et al (1998) also examined the effects of methylmercury on great egrets by dividing 16 birds

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randomly between a placebo, 0.5 mg dose group and 5 mg dose group. Birds began receiving methylmercury chloride in their food between 12 and 105 days old, and their activity levels, maintenance behaviour, and foraging efficiency were observed. Individuals in the 5 mg dose group exhibited severe ataxia after 12 weeks of exposure, and were thus euthanized. Birds in the 0.5 mg group did not significantly differ from the control group in the time required to capture live fish. However, birds in this group were significantly less likely to hunt fish and seek shade. Their activity levels were significantly lower as well. Similar results were found by Evers et al. (2008), who examined potential deleterious effects of mercury on the common loon (*Gavia immer*). They found that loons with higher mercury loads spent less time participating in high energy activities. Furthermore, loons with higher mercury loads were observed to spend less time sitting on their nests, leaving their eggs unincubated 10% of the time. Loons with lower mercury concentrations left their nests unincubated for 1% of the time.

Heinz (1979) examined the effects of methylmercury on mallard (*Anas platyrhynchos*) ducklings whose parents were fed the chemical in their diet. The affected ducklings were less responsive to maternal calls, and much more responsive to frightening stimuli in open field test. Eventually, this would have extremely deleterious effects on the birds' ability to thrive in the wild, thus causing population level effects.

However, it has been demonstrated that methylmercury can have population level effects in another manner, in the form of impairing important reproductive behaviours. A study by (Frederick & Jayasena, 2011) examined the effects of methylmercury on reproductive success of white ibises. They exposed white ibises (*Eudocimus albus*) to environmentally relevant concentrations of methylmercury (0.05 – 0.3 ppm) over a period of 3 years (from hatching to sexual maturity) and recorded changes to reproductive behaviour. They found dose related decreases in exhibition of key courtship behaviour in males, and found dose related increases of male – male pairing. Consequently, less eggs were produced, which is a potentially deleterious phenomenon to wild populations. Another study by Jackson et al., (2011) collected data on the reproductive success of Carolina wrens (*Thryothorus ludovicianus*) living alongside a contaminated river in Virginia, United States. When they compared the nesting success of birds living in the contaminated site to birds (of the same species) living on a reference site. They

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found that birds on the contaminated site were 3x more likely to abandon their nests than birds on the uncontaminated sites.

3.2 Lead

Lead, another legacy chemical, is well known for causing harmful effects. This was bought to attention three decades ago. Prior to this legislation that prohibited the use of lead paint in homes, children were at risk of ingesting paint chips with high concentrations of lead. Paint chips the size of a quarter contained enough lead to cause life-threatening symptoms in children (Rabinowitz, Leviton, & Bellinger, 1985).

Birds have been known to be particularly sensitive to the effects of lead. Furthermore, as a neurotoxicant, it has been known to cause severe behavioural alterations. A study by Gochfeld & Burger (1988) examined the effects of lead on common tern (*Sterna hirundo*) chicks. They found that chicks exposed to lead were significantly impaired in feeding, which was believed to be due to the lead disrupting the complex motor movements involved in fish handling. In 1995, they performed a similar study on 1 day old herring gull (*Larus argentatus*) chicks exposed to lead nitrate, and conducted behavioural tests to examine locomotion, balance, righting response (also known as tonic immobility), thermoregulation and visual cliff. They found that the lead nitrate exposed birds had lower locomotion and balance ability, in addition to having poorer thermoregulation ability. The righting response tests revealed that the treatment groups had a slower righting response, and also vocalized less than the controls.

Another example of lead's effect on avian behaviour comes from a study by Gorissen et al., (2005). They examined the differences in dawn singing behaviour between male great tits from a site situated within the area of a large pollution source (metallurgic smelter), and another site located 4 km eastwards from the pollution source. It was found that birds 4 km away from the polluted site had a significantly richer repertoire and had longer songs than birds living in the area of the smelter.

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3.3 Pesticides

It has been long understood that neurotoxic pesticides can cause behavioural alterations. The four main groups of pesticides (organophosphorus, carbamate, pyrethroid and organomercury fungicides) have all been found to be neurotoxic, in addition to the newer type of pesticides, neonicotinoids (Walker, 2003). Many pesticides are anticholinesterase compounds; inferring they are capable of inhibiting acetylcholinerase, an enzyme found in many nerve and muscle tissues, in addition to motor and sensory fibres, etc (Colovic et al., 2013). This is especially true of organophosphate pesticides (Colovic et al., 2013; Nicolaus & Lee, 1999). These pesticides play a role in impulse termination by the rapid hydrolysis of the neurotransmitter acetylcholine. Inhibition of this enzyme can lead to acetylcholine accumulation and hyperstimulation of nicotinic, muscarine receptors and disrupted neurotransmission (Colovic et al., 2013). This can result in a vast array of deleterious symptoms ranging from malaise to death.

A study by Busby, White, & Pearce (1990) determined behaviourial effects on a white throated sparrow (*Zonotricha albicollis*) population in New Brunswick, Canada after their habitat was sprayed twice with fenitrothion (trade name: Suminthion), an inexpensive organophosphate insecticide used worldwide. It was found that their mean brain cholinerase activity was reduced 42% on the first spray and 30% on the second spray. This was accompanied by a wide range of individual responses, with some succumbing to acute poisoning. Reported behavioural effects included inability to defend territories, disruption of incubation patterns and clutch desertion. Further effects were observed in the reproduction of birds in the spray area, with the affected birds having a reproductive success 25% of that of birds in the reference site.

Another study by Nicolaus and Lee (1999) examined the effects of consumed parathion (trade name: Folidol), an organophosphate insecticide, on red winged black birds (*Agelaius phoeniceus*). While it was observed that birds who consumed the contaminated prey did not exhibit any physiological maladaptive effects (ie outward illness or depressed acetylcholinerase activity), they did adjust their feeding behaviour by avoiding the prey species that was tainted

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with parathion. This feeding behaviour change persisted even the offered prey species was no longer tainted with parathion, indicating a long term effect on feeding behaviour had taken place.

Finally, Grue & Shipley (1981) dosed male European starlings (*Sturnus vulgaris*) with dicrotophos (trade names: Bidrin, Carbicron, Diapadrin, Dicron and Ektafos), a organophosphate insecticide, during the breeding season, and examined their activity in terms of four categories: "flight", "perched", "foraging" and "singing and displaying". Examination of the brain acetylcholinerase found it was reduced by 49.5% within 28 hours of dosing. They also found that dosed birds were less active than controls, as they spent significantly more time perched, in addition to spending significantly less time foraging, singing and displaying within 25 hours of exposure.

3.4 Pharmaceuticals

The effects of pharmaceuticals on bird behaviour is an area that has yet to be examined thoroughly. Thousands of pharmaceuticals are deposited into the sewage system annually, many of which are only partially metabolized, and are incompletely removed by water treatment processes (Jones, Voulvoulis, & Lester, 2005). Therefore, with the resulting effluent discharge to the surface water in addition to the application of sewage sludge to farmland, organisms can be exposed to a variety of pharmaceuticals through foraging (Bean et al., 2014). This class of emerging environmental contaminants are believed to be hazardous due to the fact that they are designed to alter physiology and behaviour at lower concentrations, with a high probability of affecting organisms due to evolutionarily similar regulatory processes across taxa (Bean et al., 2014). This has been shown by studies finding mortality, reproductive effects and changes in feeding and behaviour. Birds can be exposed to pharmaceuticals such as fluoxetine (trade name: Prozac) through consumption of invertebrates present at wastewater treatment plants. Some initial examples on effects on avian behaviour include a study by Whitlock et al., (2018), who examined the effects of environmentally relevant concentrations of fluoxetine (2.7 ug/day) on the courtship behaviour of wild caught starlings. It was found that males exposed to fluoxetine sang less, and were more aggressive to fluoxetine -treated females. This suggests that fluoxetine treated females were less attractive to males. Furthermore, fluoxetine treated females were

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initially more aggressive towards males, but became significantly less aggressive on the second day, while control females exhibited intermediate levels of aggression throughout.

3.5 Flame Retardants

Flame retardants (FRs) are chemical compounds that are applied to materials in order to reduce their flammability or combustion potential (van der Veen & de Boer, 2012). However, a large portion of flame retardants are additives which do not chemically bind to the mixtures they are added to, and thus have elevated potential to be released into the environment (van der Veen & de Boer, 2012) compared to those that do chemically bind to their mixture. They are a diverse group of chemicals that have a multitude of properties and effects on biota, one of which is behaviour (Guigueno & Fernie, 2017).

The effects of flame retardants on avian behaviour has not been particularly well examined, with the majority of studies focusing on the effects of brominated flame retardants (a group of legacy chemicals that have since been phased out in North America) on reproductive behaviour (Guigueno & Fernie, 2017). Fernie et al., (2008) exposed adult American kestrels (*Falco sparverius*) to a polybrominated diphenyl ether mixture named DE-71 for 75 days, beginning 21 days prior to breeding. The two treatment groups (environmentally relevant 0.3 ppm and the higher dose of 1.6 ppm) copulated less, spent less time in their nesting boxes and participated in fewer pair bonding behaviours. Sullivan et al., (2013) also examined the effects of the DE-71 mixture on American kestrel incubation, finding that kestrel pairs exposed to the contaminant had longer incubation periods and reduced incubation constancy.

Another study using American kestrels examined the behavioural effects of hexabromocyclododecane (HBCD) on their courtship, incubation and parental behaviours. Twenty kestrel pairs were exposed daily to 0.51 µg of HBCD (per bird) from 4 weeks prior to pairing until the hatching of their offspring. Exposed males and females emitted fewer 'chitter-calls' (vocalizations during courtship), and were less active than controls during courtship. Furthermore, nests of treatment birds was lower than that of controls, suggesting less consistent

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incubation. Lastly, treated males were less active in parental duties than controls, leaving their respective females to compensate.

Eng et al., (2012) examined the effects of 2,2', 4,4',5- Pentabromodiphenyl Ether (BDE-99) on zebra finch (*Taeniopygia guttata*) reproductive behaviour. They exposed birds by dosing them with their respective treatments from nestling to fledgling age. Male zebra finches exposed to the chemical sang less, making them less attractive to females.

4.0 STRESS RESPONSE

The stress response, or "fight or flight reaction" is an endocrine reaction necessary for the survival of an organism. Wild organisms live in an unpredictable environment, and therefore require the ability to make abrupt adjustments in response to environmental challenges. These acute adaptations are facilitated by the stress response.

The stress response is regulated by the hypothalamic, pituitary and adrenal axis (HPA). Upon the perception of stress, the hypothalamus is activated to secrete arginine vasopressin (AVT) and corticotropin releasing factor (CRF), which stimulates the pituitary to release adrenocorticotropin. This in turn, stimulates the release of glucocorticoids from the adrenal gland.

Corticosterone is a glucocorticoid that plays a key role in stress response (Meier & Ferrell, 1978). It is the primary glucocorticoid for birds, amphibians, reptiles and rodents. In the stress response, its primary function is to facilitate the release of glucose within the organism during times of low food intake and strenuous situations (ie: exposure to physiological stressors; Hull et al., 2007). In the short term, glucose release is highly beneficial, as it can be broken down by physiological processes to create more energy for the body. However, chronically elevated glucose concentrations can have deleterious effects on the body by damaging microvessels, consequently affecting organ function (Remage-Healey & Romero, 2001).

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4.1 Stress Response and Behaviour

Proper execution of the stress response is necessary for an organism's survival (Bonier, Moore, Martin, & Robertson, 2009), and corticosterone is often used as a measure of the successful initiation of the stress response (Busch & Hayward, 2009). Several studies have shown that its release can affect behaviour, especially in the case of young organisms. Young organisms that experience high corticosterone concentration due to environmental stress (Cockrem, 2007) or maternal transfer (the phenomenon in which a mother with high corticosterone can pass high corticosterone concentrations to her young; Hayward & Wingfield, 2004), can experience effects on behaviour persisting into adulthood (Rubolini et al., 2005). This can be manifested by altering foraging behaviour, altering reproductive decisions/success and exhibiting more fearful behaviour (extreme caution after a fear event such as predator exposure).

Stress induced fear behaviour can present itself in a multitude of ways. Organisms can become more cautious, by spending more time in hiding or being slower to investigate novel objects or areas (Jones, Satterlee, & Ryder, 1975; Kobiela et al., 2015). A common measure of fear behaviour is tonic immobility (TI, also known as the 'righting response'). It is defined as a last resort anti- predatory response, when fight, flight or freezing behaviour has failed to neutralize the threat (Gallup, 1977; Humphreys & Ruxton, 2018). It is an unlearned behaviour triggered by physical restraint, characterized by a lack of movement in response to a severe threat. The body of the organism will go limp in case of being apprehended by a predator (Fraisse & Cockrem, 2006). It has been bred into the genetics of two lines of Japanese quail (for 'long' and 'short' tonic immobility durations; Jones, Mills, Faure, & Williams, 1994). This behaviour has also been shown to be influenced by corticosterone concentrations.

As mentioned above, while spikes in corticosterone can have a short term benefit, long term elevation of the corticosterone levels can be extremely detrimental to an organism's survival (Rich & Romero, 2005). Chronically elevated corticosterone concentrations can have both physiological and behavioural effects by disrupting organs. One side effect of persistent corticosterone elevations is reaching the 'exhaustion' stage of the stress response (Rich, 2005). The exhaustion stage refers to the inability of an organism to mount a proper stress response,

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likely due to damage to the HPA axis, which renders an inability of the organism to increase corticosterone concentrations in response to a threat (Rich & Romero, 2005). This results in lower, circulating corticosterone concentrations, even when exposed to a threat, which does not initiate the fight or flight response (Cyr & Romero, 2007). Organisms in the exhaustion stage can also exhibit bolder behaviour (Cockrem, 2007; Rich & Romero, 2005).

4.2 Stress Response and Contaminants

Contaminants can affect circling corticosterone concentrations with acute and chronic exposure (Love et al., 2003; Rattner et al.,1984; Tartu et al., 2014). In the literature, contaminants can affect baseline and stress corticosterone both separately and simutaneously. This is especially the case if the contaminants is a known endocrine disruptor, such as polychlorinated biphenyls (PCBs; McCarty & Secord, 1999). There are multiple mechanisms thought to cause alterations in corticosterone levels, one being the high susceptibility of the adrenal gland to contaminant damage (Ribelin, 1984). This is attributed to the high lipid content of the adrenal cortex (Ribelin, 1984). Many contaminants are hydrophobic, therefore the adrenal gland is susceptible to their deposition and accumulation. Additionally, the adrenal gland has the potential to bioactive exogenous compounds that are deposited there. It has recently been established that adrenal mono-oxygenases (P450 family) not only participate in steroid metabolism, but also have the capacity to break down contaminants as well, thus producing toxic metabolites (Lorenzen et al., 1999).

4.3 Stress Response, Behaviour and Contaminants

As discussed above, chemicals can cause alterations in physiological processes within organisms, one of those being the HPA regulated corticosterone release (Herring, Ackerman, & Herzog, 2012). By modifying corticosterone release, they can consequently alter the resulting behaviour influenced by corticosterone (Astheimer et al., 1992). With natural populations being persistently exposed to environmental pollution, being unable to mount a proper stress response could compromise the organism's survival

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Relationships between contaminants, corticosterone and behaviour have been observed in a number of studiesScheiber et al. (2018) examined the effects of trace metals from a coal mine on barnacle goslings. They led a group of imprinted barnacle goslings (*Branta leucopsis;* collected as eggs from unpolluted sites and hatched) to feed daily on an abandoned coal mine site, where they were exposed to trace metals. A control group was led to feed on unpolluted grounds. Both test groups were put through well established stress tests (group isolations, on back restraint (tonic immobility) and individual isolation. Their feces were also sampled for corticosterone levels. They found that control goslings were calmer when isolated, and had shorter tonic immobility durations. They also excreted lower levels of the corticosterone metabolites.

Tartu et al. (2016) examined the effects of mercury on reproductive behaviour. Reproductive behaviour is extremely energetically costly. Individuals utilize life history strategies in order to cope with environmental stressors. Corticosterone plays a significant role in this phenomenon, by helping regulate reproductive decisions through the disruption of the hormone prolactin. Prolactin is another hormone involved in reproductive behaviour, by stimulating parental behaviour (ie incubation and brood provisioning). However, in response to increased corticosterone concentrations, prolactin concentrations will decrease. When prolactin levels are low for an extended period of time, this could trigger nest desertion. This study examined the effects of mercury on reproductive behaviour of Arctic Black Legged Kittiwakes (*Rissa tridactyla*). It was found that mercury levels were positively correlated with corticosterone levels, which in turn, decreased prolactin levels. It was found that male birds with higher corticosterone and mercury levels (and hence lower prolactin levels) were more likely to desert their eggs.

When it comes to corticosterone, contaminants and behaviour, wild bird populations are not the only groups we need to consider. Broiler chickens in the poultry industry are raised for meat production and consumed by the public at large. They are fed a diet based mostly on cereal grains, which can become contaminated with the vomitoxin deoxynivalenol (DON) when exposed to changes in environmental conditions such as temperature and humidity (Ghareeb, Awad, Sid-Ahmed, & Böhm, 2014). Given that broiler chickens are exposed to a variety of

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stressors (such as weighing, vaccinations and management procedures), it is integral that the effects of DON are characterized in these animals in order to determine any potential effects on meat quality. A study by Ghareeb, Awad, Sid-Ahmed, & Böhm (2014) examined this phenomenon by measuring corticosterone and stress behaviour of broiler chickens exposed to DON. It was found that chickens fed the contaminated diet, resulted in elevation of plasma corticosterone and longer tonic immobility durations (hence fear behaviour).

With numerous pharmaceuticals designed to alter behaviour of humans struggling with anxiety and depression, Bean et al. (2014) examined the potential effects of fluoxetine (trade name: Prozac) on wild caught European starlings. Birds were exposed to 1.5 ug of fluoxetine 5 days per week. It was found that birds exposed to the environmentally relevant concentrations of fluoxetine had significantly lower corticosterone concentrations in their feces, in addition to making significantly fewer visits to food trays in comparison to the controls between peak foraging times during sunrise and sunset, which is the optimal foraging model for building energy and fat reserves. While it is true that fluoxetine may have impacted an individual's sensitivity to environmental pressure, it is also plausible that the disruption to corticosterone altered the way individual fluoxetine- exposed starlings responded to environmental stimuli. Despite the apparent importance of the relationship between stress, behaviour and contaminants, few studies have delved into the subject. This has resulted in gaps in the research. This gap manifests itself as a lack of variety in behavioural tests and describes the effects of a small subset of chemicals on behaviour. Furthermore, there is little characterization of potential early life stage effects. This is an extremely important area, given the ability of both corticosterone and some types of contaminants to be passed from mother to offspring via maternal transfer.

With this research in mind, we were able to design a study utilizing neophobia, exploratory and tonic immobility endpoints, which are depicted not only in the following chapter, but in Hanas et al., 2020.

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CHAPTER 2: EFFECTS OF TRIPHENYL PHOSPHATE ON STRESS RELATED BEHAVIOUR IN JAPANESE QUAIL (*COTURNIX JAPONICA*)

1.0 INTRODUCTION

Flame retardants are chemical compounds that are applied to materials in order to reduce their flammability or combustion potential. Among the different categories of flame retardants, Organophosphate Flame Retardants (OPFRs) are one of the more commonly used internationally (EPA, 2005). They are considered as a suitable alternative to polybrominated flame retardants (Brandsma et al., 2015; Hoffman et al., 2017a; Sundkvist et al., 2010), a once ubiquitous group of flame retardants, some of who have been banned in numerous countries due to environmental persistence and association with adverse health effects. Consequently, OPFR market demands have increased, which in turn, inflated its global annual production from 1000 tonnes in 2001 to 680 000 tonnes in 2015 (Wang et al., 2015).

Within the past decade, OPFRs have become increasingly present in a variety of media. They have been detected within house dust (Brandsma et al., 2014; Hou., 2016), aquatic and avian biota (Giulivo et al., 2017; Greaves et al., 2016; Hou et al., 2016; Sundkvist et al., 2010), and in terrestrial and aquatic sediment (Giulivo et al., 2017; Wang et al., 2018). Spatial increases in concentrations of OPFRs in human urine, indicate that human exposure to these chemicals is also becoming progressively more widespread (Hoffman et al., 2017b). Therefore, understanding the potential effects of these chemicals has become a priority.

Triphenyl Phosphate (TPHP) is an emerging, lipophilic OPFR (log K_{ow} = 4.59; Figure 1) (OECD, 2015) that is classified as a priority chemical under the Government of Canada's Chemical Management Plan. It does not chemically bond to the mixtures it is part of, and therefore has elevated potential to be released into the environment. High volumes are manufactured annually, with 4,536 – 22,680 tonnes produced in 2015 within the United States alone (United States Environmental Protection Agency, 2015). In addition to being used as a flame retardant additive, TPHP is also used as a plasticizer (e.g. in PVC materials and beauty products), lubricant, and in transmission fluids, motor oils and hydraulic fuel (Organisation of

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Economic Co-operation and Development, 2002). It has been detected in house dust (Brandsma et al., 2014; Christia et al., 2018; Meeker & Stapleton, 2010), human breast milk (Sundkvist et al., 2010), human urine samples (Cequier et al., 2015) and tissues of fish (Brandsma et al., 2015; Sundkvist et al., 2010). Additionally, the compound has been found within the tissues of herring gulls and their eggs (Greaves & Letcher, 2014; Greaves et al., 2016), indicating it is passed from the mother via maternal transfer.

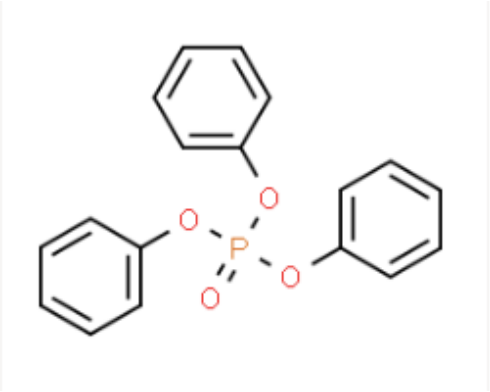

Figure 1. Structure of Triphenyl Phosphate (TPHP). Image taken from Chem Spider.

Despite the widespread occurrence of TPHP in the environment, relatively little is known about its effects on biota. Previous literature has described effects on zebrafish, in the form of larval deformities (Oliveri et al., 2015), early life stage behavioural abnormalities that persist into adulthood (Oliveri et al., 2015) and disruptions to the thyroid hormone receptor α1 (Du et al., 2016). Exposing a pregnant mouse to TPHP causes a significant increase in placental size, and alterations to the transcription of Igf1 and Irs2 genes (genes involved in insulin production) for both the mother and fetuses (Philbrook et al., 2018). Another mammalian study found that subcutaneous doses of 0.4 g/kg of TPHP caused prostration in cats (Wills et al., 1979). Endocrine disrupting effects have also been observed in humans, with testosterone levels in men being negatively correlated with higher amounts of TPHP within the dust of their respective homes (Brandsma et al., 2014; Meeker & Stapleton, 2010).

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Many contaminants are understood to influence the behaviour of organisms (Adkins-Regan et al., 2002), causing alterations in predator avoidance (Hellou, 2010), interactions with conspecifics (Hellou, 2010) and even interactions with offspring (Tartu et al., 2013). TPHP appears to be no exception. A study done by Oliveri et al. (2015) demonstrated that both developmental and acute adult exposure to TPHP resulted in hypoactivity in zebrafish. With the exception of this study, very little work has been done to further examine the link between TPHP and behaviour. This is an important data gap because contaminant-induced alterations in behaviour can have a direct impact on ecologically relevant outcomes such as growth, reproduction and survival.

The adrenal gland within the hypothalamus – pituitary – adrenal (HPA) axis is susceptible to deleterious effects resulting from exposure to lipophilic chemicals (Ribelin, 1984;Rosol et al., 2001). The adrenal gland is responsible for secreting corticosterone, the primary glucocorticoid found in birds. Corticosterone facilitates General Adaptation Syndrome (GAS) within organisms, which aids behavioural adaptation in response to a stressor (Cockrem, 2007). An organism's corticosterone levels influence and interact with a number of physiological processes that can result in a behaviour that is more adaptive to coping with the stressor. Depending on the stressor, individuals with high corticosterone levels can exhibit lower parental care (Angelier et al., 2009), increased anxiety (Shepard et al., 2000), or, in some species, increased spatial memory (Pravosudov, 2003).

Chronic exposure to a chemical can alter baseline and stress corticosterone concentrations (Rich & Romero, 2005). For example, a study by Tartu et al., (2014) examined the relationship between corticosterone and PCBs in black legged kittiwakes. It was found that birds with a higher load of PCBs tended to have higher baseline corticosterone levels, and were consequently were more likely to exhibit survival behaviours to delay breeding, or irregular incubation. This resulted in a delayed hatching date for eggs. This study represents an example of corticosterone, contaminants and behaviour interacting, resulting in an alteration of life history behaviour.

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Current research on the effects of TPHP in birds is limited to a study by Su et al. (2015), which examined its cytotoxicity and mRNA expression in chicken hepatocytes. The study found cytotoxicity occurred at exposures >10 µM of TPHP. To the best of our knowledge, there has been no research conducted on early life stage effects within a whole organism avian model exposed to TPHP.

The objectives of this study were to: 1) develop methods to assess stress related behaviours in birds exposed to contaminants, 2) determine whether exposure to varying levels of TPHP can affect corticosterone concentrations of Japanese quail chicks and lastly, 3) determine if TPHP can affect stress related behaviour utilizing classic neophobia, novel area exploration and tonic immobility tests. All three of these behavioural tests have been used previously in assessing the effects of chemical exposure on behaviour (Oliveri et al., 2015; Quinn, 2012; Swaddle et al., 2017).

2.0 METHODS

2.1 Chemicals

A standard solution of TPHP (CAS # 115-86-6) was purchased from Sigma-Aldrich (Oakville, ON, Canada) at a purity greater than 99%. Two sets of dosing solutions were prepared in organic safflower oil with different concentrations of TPHP; one set being for egg injections and the other for daily oral dosing of the chicks after they hatched. The nominal and actual concentrations of TPHP stock solutions can be found in Table 1. Actual concentrations of TPHP were analytically determined using an ultra high performance liquid chromatograph–mass spectrometer system in the laboratory of Dr. Robert Letcher (Environment and Climate Change Canada), as described in (Guigueno et al., 2019).

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Treatment	Exposure Route	Nominal Concentration (ug/mL)	Measured Concentration (ug/mL)
Vehicle (Safflower Oil)	Egg Injection, Oral	0	0.01
Low TPHP	Egg Injection	8	12.5
Mid TPHP	Egg Injection	75	68.95
High TPHP	Egg Injection	150	145.86
Low TPHP	Oral	21	19.42
Mid TPHP	Oral	216	155.89
High TPHP	Oral	429	345.52

Table 1. Nominal and actual concentrations of TPHP in stock solutions used for egg injection and oral dosing.

2.2 Study Design

Protocols and procedures regarding the handling of the chicks were approved by McGill University's Animal Care and Use Committee (under the guidelines of the Canadian Council on Animal Care) prior to the commencement of the study.

2.2.1 Eggs

Fertilized, unincubated Japanese quail eggs were purchased from Ferme Patrick Brodeur (Saint- François – du – Lac, Québec, Canada). Eggs were stored in a cool (15° - 18°C) room for 1-4 days after being received, in order to prevent development beyond the blastoderm stage (the stage the embryo is at when the egg is laid).

2.2.2 Treatments & Exposure

Eggs were randomly assigned to one of the following four treatment groups: vehicle control (organic safflower oil), Low TPHP (nominal 5ng/g), mid TPHP (nominal 50 ng/g), and high TPHP (nominal 100 ng/g). The low TPHP group reflects concentrations in wild bird eggs and tissues reported by Greaves and Letcher (2014), Greaves et al., (2016) and Lu et al., (2017), however, our more recent results suggest that this may be an underestimate (Marteinson & Guigueno et al. 2019) due to rapid metabolism of TPHP by the embryo. The medium and high dose concentrations represent 10x and 20x the low dose respectively.

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Nominal (ng/g egg or chick)	Measured (ng/g)*
Low TPHP (5 ng/g)	8.4 (eggs), 4.5 (chicks)
Mid TPHP (50 ng/g)	46 (egg), 36.4 (chicks)
High TPHP (100 ng/g)	97.2 (eggs), 80 (chicks)

Table 2. Nominal and measured concentrations in eggs and chicks
*These concentrations were measured in the day 6 chick carcass by ultra high performance liquid chromatograph–mass spectrometry, in the laboratory of Dr. Robert Letcher (ECCC).

Each quail was exposed to TPHP through two methods aimed at mimicking an exposure that would occur naturally in the wild. The first exposure took place *in ovo*, via egg injection (as previously described in Franci et al., 2018). In brief, this was performed by locating and marking the air cell of the quail egg before drilling a hole through the shell, into the air cell. Then, 10 ul of the requisite stock solution was dispensed into the egg using a repeater pipette, for final nominal concentrations of 0 ng/g (vehicle), 5 ng/g (low), 50 ng/g (mid) and 100 ng/g (high). Each egg received the same volume, regardless of mass (mean egg mass ± SE; 13.32 ± 0.07 g). The hole was subsequently covered with breathable tape (Air Pore Tape Sheet, Qiagen N.V; Venlo, Netherlands), and the egg was left upright for 45 minutes to ensure complete absorption of the dosing solution. The second set of exposures took place *in vivo*, 24 hours after hatching. *In vivo* dosing took place at the same time daily via oral route, using a pipette (see section 2.2.4 for additional details).

2.2.3 Chick Incubation and Care

Quail eggs were incubated on their side, in an Ova-Easy Advanced Series II Cabinet Incubator, type 190 (Brinsea). For the first 15 days of incubation, quail eggs were kept at 37.5°C, 55% humidity, and rotated on an hourly basis. To prevent a large number of chicks from hatching simultaneously, eggs were injected and incubated in 4 different batches. Each batch included all treatment groups, and was staggered so that incubation for batches 2, 3 and 4 began 4, 14 and 18 days after batch 1 respectively. Batches 1 and 2 included 9 eggs per treatment, and batches 3 and 4 included 12 eggs per treatment. In total, 42 eggs were injected for each treatment group in the study.

During the final 3 days of incubation, the temperature was decreased to 37.2°C, humidity was increased to 70%, and rotation of the eggs was halted. During this time, eggs were also

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checked daily for pipping. If evidence of pipping was observed, eggs were transferred into individual nesting cells for hatching.

During hatching for batches 1 & 2, we noticed that chicks were hatching approximately 24 hours earlier than anticipated, based on literature and a previous pilot study in our lab. After verifying the interior temperature of the incubator with a second thermometer, it was realized that there had been a malfunction of the incubator, and the temperature within was 1-2°C higher than stated by the digital display of the incubator. Therefore, batches 3 & 4 were switched to a different incubator. Given that batches 1 & 2 had an additional, uncontrolled 'heat stress' factor, this had bearing on how we analyzed the results (see results section for further details).

After hatching, chicks were assessed for deformities before being weighed and assigned a plastic leg band (2.8 – 3.0 mm; Red Bird Products Inc., Mount Aukum, CA, USA) with a unique number for identification. Once their down was dry, they were transferred to a 5-tier brooder purchased from (GQF Manufacturing Co. Stacked Brooder, Model #0540, Savannah, GA, USA). Each tier measured 81.3 x 96.5 x 30.5 cm and housed individuals from a single treatment group. The tier was further divided into subgroups by adding two plastic bins within the enclosure. This functioned to prevented individuals from overlapping batches from interacting.

In the brooder, chicks were kept at 34°C and a 12 hr light/12 hr dark cycle. They were given a nutritionally balanced food mixture (produced by Ferme Patrick Brodeur) and water *ad libitum*, which was refreshed twice daily when their plastic bin enclosures were cleaned.

2.2.4 Post Hatch Dosing

After hatching, chicks were allowed time to dry prior to initial measurements. Physiological measurements included tarsus length (the bone located between the tibiotarsus and phalanges of the bird), headbill (the vertical combined length of the skull and bill) and weight. Starting 24 hours after hatching, chicks were weighed daily, and had measurements taken daily. These data are reported elsewhere (Guigueno et al., in press). After their measurements were completed, chicks were given an oral dose of their designated treatment. The amount of dosing solution they received depended upon their weight that day. To administer the treatment, the

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chick's mouth was carefully opened, and the pipette was inserted in the mouth just past the glottis. Then, the treatment was dispensed orally. This took place daily, due to evidence that TPHP is rapidly metabolized (Marteinson et al., 2019).

2.3 Behavioural Tests

Behavioural tests were conducted on post hatch days 2 (tonic immobility) and 5 (neophobia and novel area exploration). Timing of the tests, lighting, set up and number of people in the room were kept consistent across batches. An additional level of the brooder was purchased to use as the testing arena, in an effort to keep the chick's surroundings as consistent as possible. A camcorder was set up to record a bird's eye view of the testing arena. For uniformity in recording, care was taken not to move the camera's position/ orientation for the duration of the study.

2.3.1 Tonic Immobility (Post Hatch Day 2)

The tonic immobility test examines the propensity of an animal to "freeze" (or "play dead"), often in response to restraint. To perform this test, a section of cardboard was folded into an M shape, with the centre angle being approximately 100 degrees (Figure 1). Once secured, a sheet of lab paper was placed over the cardboard structure to prevent contaminations between treatment groups.

Test subject selection was done by cycling through the treatment groups and randomly selecting a chick from each group until all chicks were tested (eg first chick from treatment 1, second chick from treatment 2, etc). Each individual chick was tested only once, unless extenuating circumstances (such as loud noises outside, or someone entered the room unexpectedly during the test) required the test to be repeated.

The chick was placed on its back within the main angle of the cardboard M, and two fingers were gently held against the crop for 5 seconds. After three seconds, the pressure against the bird's crop was lifted, and the bird's behaviour was observed. If the chick displayed 'freeze behaviour' (ie remained in an immobile state on its back, despite the removal of pressure on its

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crop, a timer was started in order to measure the duration of the freeze response. The frozen state was considered to have ended when the chick rolled into a standing position. If the chick did not enter an immobile state after the removal of pressure from its crop, the test was repeated up to four additional times.

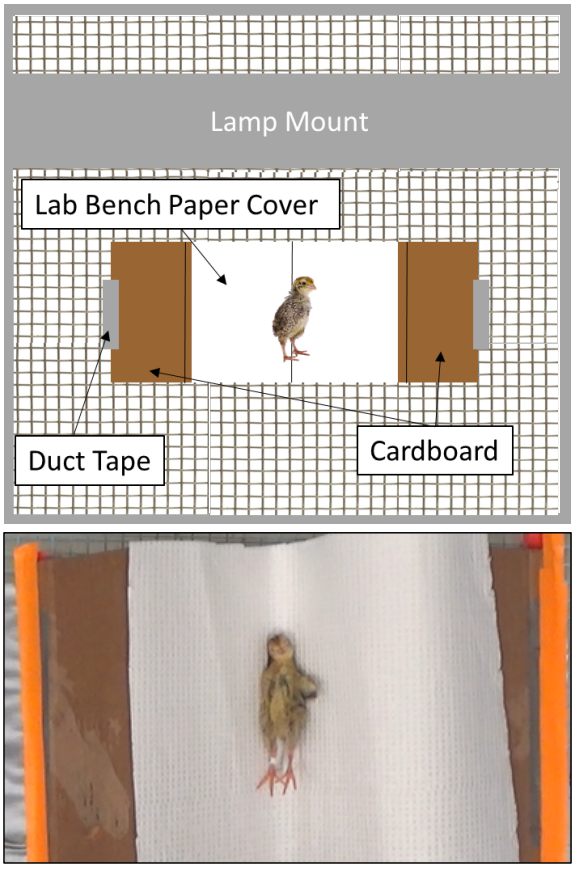

Figure 2. Arena set up for tonic immobility test. Dimensions of the arena were 81.3 x 96.5 x 30.5 cm.

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2.3.2 Neophobia and Novel Area Exploration (Post Hatch Day 5)

The goal of the neophobia and novel area exploration tests were to assess the boldness of chicks within a group of their conspecifics. The neophobia test evaluates reaction to a completely novel object, and the novel area exploration examines propensity to explore a novel area within the brooder.

As mentioned previously, extra care was taken to purchase a brooder level identical to the one that the chicks had been raised in since hatching, to minimize confounding variables. A plastic bin identical to the one that the chicks were housed in within the brooder (henceforth referred to as testing bin) was placed in the testing arena to accommodate the chicks during the trial. However, unlike their regular housing, a portion of the side of the testing bin was removed and then re-secured, to act as a door for the novel exploration trial. The wire floor of the testing arena was covered with a thin sheet of cardboard for stability. Then, white lab bench paper was taped onto the cardboard to ensure maximal colour contrast between the chicks and the floor for video recording.

Chicks were randomly sorted into testing groups comprised of one chick per treatment, and each chick was subsequently marked on the head with a different colour tape for identification and tracking purposes. Each group was given 5 minutes to adjust to the unfamiliar individuals, before a disco ball ornament (5 cm diameter) was placed and securely taped in the centre of the testing bin. After the placement of the novel object, chicks were given 7 minutes to interact with it. The endpoints examined were 1) time taken to first approach novel object, 2) the number of pecks to the novel object and 3) the amount of time spent in close proximity to the novel object.

After completion of the neophobia test, chicks were given a 5 minute reprieve with fresh food and water. The novel area exploration test was then initiated. Chicks were removed from the testing bin as a group, and held for three seconds before releasing them back into the bin, acting as a 'reset' for perception of their surroundings. Simultaneously during the reset, the door of the testing corral was removed, enabling the chicks to venture into the entire testing arena.

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They were given 5 minutes to explore the new area. Endpoints examined were 1) Time taken to first enter novel area, and 2) Percentage of time spent in novel area.

Figure 3. Arena set up for neophobia and novel area exploration tests.

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2.4 Blood Sampling (Post Hatch Day 6)

Blood sampling took place in the morning of post hatch day 6. Chicks were given extra food the previous night, to minimize any effects that may stem from a later feeding time the following morning due to the sampling taking place.

Baseline blood samples were collected from the brachial vein with a 27 gauge needle. A large team of volunteers was used to simultaneously sample blood from multiple individuals with the goal of obtaining one full micro-hematocrit capillary tube (heparinized, from Fisher Scientific) within 3 minutes of entering the room. After the blood samples were successfully collected, the chick was exposed to a stressor by putting it into a cloth bag (supplied by the McGill Bird Observatory). The bag (with the chick inside it) was hung on a hook for 30 minutes, after which the bird was removed from the bag and a second 'response' blood sample was collected from the brachial vein with a micro-hematocrit capillary tube. After the blood sample was collected, the birds were given water and returned to their respective brooder bins.

2.5 Euthanasia

On post hatch day 6, chicks were euthanized via cervical dislocation. Brain and liver tissue were sampled for molecular analyses (Guigueno et al., 2019). The remaining chick carcass was then preserved within tin foil for the analytical determination of TPHP concentrations (Marteinson et al., 2019).

2.6 Corticosterone Analysis

Whole blood was spun with a hemacentrifuge for 7 minutes. The hematocrit of each sample was read, and the plasma was extracted and stored in a -80°C freezer. A corticosterone competitive ELISA kit (produced by Enzo Life Sciences Inc, ADI-900-097, Farmingdale, NY, USA) was utilized to determine the concentration of corticosterone in the plasma.

We attempted to validate the assay for Japanese quail chicks by performing a serial dilution of a pool of the quail chick plasma and comparing this to a standard curve. Serial dilutions showed parallelism to the standard curve, however this took multiple attempts to

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obtain, as the serial dilution curve lacked linearity (see Discussion section for further comment). The successful validation provided a linearity range of 19 pg/mL to 1047.55 pg/mL, and the standard curve and serial dilution curves were not significantly different from one another ($p = 0.51$). When performing the experimental assays, the lowest value detected by the kit was 21.49 pg/mL. Samples and standard curves were assessed according to manufacturer instruction, and analyzed in duplicates.

A pooled sample was used for inter-plate comparisons. The pool was a combination of plasma from a number of different baseline and stress samples from randomly selected quail. This pool sample was used on each plate to measure the difference between plate readings. The concentration of the pools on each plate ranged from 2.65 ug/mL to 3.0 ug/mL and variability (%CV) ranged from 8-11% between plates.

2.7 Data Analysis

Means and standard error were calculated for all endpoints. Results were considered statistically significant at a p-value of < 0.05.

2.7.1 Hatching Success

Hatching success was calculated as the percentage of fertilized eggs that hatched within 24 hours of pipping. Hatching success and growth endpoints were analyzed by with SAS version 9.3 (SAS Institute Inc, Cary, NC).For hatching success, we ran a logistic regression (PROC LOGISTIC), using treatment and batch as factors.

2.7.2 Behavioural Endpoints

Data for neophobia and novel area exploration endpoints was collected using Ethovision® XT 12 Tracking Software (Noldus Information Technology INc, Leesburg Virginia). Tracking was done from a video source, and used colour marker tracking to monitor the individual movements of each quail. Statistical analysis for all behavioural endpoints was performed using R Studio Version 3.4.3 (R Foundation for Statistical Computing, Vienna, Austria).

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Tonic Immobility: Number of Attempts to Initiate Immobile State)

Data were first tested for violation of normality and equal variance. The null hypothesis for normality was rejected ($p < 0.01$), therefore the data were square root transformed. This allowed the null hypothesis for normality to be accepted ($p = 0.4593$). The transformed data was subsequently analyzed with the use of a one way ANOVA.

Tonic Immobility: Duration of Immobile State

Data were first tested for violation of normality and equal variance. The null hypothesis for normality was rejected ($p = 0.019$), even after applying square root ($p = 0.028$) and logarithmic ($p= 0.005$) transformations. Therefore, a Kruskall- Wallis Rank Sum Test was utilized to analyze the data.

Neophobia: Time to Approach Novel Object

Time to approach novel object was defined as the time from the start of the trial until the chick's head (ie colour marker) entered the novel object zone. The novel object zone was a defined within Ethovision® as within 5 cm from the novel object. Data were first tested for violation of normality and equal variance The null hypothesis for normality was rejected ($p < 0.001$), therefore the data were logarithmically transformed. This allowed the null hypothesis for normality to be accepted ($p = 0.308$). The transformed data was subsequently analyzed with a one way ANOVA.

Neophobia: Time Spent Near Novel Object

Time spent near the novel object was defined in Ethovision® as the amount of time the chick's head spent within the novel object zone. Distribution of data was noted to be linear. Consequently, a general linear model was run, with treatment as a factor. Variances between treatment groups were compared to the F distribution using the F test.

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Neophobia: Number of Pecks

A peck was defined as the chick's bill making contact with the novel object. Distribution of the data was noted to be poisson, therefore a Poisson regression model was used to analyze the number of pecks to the novel object (with treatment as a factor). Bonferroni post hoc test was used for comparisons. Variances between treatment groups were compared to the F distribution using the F test.

Novel Area Exploration: Time to Enter the Novel Area

The latency to enter the novel area was defined as the time between the start of the test, and when the bird's head first crossed from the familiar area into the novel area. Data were first tested for violation of normality and equal variance. The null hypothesis for normality was rejected ($p < 0.0001$), even after applying square root ($p < 0.001$) and logarithmic ($p = 0.0017$) transformations. Therefore, a Kruskall Wallis Rank Sum test was utilized to analyze the data.

Novel Area Exploration: Percentage of Time Spent in Familiar Area

The percentage of time spent in the familiar zone was defined as the proportion of time during the 5 minute testing period that the individual spent in the familiar area. Data were first tested for violation of normality and equal variance. The null hypothesis for normality was rejected ($p < 0.001$), even after applying square root ($p = 0.003$) and logarithmic ($p = 0.0014$) transformations. Therefore, a Kruskall Wallis Rank Sum test was utilized to analyze the data.

Corticosterone

Means and standard deviations were calculated for each treatment group. Time taken to obtain baseline blood sample versus baseline corticosterone concentration was plotted, and the regression was assessed. Response corticosterone values were calculated as baseline corticosterone concentrations subtracted from stress corticosterone concentrations.

As mentioned above, to analyze corticosterone concentrations for the baseline and stress samples for each chick, we validated and utilized an Enzyme Immunoassay kit for corticosterone. Corticosterone concentration values were obtained utilizing R studio to create a

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4- parameter logistic curve with the values of the standards and using this to interpolate values along the standard curve (method recommended by manufacturer).

3.0 RESULTS

3.1 Hatching

Hatching success was assessed as the percentage of fertilized eggs that successfully hatched within 24 hours of pipping across all 4 batches. Batches 1 and 2 hatched after 17-18 days of incubation, approximately 24 hours earlier than expected. Batches 3 and 4 hatched at the expected time of 18-19 days. Given that higher incubation temperatures have previously been shown to cause early hatching (Hepp et al., 2011), we concluded that this effect was associated with the elevated incubation temperature experienced by Batches 1 & 2 (see methods). Despite this, hatching success amongst batches was not significantly different ($p = 0.30$; see Guigueno et al., 2019 for further information).

Overall, hatching success for chicks was 43%. Hatching success was highest (53%) for the vehicle treated embryos, but this value was not significantly different from the values for the low (34%), mid (47%), and high (40%) treatment groups (Table 1).

Treatment	**Injected**	**Infertile Eggs**	**Hatched**	**Hatching Success (%)**
Vehicle	42	6	19	53
Low	42	7	12	34
Mid	42	10	15	47
High	42	7	14	40
Total	**168**	**30**	**60**	**43**

Table 3. Hatching success across treatment groups (includes all batches). No significant differences were observed across treatment groups ($p = 0.44$).

3.2. Behavioural Tests

Starting at 2 days post hatch, chicks were put through three different behavioural tests: Tonic immobility (post hatch day 2), neophobia (post hatch day 5) and novel area exploration (post hatch day 5).

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As mentioned above, chicks from Batches 1 & 2 hatched nearly 24 hours earlier than expected, and we suspect that this was due to an incubator malfunction which set the temperature higher than intended. In addition to early hatching (Hepp et al., 2011), elevated heat during incubation has previously been shown to result in lower baseline corticosterone levels in hatched chicks (DuRant et al., 2010). To test for potential impacts of the accidental heat stress on behaviour, we compared corticosterone levels and behavioural responses in vehicle-treated individuals from Batches 1 and 2 to individuals in Batches 3 and 4. Chicks from Batches 1 and 2 had significantly lower levels of baseline corticosterone than chicks from Batches 3 and 4 (Figure 1A). While we did not see any differences for tonic immobility and novel area exploration (data not shown), we did consistently observe a trend of bolder behaviour from chicks in Batches 1 and 2 when examining the neophobia endpoints. Specifically, we observed significant differences in the amount of time spent near the novel object ($p = 0.016$; Figure 1B), and the number of pecks distributed on the novel object ($p = 0.013$; Figure 1C).

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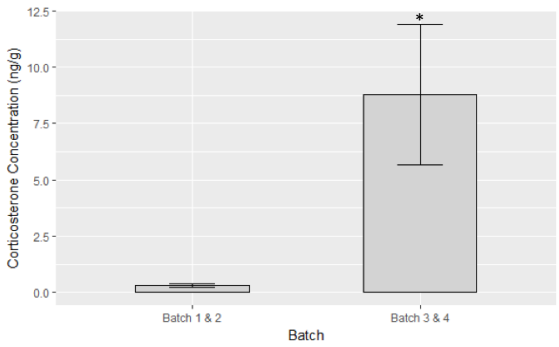

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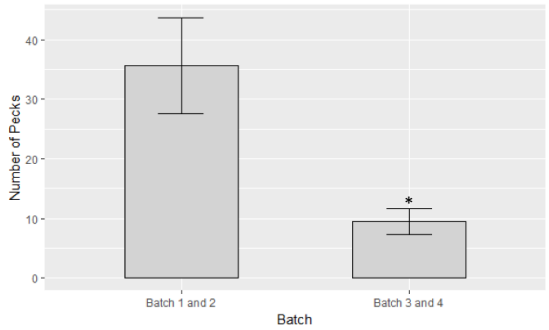

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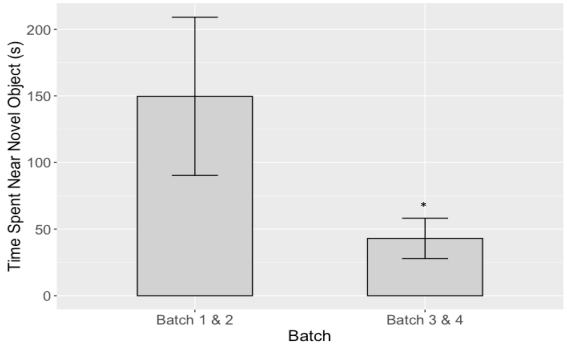

Figure 4. Comparison of vehicle-treated individuals from Batches 1, 2 (n = 2) and Batches 3,4 (n = 8) for endpoints A) 'Baseline Corticosterone' ($p = 0.04$), B) 'Number of Pecks' and C) 'Time Spent Near Novel Object' ($p = 0.016$) (Error bars represent standard error). Asterisk indicates the existence of significant differences of <0.05.

Given these results, we assessed TPHP related behavioural effects on chicks from Batches 1&2 and 3&4 separately. There were no significant effects of TPHP on behavioural endpoints in chicks from Batches 1&2 .Thus, all behavioural and corticosterone data below relate to Batches 3& 4 only.

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3.2.1 Tonic Immobility

On post hatch day 2, we tested the fear response of Japanese quail chicks by examining their propensity to freeze in response to a stressor. Thirty-two out of 34 chicks (94%) displayed freeze behaviour within 5 attempts after being restrained on their backs through gentle pressure to the crop. The two chicks that didn't freeze within 5 attempts were excluded from further analysis. There was no significant difference observed in the number of attempts to initiate freeze behaviour across treatment groups ($p = 0.3$). Chicks from the vehicle, low, mid and high groups required a mean (± standard error) of 2.5 ± 0.2, 3.8 ± 0.3, 3.7 ± 0.4 and 2.6 ± 0.2 seconds respectively (Figure 2B).

The average duration of the immobile state (± standard error) was 25.7 ± 4.2 seconds across all treatment groups. No significant differences existed between groups ($p = 0.1$). Chicks from the vehicle group had a mean immobile state duration of 21.8 ± 2.4 seconds, while chicks from the low, mid and high group display mean immobile state durations of 29.9 ± 6.58 seconds, 22.0 ± 3.3, 30.0 ± 6.7 respectively (Figure 2A).

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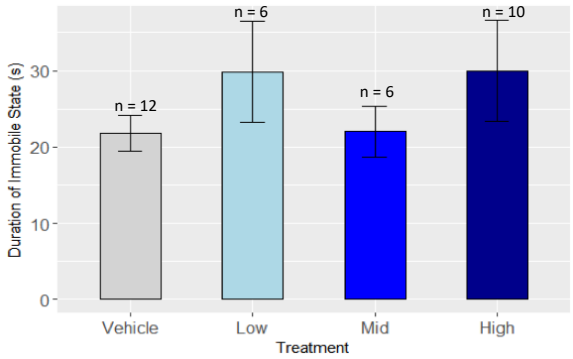

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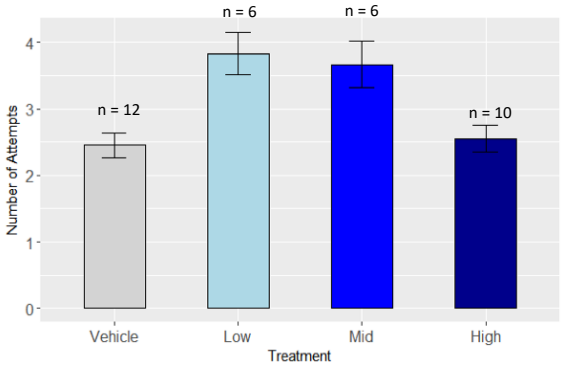

Figure 5. Effects of TPHP on tonic immobility. A) Mean duration of immobile state in Japanese quail chicks (n = 34; $p = 0.96$) exposed to varying levels of TPHP or a vehicle control, and **B)** Mean number of attempts to initiate freeze response (n = 34; $p = 0.255$) No significant differences were observed across treatment groups. Error bars represent standard error.

3.2.2 Novel Area Exploration

On post hatch day 5, we tested the propensity of Japanese quail to explore novel environments. In total, 12 out of the 24 chicks (50%) did not enter the novel area. For the individual chicks that entered the novel zone, the time taken to enter the novel object zone varied between 109.5 ± 42.7 and 155.6 ± 35.4 across all treatments. There was no significant difference observed between treatment groups (Figure 6; $p = 0.96$). Furthermore, chicks from different treatment groups did not have an increased propensity to enter the novel object zone ($p = 0.8$) or be the first individual to enter novel object zone ($p = 0.9$).

The percentage of time spent in the familiar area varied between 66.9 ± 8.3 and 52.0 ± 10.8 % across all treatments. There was no significant difference observed between treatment groups (Figure 6b; $p = 0.98$).

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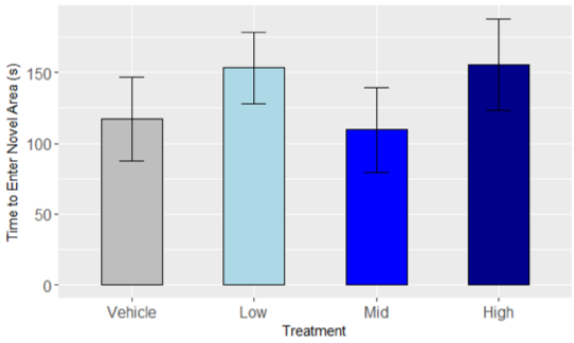

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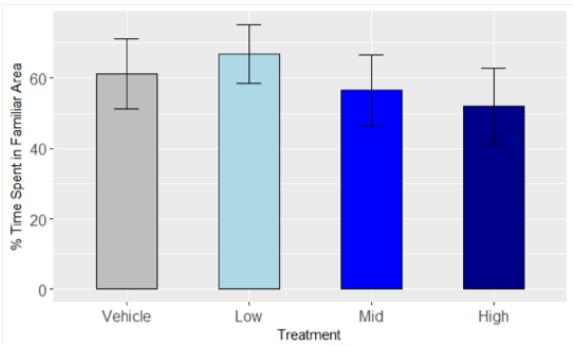

Figure 6. Effects of TPHP on novel area exploration. A) Latency to enter the novel area (n = 8 for each treatment; p = 0.96) and **B)** Total percentage of time spent in the familiar area in Japanese quail chicks exposed to varying levels of TPHP and a vehicle control. There were no significant differences observed across treatment groups (n = 8 for each treatment ; p = 0.98). Error bars represent standard error.

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3.2.3 Neophobia

On post hatch day 5 we tested the reaction of Japanese quail chicks upon introduction to a completely novel object. Mean approach time varied between 55.7 ± 11.4 and 114.76 ± 30.1 seconds across all treatment groups. No significant differences were observed across treatment groups (Figure 4A; $p = 0.94$).

The duration of time spent in close proximity to the novel object was also examined. Mean duration of time spent near the novel object varied between 43.0 ± 7.6 and 89.7 ± 12.2 seconds across all treatment groups.

The number of pecks received by the novel object was assessed. Each chick pecked the object, and a significant dose-dependent increase in pecks was observed (Figure 6C; $p < 0.001$). In order of increasing mean pecks, the vehicle group administered the least number of pecks (9.5 ± 1.4), followed by then the low treatment group (10.3 ± 0.9), mid treatment group (14.7 ± 1.5) and high treatment group (18.0 ± 2.8).

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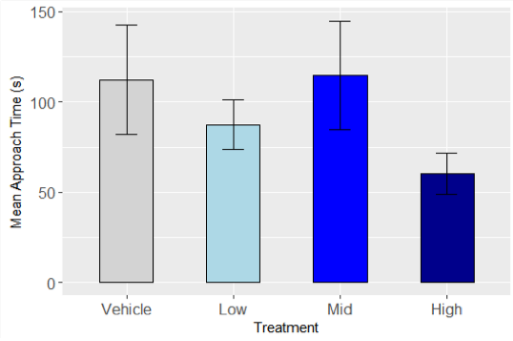

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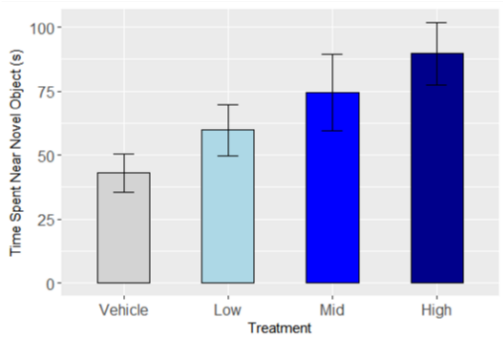

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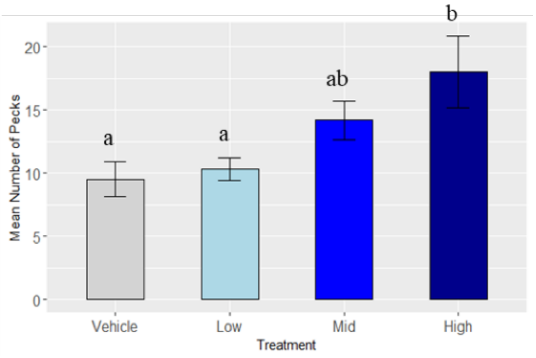

Figure 6. Effects of TPHP on neophobia. A) Mean time to first approach the novel object ($p = 0.942$) **B)** Mean amount of time spent near the novel object ($p = 0.54$), and **C)** Number of pecks upon a novel object in Japanese quail (chicks exposed to varying levels of TPHP or a vehicle control ($p < 0.01$). Significant differences were observed between the vehicle and high treatment ($p < 0.01$), and the vehicle and low treatment ($p < 0.01$). n = 8 for each treatment. Error bars represent standard error.

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3.3 Corticosterone

On 6 days post hatch, levels of corticosterone were measured in Japanese quail chicks before and after exposure to a stressor. Although we aimed to obtain the baseline blood sample for individual chicks within 3 minutes of entering the room, 44% of the 45 individuals we were able to draw blood from were sampled past the 3 minute mark (n= 14 for vehicle, n = 7 for low, n = 14 for mid and n = 10 for high). Individuals sampled past the 3 minute mark had a mean baseline corticosterone level of 9.4 ± 1.7 ug/mL. This was 5.5x higher than the mean baseline corticosterone concentration of those sampled within the 3 minute mark (1.7 ± 0.49). Furthermore, a significant association between corticosterone levels and sampling time was observed even for those individuals sampled within three minutes (correlation coefficient of 0.27). Those sampled past the 3 minutes however, had a correlation coefficient of 0.48 when compared with the sampling time (Figure 8).

In individuals that were initially sampled within 3 minutes, the stress corticosterone concentrations were higher than the baseline corticosterone concentration 88% of the time. There were no significant differences in corticosterone concentrations amongst treatment groups for the baseline ($p = 0.628$), stress ($p = 0.131$) samples, nor the stress response (stress concentration – baseline concentration; $p = 0.34$; Figure 9). Baseline values varied between 0.42 ± 0.1 and 2.9 ± 0.95 ug/mL, while stress samples encompassed values between 2.3 ± 0.61 and 11 ± 1.9 ug/mL. The difference between baseline and the stress samples varied between 2.0 ± 0.4 and 7.65 ± 1.04 ug/mL (Figure 10).

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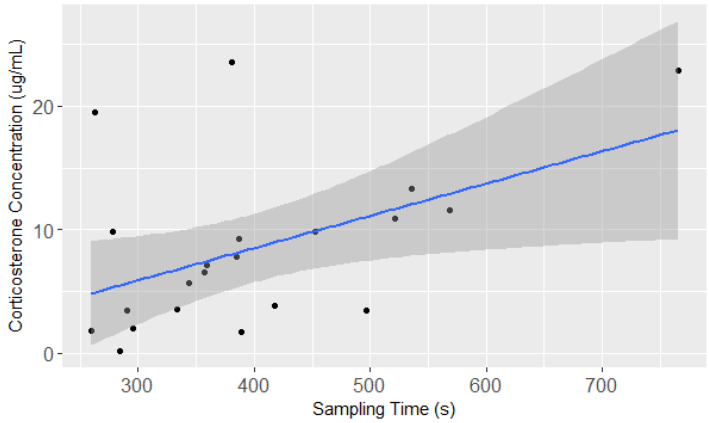

Figure 8. Corticosterone samples taken past 3 minutes (y = 0.037x – 2.86).

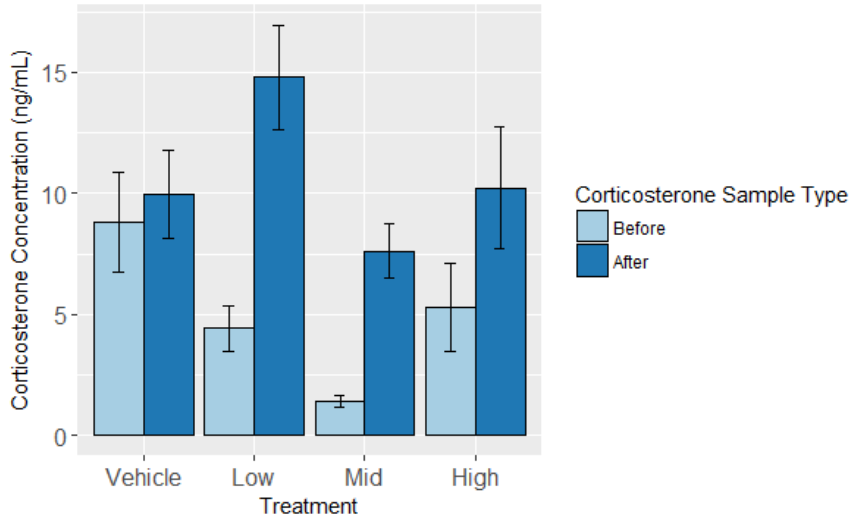

Figure 9. Effects of TPHP on corticosterone concentrations. Mean baseline and stress corticosterone concentrations (ug/mL) across in quail chicks exposed to varying levels of TPHP or a vehicle control. No significant differences were found in baseline values ($p = 0.63$) or stress values ($p = 0.13$) across treatment groups. Error bars represent standard error.

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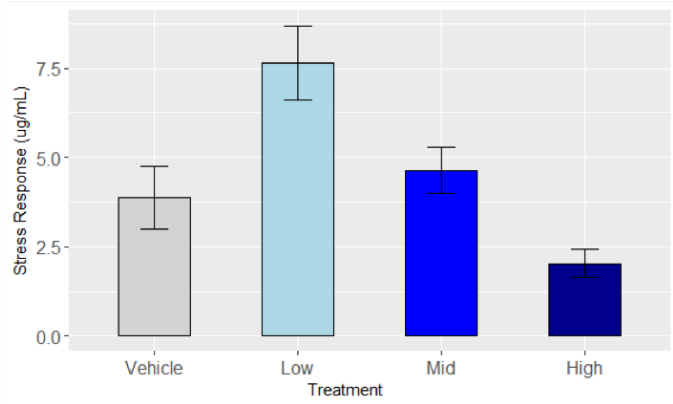

Figure 10. Mean stress response (change in in corticosterone levels) in Japanese quail chicks across treatments. No significant effects were observed across treatment groups (p = 0.342).

4.0 DISCUSSION

In the current study, we examined the effect of early life exposure to TPHP on stress-related behaviours in Japanese quail chicks. The results obtained suggest that TPHP at concentrations 20x effect on the boldness of birds, expressed as an absence of neophobia. No associations between TPHP exposure and behavioural effects were observed for the tonic immobility and novel area exploration tests.

4.1 Tonic Immobility

The tonic immobility test (also known as the 'righting response', 'thanatosis' or 'animal hypnosis') is a relatively common type of behavioural test used for a variety of species such as birds and sharks (Watsky & Gruber, 1990). However, it is predominantly employed by the poultry industry on adult and young fowl in order to research the welfare of domesticated birds, by using it as a measure of potential effects on egg laying and growth (Quinn, 2012). Tonic immobility is an extreme fear response and is thought to be associated with potential alterations

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to the autonomic nervous system and hypothalamus, both of which are involved in the expression of stress in most vertebrates (Quinn, 2012).

In an ecological context the tonic immobility test can be useful for predicting how organisms will react when faced with a predator (Edelaar et al., 2012).Based on a literature search, a single study was found that examined the effects of toxicants on the tonic immobility response; it examined the effects of deoxynivalenol (DON) mycotoxin on broiler chicken chick fearfulness (Ghareeb et al., 2014). The authors found that chicks exposed to higher levels of DON mycotoxin were rendered immobile for an increased duration of time compared to the control chicks. Another study by Deo, Blaney, & Dingle (1998) examined the tonic immobility of chickens exposed to aflatoxin in their feed. The authors found that aflatoxin exposed decreased the duration of immobility.

In the current study, no significant differences were obtained across treatment groups for two tonic immobility endpoints; 'number of attempts to initiate immobile state' and the 'duration of immobile state'. Most individuals performed well at this test; 94% of the chicks displayed freeze behaviour within 5 attempts. Among quail chicks, freeze times varied dramatically, from 1.7 to 99.4 seconds. In the vehicle control group, the average freeze duration was 21.0 seconds. This average duration is lower than the average durations obtained by Launay, Mills, & Faure (1993) and Calandreau et al., (2011), who observed average freeze durations with their control birds of 51.3 and 113.8 respectively. This could be attributable to the larger sample sizes used by these studies (400 and 16 respectively), or due to the older ages of the chicks (both groups of chicks in the aforementioned study were 1 week old. In this study, tonic immobility was performed at 2 days old). The difference in freeze duration across studies can also be connected to differences at the genetic level between individual birds, as researchers have been able to successfully reproduce lines of Japanese quail that have been diverged into LTI and STI, which stand for 'long tonic immobility' and 'short tonic immobility' respectively (Mills & Faure, 1991). Within individuals, the tonic immobility test is one that can produce consistent results, and appears to produce dependable correlations between performance and boldness (Jones, 1988), although we did not do this in this study. Furthermore, an individual's performance on this test is based on their personality, ie the individual organism's notion of stress is a strong

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predictor on how it will respond (Edelaar et al., 2012; Quinn, 2012). A way to account for inter-individuality is to increase sample size, and/or number of observations (Fay & Gerow, 2013). Due to the small sample size of this study, it may have been prone to strong interference stemming from individual uniqueness. This study had a statistical power of 0.45. To improve statistical power and account for unique individuals, future studies should consider increasing sample size.

Similar studies in the future should aim to take genetic and individual differences into account. This can be done by using quail that have been bred specifically for STI and LTI, in addition to increasing the number of organisms participating in the study to reduce variation. Furthermore, future studies should take the opportunity to verify the test by doing the test multiple times on individuals, and see if similar freeze durations were presented.

4.2 Novel Area Exploration Test

The novel area exploration test is designed to measure an individual's tendency to explore new surroundings. To survive, animals must have a level of familiarity with their environment (Newberry, 1999; Verbeek, Drent, & Wiepkema, 1994). Through exploration of their surroundings, they learn the location of essential resources such as food, water and shelter. This makes an individual's performance in this test a good indicator of their potential fitness, for as a general rule, the more an animal explores, the easier they are able to adapt to environmental change (Verbeek et al., 1994).

Exploration behaviour appears to be one of the most common behavioural endpoints examined for behavioural ecotoxicology studies. It has been used to examine the behavioural alterations of zebrafish exposed to TPHP (Oliveri et al., 2015), zebrafinch exposed to methylmercury, rats exposed to Firemaster® 550 (Patisaul et al., 2013), and sheep exposed to sewage (Erhard & Rhind, 2004). All of these aforementioned studies found significant effects of contaminants on exploration behaviours.

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In the novel area exploration test for this study, chicks were given the opportunity to explore a previously restricted area of the brooder. They were removed from the container while the door was opened, and after three seconds, were released back into the container. No significant differences were observed for any of the endpoints ("percentage of time spent in the familiar zone", and "latency to enter novel zone"), nor an interpretable trend. However, this result should be interpreted with caution given several limitations associated with the test design. In our study, 50% of chicks entered the novel area within 3 minutes. Although it is possible that the chicks who remained in the original compartment did so because of a reluctance to explore the novel area, our observations suggest that this was not the case. One factor likely contributing to the lack of movement was the external room temperature where the tests were conducted. Many of the chicks who remained in the familiar area were huddling in a far corner of the testing container, exhibiting very little movement. In many young domestic fowl species, huddling represents a form of behavioural temperature regulation. The chicks did not have access to a heat source during the behavioural trials, despite having become accustomed to having easy access to one in their housing brooder. As Japanese quail are not fully capable of internal thermoregulation until two weeks of age (Spiers, McNabb, & McNabb, 1974), chicks were more likely to spend extended amounts of time thermoregulating, rather than engaging in other activities, a finding supported by Pedersen & Steen, (1979). Future studies should provide the quail chicks with a heat source, or ensure that the testing room is the ambient temperature that fulfills the husbandry requirements associated with the age of the quail chick.

Secondly, the removal of the door of the familiar area may not have been obvious to the quail chicks, given that the container they were housed in was clear plastic. Therefore, if none of the chicks attempted to enter the novel zone, it may not be evident to the quail chicks that they could venture into the novel zone. Future test should ensure that all housing containers, in addition to the testing container have a solid door area, to better signal to chicks they are able to exit the container.

4.3 Neophobia

Neophobia tests are designed to examine an organism's cognitive assessment of novel stimuli (Greggor & Clayton, 2016). Depending on the study design, these tests can be used to

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provide information on a range of behaviours, such as anxiety and predatory wariness. Therefore, the results can have important implications for an organism's fitness. In past studies, the use of neophobia trials has been more common in the field of psychopharmacology (Greggor, Thornton, & Clayton, 2015), where the tests help determine the effects of certain drugs on endpoints such as anxiety and memory. However, despite the fact that many contaminants have been shown to be neurotoxic, it is not common for studies to measure neophobia-associated endpoints. A few exceptions are studies by Swaddle et al., (2017) and Zahara et al. (2015). Swaddle examined the effects of dietary methylmercury on a range of behaviours in zebrafinch, one of them being novel object neophobia. The authors examined the behaviour of the zebrafinches in their time to approach a food dish with or without a novel object present nearby. In this case, no significant effects on neophobic behaviours were observed. In a similar fashion, Zahara investigated the effects of PCBs in juvenile, European starlings. Birds were placed daily in a cage with food dishes. However, each day, they would change an aspect of the cage, ie: cage entry point, food dish position and food dish colour. The test was considered to be successfully completed by the bird if it fed from the food dishes. Like the aforementioned study, no significant differences were noted across treatment groups.

The current study measured neophobia by exposing the quail chicks to a completely novel object, and observing their behaviour. Overall, our results indicate that birds exposed to higher doses of TPHP were more likely to exhibit bold behaviour in comparison to the lower dose groups and the control. 'Number of pecks' to the novel object showed a significant difference across treatment groups, a similar, dose dependent trend was observed for a related measure, 'amount of time near novel object', but this result was not significant. All chicks approached the novel object within 6 minutes, and this time was not significantly different between groups. No trend was observed across treatments for this particular endpoint.

As addressed previously, performance on these behavioural tasks is influenced by the type of behaviour individual animals are predisposed to. Once again, future studies should consider increasing sample size to account for inter-individual variation. This study had a statistical power of 0.45, suggesting that there are aspects of the study that could be improved upon to make it more effective at detecting potential false positives and false negatives.

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4.4 Corticosterone

Corticosterone values were measured for baseline and stress blood samples from the Japanese quail chicks. However, validating the kit (Enzo Life Science Corticosterone EIA) proved to be difficult, and generated inconsistent values. Serial dilutions of the quail plasma produced curves with visibly irregular slopes in between validation plates. Furthermore, our attempt at sampling within 3 minutes was not successful, and we saw a relationship between time since sampled and corticosterone concentrations, which could lead to difficulty interpreting the results. The fact that 12% of chicks (5 of 45) demonstrated higher baseline corticosterone values compared to their respective stress corticosterone values, is an indication that the quail may have been stressed prior to sampling. Additionally, after further investigation, it was discovered that Japanese quail chick plasma is not a good candidate for EIAs due to the higher lipid concentration in their plasma, which can alter the results of the assay (Selby, 1999). The corticosterone results should therefore be interpreted with caution, and will not be included in a manuscript based on this work.

In spite of these technical issues we were able to obtain values for corticosterone that were within the same range of previously reported values. Average baseline corticosterone concentration for the control birds was 0.42 ± 0.47 ng/ mL in six day old quail, while a study by Hazard et al., (2005) found an average baseline corticosterone concentration of 0.8 ± 0.2 – 0.8 ± 0.3 ng/ mL in their four week old birds. However, according to this study, baseline Japanese quail values increased until the birds reached six weeks of age, which was when they hit maximum baseline

4.5 Effect of Heat Stress

For the statistical analyses of TPHP related effects presented above, we did not include batches 1 and 2, which were the batches incubated at a slightly increased temperature due to an incubator malfunction. This was due to an observed strong effect of the nominal heat stress on not only stress related behaviour, but corticosterone levels as well. In this section, we further discuss and clarify the effect of heat stress on the behavioural endpoints measured in this study. This was accomplished by comparing behavioural test performance in vehicle chicks from

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batches 1 and 2 (hereafter referred to as heat stress batches) to the vehicle chicks from batches 3 and 4 (hereafter referred to as non-heat stress batches).

4.5.1 Heat Stress and Tonic Immobility, Novel Area Exploration, Neophobia

When tonic immobility endpoints (duration of time spent immobile and number of tries required to initiate immobility) were compared between vehicles from the heat stressed batches and non-heat stressed batches, no significant differences were observed. The effects of heat stress during incubation on tonic immobility has not been previously studied, nor cited in the literature. A similar result was found when comparing the novel area exploration endpoints of the heat stressed batches and non-heat stressed batches. As with tonic immobility, there are no studies that currently examine the effects of heat stress on novel area exploration.

However, in contrast, when neophobia endpoints were compared between heat stressed batches and non-heat stressed batches, it was found that the heat stressed batches administered significantly more pecks upon the novel object, in addition to spending more time near it than the non-heat stressed batches. While this phenomenon has not yet been tested in birds, it is reminiscent of a study done by Siviter et al. (2017), who incubated bearded dragon eggs at two different temperatures. It was found that the bearded dragon hatchlings incubated at higher temperatures displayed bolder tendencies in neophobia (ie: spent significantly more time near the novel object compared to those incubated at a lower temperature).

4.5.2 Heat Stress and Corticosterone

As mentioned above, the heat stressed batches exhibited lower baseline corticosterone levels in comparison to the non-heat stressed batches. These findings are supported by DuRant et al., (2010), who determined that slightly higher incubation temperatures in wood ducklings resulted in lower baseline and stress corticosterone levels compared to those incubated at normal temperatures. Potential effects of incubation temperature on boldness behaviour have not been reported in birds, however have been tested in reptiles.

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Because the heat stressed batches exhibited statistically significantly bolder behaviour in neophobia trials, it is possible that they have lower baseline and stress corticosterone due to disruptions of mechanisms associated with the HPA axis. The negative relationship between boldness and corticosterone concentrations has frequently been frequently documented in previous studies (Baugh et al., 2012).

4.6 Conclusion

The results obtained suggest TPHP can influence boldness in the Japanese quail at concentrations that, when metabolism is considered, may be experienced by wild birds. This research builds on our understanding that TPHP is capable of altering behaviour. Prior to this study (to our knowledge), studies of TPHP's potential effect on behaviour focused on fish. By continuing to establish behavioural phenotypes for a variety of chemicals for numerous types of sentinel species, we can detect potential, subtle, deleterious effects prior to widespread mortality. This would enable the design of a more sensitive toxicity test before products are commercially used.

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GENERAL DISCUSSION

Behaviour is the result of complex external and internal interactions between an organism and its environment. It has evolved to be plastic, to facilitate adaptations to acute and chronic events with the goal of maximizing the organism's fitness within their physical and psychological limits. Thus, it is unsurprising that it can be especially susceptible to alterations via chemical exposure, especially those that are neurotoxic in nature.

We used behaviour to observe sublethal effects of TPHP on birds, observing a significantly higher number of pecks given by chicks in the high exposure group during the neophobia trials. While concrete conclusions were not able to be derived from the corticosterone data, the exhibition of bolder behaviour by the chicks in the high exposure group warrants further investigation into the potential effects of TPHP on circulating corticosterone concentrations.

In other papers produced from the overall project, it was also determined that TPHP is rapidly metabolized by Japanese quail, and varies depending on the stage of chick development (Marteinson et al., 2019). Therefore, it is possible that TPHP exposure in the environment has been underestimated. Other branches of the project explored potential impacts TPHP could have on morphology, physiology and behaviour. Additionally, these effects can vary between sexes. For example, TPHP exposure enhanced thyroid gland structure in high TPHP males, however suppressed thyroid gland structure and activity in all TPHP exposed females (Guigueno et al., 2019). Females in the high TPHP treatment also experienced suppressed circulating free triiodothyronine. Additionally, compared to controls, chicks in the mid and high TPHP treatments experienced significantly decreased metabolic rate ($\leq$ 13%) and growth ($\leq$ 53%) (Guigueno et al., 2019). Mid TPHP males and high TPHP females were significantly smaller. TPHP was found to also increase aggressive behaviour in low TPHP chicks, by increasing the number of pecks they gave their conspecifics (Hanas et al., 2020).

While we have shown above that behaviour is an intrinsically useful tool for assessing chemical exposure, it does not come without its challenges. Some issues that could be improved upon in future studies could be making the door in the exploratory test more obvious, by giving it

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a solid colour or different texture. This would make it more obvious when it is removed from a chick's environment, which may make them more interested in exploring the novel area. Another aspect that should be considered is the temperature of the room when utilizing quail chicks that have yet to develop proper feathers for thermoregulation. This potentially confounding factor could be minimized by the addition of a heating lamp over the testing arena, or increasing the temperature within the room the testing arena is located in. Future studies could also take more specific behaviours into account, such as predator vigilance.

It was interesting that the strongest effects were observed with faulty incubation temperatures, with the batches incubated at higher temperatures demonstrating bolder behaviour compared to those incubated at normal temperatures. While unplanned, this may open an avenue of further investigation into possible effects of global warming on resulting offspring behaviour, and perhaps even potential interactive effects between chemical exposure and global warming effects (both of which are highly relevant environmental threats in this day and age). While few studies have examined this phenomenon in birds, there have been several done in reptiles ((Siviter et al., 2017). We were unfortunately unable to accomplish this with our data, as we do not know the extent of the temperature fluctuation within the faulty incubator.

While unexpected, the incubator malfunction presented us with the opportunity to validate the behavioural tests. We found that chicks incubated at higher temperatures had a lower baseline corticosterone concentration in comparison to chicks incubated at the recommended temperatures. This finding was supported by the results obtained in the neophobia behavioural tests, which found chicks incubated at the higher temperatures displayed bolder behaviour.

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CONCLUSION AND SUMMARY

When designing and researching this thesis, we set out to develop methods to assess stress related behaviours in birds exposed to contaminants, determine whether exposure to varying levels of TPHP can affect corticosterone concentrations of Japanese quail chicks and determine if TPHP could impact avian stress behaviour utilizing classic neophobia, novel area exploration and tonic immobility tests. By observing a significant increase in the number of pecks upon the novel object, we were able to demonstrate that early life stage exposure to chemicals can influence an organism's behaviour. Alterations of behaviour can result in decreased fitness, as it can lower the organism's success coping with acute environmental threats such as inclement weather, predators, resource shortages and obtaining a mate.

Due to the results of this study (in addition to other deleterious results found in other studies), I believe TPHP poses a risk to wildlife in high concentrations. With TPHP being so widespread, and continually leached into the environment, it is important to thoroughly define a framework not only to determine the extent of the threat it poses, but also to be able to monitor sites of heavier exposure for deleterious effects. If TPHP does increase boldness in birds, we may see changes in predator/prey interactions and organization of individuals living in social groups.

Animal behaviour is a highly flexible, valuable tool to assess potential occurrences of chemical exposure. Its ability to integrate both biochemical and physiological processes, in addition to acting as an early warning signal, open up numerous alternative methods to assess the severity of environmental threat. Despite its multiple benefits, behavioural endpoints also have their challenges. They can be time consuming to perform, and large sample sizes are required in order to correct for individual personalities. Behaviour offers researchers a unique opportunity to observe the 'big picture' on contaminant exposure. By observing behaviour, we can see potential fitness alterations of individuals, their population, and by proxy their ecosystem.

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