Avian Early-Life Stage Toxicity Testing as an Alternative to Traditional Methods

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December 2023

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science

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Abstract

The assessment of chemical toxicity is crucial for protecting the environment and human health. However, traditional testing methods are costly, time-consuming, and raise ethical concerns. Overcoming these challenges requires adopting humane and efficient approaches, and thus there is regulatory demand in Canada (and worldwide) for new approach methods (NAMs) that are cost-effective, time-saving, and ethical.

One such alternative method is the early-life stage (ELS) toxicity test using prehatch and fertilized avian embryos, which has been established by Farhat and colleagues from Environment and Climate Change Canada as a promising alternative to traditional avian testing. However, for this method to align with the requirements of both horizontal and vertical legislation, there is a need for more case studies involving diverse chemicals, as well as continued focus on improving technical aspects of the method. Accordingly, this thesis presents two studies: Study 1: a comparative analysis between results derived from the avian ELS toxicity testing method and traditional avian toxicity (AT) testing methods; and Study 2: an exploration of methods to measure embryonic survival in a non-invasive manner.

In study 1, the correlation between avian ELS testing and traditional AT testing was explored through a study of six pesticides: endrin, ethoprophos, carbofuran, trichlorfon, permethrin, and glufosinate-ammonium. From the ELS test I measured the following variables: LD50, LOEL, BMD. When these variables were compared to toxicity data on these same pesticides derived from traditional AT, there were poor correlations calculated. Despite such findings, a wealth of novel data was accrued for pesticides of environmental concern, along with technical information on

the quality of the ELS method (e.g., mortality in controls). Even though the concordance between the two approaches was poor, such studies are needed to increase understanding of the applicability domain of ELS testing as an alternative to traditional avian acute toxicity assessment.

In study 2, novel techniques and technologies were explored to characterize embryonic survival non-invasively, focusing on monitoring embryonic heartbeat using piezoelectric sensors as an indicator of survival. However, the selected sensors did not yield expected signals from the embryos, emphasizing the necessity for alternative methods to the ones explored here to effectively monitor embryonic heart rates in avian ELS testing.

Overall, this thesis provides data necessary to increase understanding of the avian ELS test method, including pilot results on how embryonic survival may be studied in a non-invasive manner. The findings and insights gained from this study contribute to help furthering our understanding of the applicability domain of ELS testing as an alternative approach to traditional AT testing as well as technical performance of the method, while also suggesting areas for additional research.

Résumé

L'évaluation de la toxicité chimique est cruciale pour protéger l'environnement et la santé humaine. Cependant, les méthodes traditionnelles de test sont coûteuses, chronophages et soulèvent des préoccupations éthiques. Surmonter ces défis nécessite l'adoption d'approches humaines et efficaces, d'où la demande réglementaire au Canada (et dans le monde entier) pour de nouvelles méthodes d'approche (NAM) rentables, gain de temps et éthiques.

Une méthode alternative est le test de toxicité précoce du stade embryonnaire (ELS) utilisant des embryons aviaires pré-éclosion et fécondés, établi par Farhat et ses collègues d'Environnement et Changement climatique Canada comme une alternative prometteuse aux tests aviaires traditionnels. Cependant, pour que cette méthode soit conforme aux exigences de la législation horizontale et verticale, il est nécessaire de réaliser davantage d'études de cas avec divers produits chimiques, ainsi que de se concentrer continuellement sur l'amélioration des aspects techniques de la méthode.

En conséquence, cette thèse présente deux études : Étude 1 : une analyse comparative entre les résultats issus de la méthode de test de toxicité ELS aviaire et les méthodes traditionnelles de test de toxicité aviaire (AT) ; et Étude 2 : une exploration de méthodes pour mesurer la survie embryonnaire de manière non invasive.

Dans l'étude 1, la corrélation entre le test ELS aviaire et le test AT traditionnel a été explorée à travers une étude de six pesticides : endrine, éthoprophos, carbofuran, trichlorfon, perméthrine et glufosinate-ammonium. À partir du test ELS, j'ai mesuré les variables suivantes : DL50, LOEL, BMD. Lorsque ces variables ont été comparées aux données de toxicité sur ces mêmes pesticides

dérivées du test AT traditionnel, des corrélations faibles ont été calculées. Malgré de telles constatations, une richesse de nouvelles données a été accumulée pour les pesticides préoccupants pour l'environnement, ainsi que des informations techniques sur la qualité de la méthode ELS (par exemple, la mortalité dans les témoins). Bien que la concordance entre les deux approches soit faible, de telles études sont nécessaires pour augmenter la compréhension du domaine d'application potentiel du test ELS en tant qu'alternative à l'évaluation traditionnelle de la toxicité aiguë aviaire.

Dans l'étude 2, des techniques et des technologies novatrices ont été explorées pour caractériser la survie embryonnaire de manière non invasive, en se concentrant sur la surveillance du battement cardiaque embryonnaire à l'aide de capteurs piézoélectriques comme indicateur de survie. Cependant, les capteurs sélectionnés n'ont pas donné les signaux attendus des embryons, soulignant la nécessité de méthodes alternatives pour surveiller efficacement les fréquences cardiaques embryonnaires dans le test ELS aviaire.

En résumé, cette thèse fournit des données nécessaires pour augmenter la compréhension de la méthode de test ELS aviaire, y compris des résultats pilotes sur la manière dont la survie embryonnaire peut être étudiée de manière non invasive. Les résultats et les enseignements tirés de cette étude contribuent à approfondir notre compréhension du domaine d'application du test ELS en tant qu'approche alternative aux tests AT traditionnels, ainsi qu'à la performance technique de la méthode, tout en suggérant des domaines de recherche supplémentaires.

Acknowledgements

First and foremost, I would like to express my gratitude to my supervisor, Niladri Basu. I am thankful not only for his support in providing me with the opportunity to work on this interesting program, but also for his patience in helping me navigate a new study process in a new environment. I am also grateful to my committee members, Jessica Head and Crump Doug, for their valuable insights, kind words and warm encouragement.

I would also like to extend my thanks to my colleague, Emily Boulanger. I have learned a great deal from her. She has been incredibly dedicated and patient, teaching me important skills such as egg candle, egg injection, and egg dissection, and sharing her extensive knowledge of the testing procedures. Without her assistance, I would not have been able to complete my program so smoothly.

I am deeply indebted to Aimen Akbar, Muhammad Asif, and Ramela Koumrouyan for their help and collaboration throughout the study. Special thanks to Ramela Koumrouyan for her dedication and professionalism as my fitness trainer. I would also like to express my gratitude to Iris Xu for her assistance with statistical analysis and to Jenny Eng for her valuable support in program processing.

Furthermore, I am grateful to Aylish Marshall for her kind explanations regarding the treatment of quail RNA samples. I would also like to extend my thanks to Hugo Marchand and Nicolas Decelles, with whom I had a great time conducting experiments. Additionally, I would like to thank Krittika Mittal, Sophie Emberley-Korkmaz, Katie Chone, Jonathan Sangiovanni, Yeonseon Jeon for their unwavering support whenever I needed their help. Thank you all so much!

On a personal note, I would like to thank my husband for his unwavering love and support. I am also grateful to my daughter for her exceptional ability to manage her own responsibilities, allowing me to focus on my studies.

Finally, I want to express my heartfelt thanks once again to Jessica Head and Niladri Basu. I am truly thankful to Jessica for introducing me to Niladri Basu, and to Niladri for providing me with this invaluable learning opportunity. It has allowed me to have a beautiful, fantastic, and memorable experience that I will cherish for the rest of my life.

Key Terms Used in the Thesis

- Apical endpoint: Traditional, directly measured whole-organism outcomes of exposure in in vivo tests, generally death, reproductive failure, or developmental dysfunction (Villeneuve & Garcia 2011).
- Benchmark dose (BMD): A BMD is a statistical lower confidence limit for a dose that produces a predetermined change in response rate of an adverse effect (called the benchmark response or BMR) compared to background. Unlike the NOAEL, the BMD takes into account dose-response information by fitting a mathematical model to dose-response data (USEPA 1995).
- Mode of action (MOA): The sequence of key events and cellular and biochemical events (measurable parameters), starting with the interaction of an agent with the target cell, through functional and anatomical changes, resulting in cancer or other adverse health effects (USEPA2005; Boobis et al 2008; USEPA 2009)
- Adverse outcome pathways (AOPs): A linear sequence of events from the exposure of research animals (or humans) to a potentially toxic substance that results in a molecular initiating event that may lead to early cellular events and, ultimately, an apical effect, e.g., an observable outcome / phenotypic effect. In contrast to MoA,, AOPs are not substance-specific and therefore do not include metabolism considerations. AOPs can help address the biological plausibility of a MoA. (ECETOC 2017).
- Toxicity pathway: Cellular response pathways that, when sufficiently perturbed in an intact animal, are expected to result in adverse effects (NRC 2007).

- Mechanism of action: Denotes the sequence of events leading from the absorption of an effective dose of a chemical to the production of a specific biological response in the target organ. Understanding a chemical's mechanism requires appreciation of the causality and temporal relationships between the steps leading to a particular toxic endpoint, as well as the steps that lead to an effective dose of the chemical at the relevant biological target(s) (Schultz 2010).
- Lowest-observed-adverse-effect level (LOAEL): The lowest amount, dose or concentration of an agent, found by experiment or observation, that causes an adverse alteration of morphology, functional capacity, growth, development or life span in an organism, system or (sub)population. Methods vary for identifying a LOAEL, but often apply statistical significance as a criterion(CCME 2020).
- No-observed-adverse effect level (NOAEL): An exposure level at which there are no statistically or biologically significant increases in the frequency or severity of adverse effects between the exposed organisms or population and the appropriate control. Some effects may be produced at this level, but they are not considered to be adverse. Methods for identifying a NOAEL vary, but often apply statistical significance as a criterion(CCME 2020).
- Horizontal legislation: Laws, requirements and/or international treaties, which are specifically aimed at (the reduction of) animal testing and are intrinsically cross-sectoral in nature (Vonk et al. 2015).
- Vertical legislation: Laws, requirements and/or international treaties which are specifically aimed at a single sector, such as medicinal products or chemical substances. This kind of

legislation generally touches the topic of animal testing in a more indirect way (Vonk et al. 2015).

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Preface and Contribution to the Knowledge

This thesis is written in the manuscript format specified in McGill's "Thesis Guidelines: Preparation of a Thesis". Chapter 1 serves as a general introduction, providing the motivation, objectives, and outlines of the thesis. Chapter 2 conducts a comprehensive literature survey for each experimental work, offering a comprehensive overview of the existing research relevant to the topics explored in this thesis. Chapters 3-4 present the introductions, materials and methods, results, discussions, and conclusions for each of the original studies. Chapter 5 offers a comprehensive discussion and identifying future research needs.

The thesis makes significant contributions to knowledge in the following areas:

In Chapter 3, the correlation between traditional avian toxicity (AT) testing and avian early-life stage (ELS) toxicity testing is investigated. This study aimed to determine if avian ELS toxicity testing can serve as a promising alternative to traditional AT for LD50 determination. To our knowledge, this is the first study to test pesticides of notable concern (endrin, ethoprophos, carbofuran, trichlorfon, permethrin, and glufosinate-ammonium) using the Japanese quail ELS method. The LD50 values obtained from this method were compared with those derived from the traditional Japanese quail acute toxicity method, evaluating the correlation between the two approaches. This comparison is essential in assessing the potential of avian ELS testing to replace traditional AT testing in acute toxicity evaluation and risk assessment.

In Chapter 4, non-invasive techniques for monitoring embryonic heartbeats were discussed as a way to overcome the limitation of the avian ELS toxicity method. To my knowledge this was the first time piezoelectric sensors were used to detect embryonic heartbeat in avian ELS testing. The

results indicated that the selected sensors were unable to detect the embryonic heartbeat signals effectively, highlighting the challenges in using this specific configuration. The research emphasizes the need for alternative approaches to accurately monitor embryonic heart rates during incubation. It suggested exploring different sensors types, configurations, or amplifiers, as well as considering advanced imaging or lighting techniques to improve signal detection.

The findings from these studies contribute to the understanding of avian ELS toxicity testing, its potential domains of applicability, and introduce a new direction for non-invasive monitoring. In doing so, this work provides valuable insights that may help to improve ecotoxicity evaluation practices and enhance risk assessment strategies.

Chapter 1

1. Introduction

1.1 Thesis Motivation, Objectives, and Hypothesis

Avian toxicity testing is essential for assessing the potential risks posed by pesticides to avian species. Traditionally, young or adult birds have been used as the primary test subjects in toxicity investigations. However, regulations are changing to promote alternatives since traditional testing strategies in the field of toxicology are expensive, time-consuming and raise ethical concern about animal usage. One such alternative is avian early-life stage (ELS) toxicity testing, which has emerged as an alternative method to assess the potential toxicity of test substances at both the molecular and apical levels. While there is interest in this method, the correlation between ELS and traditional avian toxicity (AT) testing, particularly in evaluating acute toxicity (LD50), is still unresolved.

This thesis aims to explore the relationship between avian ELS toxicity testing and traditional AT testing through a comparative study, while also advancing our ability to measure embryonic survival non-invasively.

1.2 Specific Aims

Aim 1: To characterize the correlation in apical measures between avian ELS toxicity testing and traditional AT testing based on a comparative study of pesticide toxicity.

The objective of Aim #1 was to evaluate the suitability of avian ELS testing as an alternative to traditional AT testing for acute toxicity evaluation. A comprehensive set of experiments using the ELS testing method was conducted to assess the responses of avian embryos to six pesticides with diverse mechanisms of action and potencies. The results obtained from the testing approach were compared with those from traditional AT testing and analyzed to determine the degree of correlation between ELS and traditional AT testing. This investigation will provide valuable insights into the relationship between ELS and traditional AT testing, contributing to the ongoing discussion concerning the applicability domain of ELS testing as an alternative approach to traditional avian acute toxicity assessment as well as its technical performance.

The specific hypothesis underlying Aim #1 was that there is a strong correlation between avian ELS testing and traditional AT testing for acute toxicity evaluation (I.e., LD50 values), with a correlation coefficient greater than 80%.

Aim 2: To enhance the ability to measure embryonic survival for avian ELS toxicity testing in a non-invasive manner.

The objective of Aim #2 was to improve the embryonic survival detection method for avian ELS toxicity testing. I aimed to overcome the limitations associated with the current manual candling process used for mortality assessment which are time consuming, may require subjective judgment, and risk damaging the eggshell. The Aim #2 objective was to explore alternative methods for real-time and simultaneous assessment of embryonic survival, so as to improve accuracy and efficiency in the evaluation of toxicity. By optimizing the assessment process, I aimed to establish a reliable and robust method for determining embryonic survival in avian ELS toxicity

testing, thereby contributing to the refinement and advancement of the ELS toxicity testing protocols.

The specific hypothesis is that the alternative method can detect avian embryo mortality in real-time and simultaneously, with an accuracy reaching 95%.

Chapter 2

2. Literature review

2.1 Limitations of traditional toxicity testing strategies

Toxicity testing plays a critical role in chemical management programs worldwide. It is essential for assessing the potential risks associated with various chemicals, including pesticides, industrial compounds, and pharmaceuticals. Traditional animal testing has long been the cornerstone of toxicity assessment and regulatory decision-making (LaLone et al. 2021). It involves the use of live animals, typically rodents, fish, birds, and mammals, to determine the potential adverse effects of chemicals on various physiological systems. Despite its widespread use, the current status of traditional animal testing is marked by several challenges and limitations (Krewski et al. 2010).

One of the primary concerns associated with traditional animal testing is the extensive number of chemicals that require toxicity data. A survey encompassing 19 countries and regions has shown that, within the last 30 to 40 years, approximately 350,000 chemical substances have been registered for production and large-scale utilization (Muir et al, 2023). The European Union's Registration, Evaluation, and Authorization of Chemicals (REACH) program have released its first set of registered substances, which includes over 140,000 entries. Although the number is substantial, only a small fraction of these chemicals has undergone adequate assessment regarding their potential for human or ecological toxicity (Krewski et al. 2010; Judson et al. 2009). This lack of comprehensive data for chemicals in our environment and society has been extensively documented (Allanou et al., 1999; NRC, 2007; U.S. EPA 1998). The substantial number of existing chemicals without sufficient toxicity information, combined with the increasing influx of new chemicals submitted for registration, such as an annual submission of approximately 1500 to 2000

new industrial substances in the United States for evaluation under the Toxic Substances Control Act, presents a considerable challenge for chemical management programs (Capela et al., 2020). However, the traditional approach of conducting animal tests to evaluate the potential toxicity of chemicals is time-consuming (Krewski et al. 2010). For instance, reproductive studies in avian species can take up to two years to complete (OECD 2017), as they involve carefully nurturing breeding pairs, monitoring reproductive cycles, and assessing offspring development and health(USEPA 2012a; OECD 1984). It has been estimated by researchers that the traditional testing of a single chemical can require a time frame of up to four years (Basu et al. 2019). This extended timeframe not only delays the availability of toxicity data but also hinders the timely assessment of chemicals, potentially impacting chemicals management programs and regulatory decision-making processes. Therefore, relying on traditional strategies for assessing tens of thousands of chemicals is impractical (Judson et al. 2009).

Furthermore, the reliance on whole animal testing in traditional methodologies can incur significant costs (Krewski et al. 2010). For example, in a review of various ecotoxicity testing methods, researchers estimated that the traditional testing of a single chemical can involve expenses ranging from $1 to $20 million (Basu et al. 2019). Conducting multiple tests across different species, endpoints, and life stages amplifies the monetary burden. In the case of traditional avian toxicity testing, various protocols such as reproductive studies, sub-acute dietary toxicity studies, and acute oral toxicity studies are employed, demanding substantial financial resources and a large number of birds (OECD 2017). These costs encompass the housing and care of the avian subjects, the execution of experiments, and the analysis of resulting data. As a result,

the cumulative financial implications associated with traditional animal testing impose considerable constraints on its feasibility and practicality (NRC 2007).

In addition to financial concerns, the utilization of a large number of animals in traditional animal testing raises ethical concerns surrounding animal welfare (NRC 2007). The necessity to generate statistically significant data often leads to the use of a substantial number of animals. Each year, over 115 million animals are subjected to experimentation worldwide (Akhtar 2015), which can be ethically challenging. Animal welfare advocates and scientific communities recognize the need to minimize and, whenever possible, replace the use of animals in experiments (Festing & Wilkinson 2007). There is a growing social and ethical pressure to address the concerns related to the use of animals in toxicity testing and to explore alternative methods that are more humane, scientifically valid, and efficient (Ferdowsian & Beck 2011; Goldberg & Frazier 1989).

Finally, traditional testing methods primarily focus on identifying the apical toxic effects of chemicals across various species. As scientific understanding has progressed, these methods have evolved by expanding protocols and introducing new tests (NRC 2007). For instance, with a deeper comprehension of chemical interactions, additional endpoints such as endocrine disruptor testing may have been included. However, this patchwork approach has led to a system burdened by high testing costs, extensive use of laboratory animals, and prolonged timelines for data generation and review. Furthermore, this approach provides little information on modes and mechanisms of action underlying toxicity (NRC 2007). Consequently, valuable information regarding specific molecular targets and the sequence of events leading to toxicity may be missed. This limits our ability to fully understand and assess the underlying mechanisms of toxicity and predict the

potential adverse effects across species and/or utilize the data in a chemical read-across approach (NRC 2007; Villeneuve & Garcia-Reyero 2011).

In conclusion, traditional approaches in avian toxicology have proven to be time-consuming, costly and have raised ethical concern regarding the use of animals. These approaches have failed to deliver a completely satisfactory resolution to the fundamental problem of simultaneously addressing the four key objectives: "depth of testing; breadth of testing; animal welfare; and conservation of testing resources" (NRC 2007).

2.2 Regulatory Shift towards Alternative Testing Methods

Due to the significant challenges and limitations associated with traditional toxicity testing, regulations and directives are being enacted to drive a shift towards alternative testing strategies (Zaunbrecher et al. 2017). Notably, regulatory bodies in the United States, Europe, and Canada have recognized the need to reduce animal testing and promote the development of more advanced and humane testing approaches. These changes reflect a growing understanding of the limitations and ethical concerns associated with traditional animal testing.

In 2019, a landmark directive was signed by the United States Environmental Protection Agency (USEPA) Administrator, Andrew Wheeler, emphasizing the prioritization of efforts to reduce animal testing. This directive also included a significant financial commitment, with $4.25 million being allocated to advance research and development in alternative test methods and strategies that aim to reduce, refine, and ultimately replace vertebrate animal testing. Additionally, the

USEPA directive sets a goal to eliminate all mammal study requests and funding by 2035, highlighting a clear commitment to transitioning away from traditional animal testing practices (USEPA, United States Environmental Protection Agency).

Similarly, the European Commission issued the directive 2010/63/EU in 2010, which serves as a pivotal framework for animal research and emphasizes the Three Rs principle: replace, reduce, and refine the use of animals for scientific purposes (European Commission, 2010). This directive underscores the importance of exploring alternative methods and strategies that can effectively replace the need for animal testing or significantly reduce its reliance. It represents a forward-looking approach that aligns with the global trend towards more ethical and scientifically advanced testing practices.

Furthermore, the Canadian government has also recognized the need to prioritize alternative methods and strategies in testing and assessing substances. In 2021, Bill S-5 was proposed to amend the preamble of the *Canadian Environmental Protection Act 1999*, explicitly acknowledging the importance of promoting the development and timely incorporation of scientifically justified alternative methods and strategies to minimize or replace the use of vertebrate animals (House of Commons of Canada). This proposed amendment reflects a proactive stance towards embracing alternative testing approaches and reflects a commitment to reducing the impact on animal welfare while ensuring a robust assessment of substances.

These regulatory changes and directives represent a significant paradigm shift in the field of toxicity testing. They demonstrate a clear recognition of the limitations and ethical concerns associated with traditional animal testing and signal a regulatory revolution. The growing advocacy

for alternative testing methods and the financial investments being made in research and development highlight a concerted effort to drive innovation, improve scientific validity, and reduce the reliance on animal testing. This regulatory revolution sets the stage for a transformative era in toxicity testing, paving the way for the implementation of more advanced, efficient, and ethically sound testing approaches that can meet the demands of chemical risk assessments in the 21st century.

2.3 Current Status of Alternative Testing

The transition from traditional animal testing to alternative methods is driven by various factors, including regulatory push, advancements in scientific understanding, technological innovations, and societal demands (NRC 2007). Scientists and researchers have been exploring and developing a wide range of alternative testing approaches, such as in vitro methods, computational models, and embryo-based models, to overcome the limitations of traditional animal testing (Basu et al. 2019). These alternative methods offer the potential for more accurate, reliable, and cost-effective toxicity assessments, while also addressing the ethical concerns associated with animal use (NRC 2007).

One significant progress in alternative testing methods is the growing adoption of in vitro assays. The term "in vitro" originates from the Latin phrase, which refers to “the technique of performing a given procedure in an artificial environment outside the living organism” (Dhawan & Kwon 2017), typically using cultured cells or tissues. In vitro assays offer several advantages, including increased cost-effectiveness, faster turnaround times, enable high-throughput screening of large chemical

libraries and the ability to examine specific cellular responses or mechanisms of toxicity (Aslantürk 2018). The cellular and molecular events observed in vitro can be correlated with the physiological reactions in vivo, helping us understand why a particular response is obtained in one species but not in another. Thus, selecting an appropriate in vitro assay allows for better prediction of in vivo outcomes and the investigation of specific events and endpoints. However, toxicity is a complex process, often organ-specific and with a cell-specific etiology (Dhawan & Kwon 2017). Many toxic processes require communication between cells and extracellular matrix (ECM) proteins. Therefore, in vitro systems utilizing immortalized cell lines grown on 2D surfaces may not adequately serve as toxicological screens, as they fail to discriminate these differences and lack the necessary cell ligands for ECM-cell adhesion molecule interaction. Furthermore, the impact of the presence of the blood-brain barrier on the biokinetics of chemicals and the evaluation of neurotoxicity in neuronal systems are not considered in vitro models (Dhawan & Kwon 2017). Additionally, many in vitro assays fail to account for the potential effects of metabolic transformation, which can result in decreased sensitivity (in the case of in vivo metabolic activation), a high incidence of false positives (in the case of in vivo inactivation), or false negatives (in the case of in vivo bioactivation) (Tollefsen et al 2014). Limitations associated with the exposure design can also be observed in vitro test systems (Scholz et al. 2013). For example, plastic multi-well plates, commonly used in cell-based test systems, have the potential to adsorb lipophilic compounds (log Kow ≥ approximately 3). This can impact exposure concentrations, particularly for chemicals with low water solubility. Additionally, there can be significant losses of test compounds due to volatilization from the exposure vessels. In cell culture systems, the binding of the compound to medium ingredients (such as serum proteins) can further reduce its bioavailable

concentration (Scholz et al. 2013). Therefore, in vitro models are not yet technologically advanced enough to fully replace animal tests.

Another promising avenue in alternative testing is the use of computational models and predictive toxicology. These methods, including quantitative structure-activity relationship (QSAR) models, structural alerts and rule-based models, and read-across approaches, complement in vitro and in vivo toxicity assessments, offering potential benefits such as minimizing animal testing, reducing costs, saving time, and improving toxicity prediction and safety assessment (Dhawan & Kwon 2018; Raunio 2011, Krewski et al 2010; Hartung et al. 2013). In particular, in silico toxicity models provide a unique advantage by enabling the prediction of chemical toxicity before synthesis. This capability aids in the development of novel chemical compounds with desired properties (NRC 2007; Rovida et al 2015). Computational models are based on the principle of similarity, which suggests that chemicals with similar structures are likely to exhibit similar activity (Tong et al. 2003). By leveraging existing data, advanced computer algorithms and models, computational models can simulate and predict the biological activity, potential toxicity, and metabolism of chemicals based on their structure, physical-chemical properties, and interactions with biological targets (Dhawan & Kwon 2018). This interdisciplinary field combines physics, chemistry, biology, mathematics, computer science, and informatics with toxicology, harnessing computational tools to examine the adverse effects of chemicals (Benigni et al. 2013). It provides valuable insights and facilitates informed decision-making in toxicity assessments. This paradigm shift can contribute to a more comprehensive approach to risk assessment (Dhawan & Kwon 2018). However, the current limitations of these approaches have prevented their widespread use in regulatory settings (Hartung & Hoffmann, 2009). This is mainly due to the inherent challenge of finding an exact

formula that can accurately describe various aspects of the chemical universe based solely on chemical structure. Additionally, the quality issues associated with animal input data and the relatively small size of available datasets have further complicated these efforts (Hartung, 2019).

In addition to in vitro assays and computational models, the utilization of non-animal test organisms further expands the range of alternative testing methods in toxicology. For example, zebra fish embryos or avian early-life stages (during non-protected life stages) provides valuable insights into the potential hazards of chemicals, enabling the identification of toxicological effects and the evaluation of their underlying mechanisms while minimizing the use of whole animals. These developing organisms are not considered live animals until they have exhausted their yolk sacs. Nevertheless, at this stage, many of the biological processes found in their adult counterparts are already established (Basu et al 2019). These alternative models provide a bridge between in vitro cell-based assays and whole-animal studies, offering a balance between complexity and reduction in animal use (Butler et al 2022). As a result, non-animal test organisms contribute to the development of more ethical, efficient, and scientifically relevant approaches in toxicity testing, ultimately enhancing chemical safety assessment and promoting the advancement of regulatory practices.

Moreover, there is a growing emphasis on the use of alternative testing methods that incorporate the principles of systems toxicology and adverse outcome pathways (AOPs). Systems toxicology approaches aim to comprehensively evaluate the interactions between chemicals and biological systems, considering multiple levels of biological organization (Heijne et al. 2005). By integrating data from various sources, such as genomics, transcriptomics, proteomics, and metabolomics,

systems toxicology approaches provide a more holistic understanding of chemical toxicity and enable the identification of key molecular events and pathways involved in adverse outcomes (Heijne et al. 2005; Sturla et al. 2014).

In summary, the current status of alternative approaches to animal testing in the field of toxicity assessment is promising and rapidly accelerating. The use of in vitro assays, computational models, and non-animal models showcases the diverse range of strategies being explored. These approaches have the potential to improve the efficiency, reliability, and ethical considerations of toxicity testing. However, further research is necessary to address their limitations and areas of uncertainty. It is crucial to conduct validation processes to substantiate their ability to truly replace traditional animal testing. These efforts are essential for enhancing chemical safety assessment and reducing the reliance on animal use in both research and regulatory practices (Schmeisser et al. 2023).

2.4 Avian Early-Life Stage (ELS) Toxicity Testing

Avian ELS toxicity testing for modern regulatory toxicology applications, established by Farhat and colleagues from Environment and Climate Change Canada, has gained recognition as a valuable alternative to traditional avian testing methods (Farhat et al. 2020). Numerous studies have demonstrated its efficacy in assessing chemical toxicity and its potential to provide comprehensive information on molecular effects and toxicity pathways (Desforges et al., 2021; Legrand et al. 2022; Franci et al; 2018; Farhat et al. 2020). This testing approach is cost-effective, accessible to a wider research community, and aligned with modern regulatory objectives (Farhat et al. 2020). Unlike

adult exposures that require specialized facilities, conducting experiments on avian embryonic development only necessitates a standard poultry incubator and basic laboratory supplies. Additionally, the well-documented avian embryonic development in model species such as Japanese quail (*Coturnix japonica*) and chicken (*Gallus gallus*), along with their fully annotated genomes, allow for complementary genomic analyses (Farhat et al. 2020).The integration of transcriptomics, proteomics, metabolomics, and exposure data in avian ELS testing facilitates a more comprehensive understanding of the mechanisms and modes of action underlying toxicity, extending beyond focusing solely on apical, phenotypic outcomes. (Desforges et al. 2021; Basu 2019).

Although Avian ELS toxicity testing has been identified as a promising alternative to traditional avian toxicity (AT) testing, the concordance between avian ELS toxicity testing and traditional AT testing in terms of acute toxicity determination has not been well-established. It is crucial to understand the extent to which these two testing methods correspond to each other to validate the reliability and applicability of avian ELS toxicity testing in regulatory decision-making.

Furthermore, the current avian ELS approach has a significant limitation. It relies on manual candling, which involves visually inspecting each individual egg for mortality or survival. This process is time-consuming and raises concerns about the potential impact on human health due to prolonged working hours (Vargas, 2018). Moreover, eggs with dark shell pigmentation present challenges for accurate readings, and the subjective judgments of different individuals can further complicate the assessment process. Additionally, the candling process itself increases the risk of dropping or cracking eggshells. To overcome this limitation, the adoption of advanced equipment

capable of real-time assessment of embryonic survival or mortality, while simultaneously evaluating multiple embryos, becomes necessary.

In conclusion, the limitations and challenges associated with traditional toxicity testing strategies have led to a paradigm shift towards alternative testing methods. The current status of alternative testing methods shows promising advancements in the field of toxicity assessment. In vitro assays, computational models, and non-animal test approaches offer potential benefits such as cost-effectiveness, faster turnaround times, and a reduction in animal use (Schmeisser et al. 2023). However, the adoption of alternative methods has been limited to date. The primary obstacle is not the absence of viable methods but the need to establish scientific confidence in alternative methods for regulatory use: "fitness for purpose, technical characterization, data integrity and transparency, and independent review."(van der Zalm et al 2022) It is important to acknowledge that further research and validation are necessary to fully establish the scientific confidence of any propose alternative testing method. The field is still evolving, and challenges remain in terms of technological advancements, standardization, and the integration of alternative methods into regulatory frameworks (Schmeisser et al. 2023).

With continued research, alternative testing methods have the potential to revolutionize toxicity testing, leading to improved decision-making, reduced reliance on animal testing, and ultimately, better protection of human health and the environment (Schmeisser et al 2023).

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Preface to Chapter 3

In this chapter, I aimed to examine the correlation between apical data derived from avian early-life stage (ELS) toxicity tests that I performed myself with data from traditional avian toxicity (AT) testing as found in databases. My broad objective was to comprehensively analyze the relationship between these two testing methods regarding acute toxicity assessment.

I conducted avian ELS testing on six pesticides previously assessed using the traditional AT method. By comparing apical outcome measures from both methods, I was able to derive valuable insights into the applicability of ELS testing as an alternative approach to traditional avian acute toxicity assessment.

This chapter provides detailed information about the experimental procedures, data analysis, and results of our comparative study. It also discussed the implications of the observed correlations and the challenges associated with adopting avian ELS testing as an alternative method for avian acute toxicity assessment.

This chapter is authored by Yanan Zhang and coauthored by Niladri Basu, Jessica Head, Emily Boulanger, Doug Crump.

Yanan Zhang contributed to the study by designing experiments, performing experimental tasks, conducting statistical analysis, interpreting data, crafting the results and discussion, and writing the manuscript.

Dr. Niladri Basu made significant contributions to experimental design and edited the chapter. Jessica Head, Emily Boulanger, and Doug Crump contributed to the conception and design of the experiments. Emily Boulanger assisted in the experimental performances.

Chapter 3

3.Evaluating Avian Early-Life Stage Toxicity Testing as an Alternative to Traditional Avian Toxicity Testing: A comparative study of pesticides.

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3.1 Abstract

Traditional avian toxicology test approaches are time-consuming, expensive, and require many animals. As such, regulatory bodies are seeking New Approach Methods (NAMs) as alternative testing methods. The avian early-life stage (ELS) test using pre-hatched embryos has emerged as a promising alternative to traditional avian toxicity (AT) tests, though it has evaluated relatively few chemicals. In this study, I examined six pesticides (endrin, ethoprophos, carbofuran, trichlorfon, permethrin and glufosinate-ammonium; all were tested at 6-8 concentrations spanning 3-4 orders of magnitude from 0.01 to 320μg/g egg) using this method, and compared the derived LD50, LOEL, and BMD data against results from traditional AT testing housed in databases. The mortality in the controls was <10% suggesting that the method performed well. In general, there was no correlation between the two methods. Notably the rank-order toxicity varied with glufosinate-ammonium being most toxic in the ELS test and least toxic in the AT, with the opposite situation observed for endrin. The differences in toxicity observed between the two methods is not clear, though could be attributed to disparities in the metabolic and elimination processes of avian embryos versus young or adult birds, and perhaps physical-chemical properties of pesticides leading to varying uptake in the model systems. The study contributes to the ongoing

discussion on the technical performance of the avian ELS method as well as its domains of applicability and biological relevance.

Keywords: Japanese quail; Egg injection; Alternative toxicity testing; Early life stage; Median lethal dose; Lowest-observed-effect level; Benchmark dose

3.2 Introduction

Toxicity testing plays a critical role in chemical management programs worldwide. It is essential for assessing the potential risks associated with various chemicals, including pesticides, industrial compounds, and pharmaceuticals. Traditional animal testing has long been the cornerstone of toxicity assessment and regulatory decision-making (LaLone et al. 2021). It involves the use of live animals, typically rodents, fish, birds, and mammals, to determine the potential adverse effects of chemicals on various physiological systems. Despite its widespread use, the current status of traditional animal testing is marked by several challenges and limitations. These include significant cost implications, time-intensive procedures, and the substantial utilization of animals, as outlined in Table 1. (Krewski et al. 2010).

Table 1. Monetary cost (in USD), number of animals needed, and test duration (in weeks) of traditional (whole animal) test

Species	Test	Guideline	Monetary cost (USD)			No. of Animals	Time (weeks)	References
			Median	Minimum	Maximum			
Fish	Short term reproduction	OECD 229	104,922	46,400	150,800	72	8	Willett et al. 2011; OECD 2017
Fish	Sexual development	OECD234	161,820	127,600	185,600	350	12	OECD 2017
Fish	Lifecycle toxicity	EPA 850.1500	291,450	112,980	447,760	320	8	OECD 2017
Fish	Multi-generation	OECD 240/USEPA 890.2200	411,800	295,800	580,000	324	20	OECD 2017
Fish	Bioaccumulation	OECD 305	115,650	67,690	167,355	70	6	Bottini and Hartung 2009
Avian	Reproduction	OECD 206	116,000	116,000	116,000	120	24	OECD 2017
Avian	Multi-generation	EPA 890.2100	319,000	319,000	319,000	240	38	OECD 2017
Avian	Acute oral toxicity	OECD 223	120,000	120,000	120,000	70	6	Rovida and Hartung 2009
Amphibian	Metamorphosis	USEPA 890.1100 /OECD 231	87,000	58,000	111,360	320	5	Willett et al. 2011; OECD 2017
Amphibian	Growth and development	OECD 241	250,560	58,000	443,120	80	20	OECD 2017

OECD: Organization for Economic Cooperation and Development;
USEPA: United States Environmental Protection Agency

Due to the significant challenges associated with traditional testing, regulations are changing in many places worldwide. Notably, regulators in the USA, Europe and Canada have issued official directives to reduce animal testing and promote the development of alternative testing strategies. For example, in 2019, the US EPA signed a directive to "prioritize effort to reduce animal testing" and to "eliminate all mammal study requests and funding by 2035" (USEPA (United States Environmental Protection Agency)). Similarly, the European Commission issued a directive 2010/63/EU in 2010, which emphasizes the principle of the Three Rs: replace, reduce and refine the use of animals for scientific purposes (European Commission 2010). Furthermore, in 2021, Bill S-5 was proposed to amend the preamble of the Canadian Environmental Protection Act 1999 to recognize "the importance of promoting the development and timely incorporation of scientifically justified alternative methods and strategies in testing and assessing substances to reduce, refine or replace the use of vertebrate animals" (House of commons of Canada). Taken together, these changes indicate that the field of toxicity testing is undergoing a regulatory revolution and there is now palpable demand for New Approach Methods (NAMs) (van der Zalm et al 2022).

Avian models are an invaluable test species for the assessment of aquatic and terrestrial ecosystems health (Farhat et al. 2020). In the United States, the European Union and Canada, rigorous evaluation precedes the distribution, sale, and use of pesticides to ensure their compliance with safety standards aimed at protecting the environment. Mandatory data for pesticide evaluation includes toxicity assessments on avian species, encompassing short-term acute, subacute, reproductive, simulated field, and full-field studies. These studies are

systematically organized in a hierarchical or tiered structure that progresses from fundamental laboratory tests to applied field assessments. The outcomes of each tier of tests undergo comprehensive evaluation to ascertain the potential of the pesticide to induce harmful effects and to determine the necessity for further testing (U.S EPA 2023; EFSA 2023; CCME 1996). International test guidelines, such as OPPTS 850.2100, 850.2200, 850.2300, 850. 2500, OECD205, OECD206, and OECD223, recommend using juvenile or adult birds for acute, subacute, reproductive or field testing for chemicals. These assessments typically involve determining apical endpoints indicative of adverse outcomes, such as the lethal dose or concentration that kills 50% of the test organisms (LD50 or LC50, respectively), or benchmark measures such as the no observable effect concentration (NOEC) or lowest observed effect concentration (LOEC). However, recent conclusions from researchers suggest that subacute dietary studies may not always be necessary for most pesticides, as acute oral studies are sufficient for assessing avian risk in 99% of cases (Hilton et al. 2019; Bone et al. 2022). Traditional avian testing is costly, time-consuming, and of ethical concern (Farhat et al., 2020; Basu et al., 2019), especially when one considers that multiple tests are often required that cover different life stages or endpoints. Further, such studies may not provide a complete understanding of the mechanisms or modes of action underlying toxicity.

In comparison with traditional avian toxicity testing, alternative avian methods are hailed as being more efficient, cost-effective, and ethical (Figure 1). Avian early life stage (ELS) toxicity testing using pre-hatched embryos has emerged as a promising alternative to traditional avian studies (Farhat et al. 2020). Briefly, dosing solutions are injected into the air cells of fertilized avian eggs, which are then incubated in a controlled environment. At the end of incubation, the embryos are

evaluated for apical effects such as mortality, deformities, body weight, and tissues may be harvested for deeper biochemical, histological, or molecular analyses. The advantages of this approach are several-fold. First, the avian embryo test has demonstrated high technical performance, with control group survival rates consistently exceeding 90% (Farhat et al. 2020). Thish meet the cretiria for test validation required in the US EPA avian acute oral toxicity test and avian dietary toxicity test (US EPA 2012b; US EPA 2012c). Second, the test is simple to perform and requires minimal space and equipment. Furthermore, the test is initiated on embryo day (ED) 0 (prior to incubation) and terminated at ED 9 (or prior to –hatch), making it faster, less costly, and more ethical than traditional avian tests. Avian embryos may be considered a non-protected life stage as they are contained within the egg and depend on their yolk sac for sustenance (Farhat et at. 2020). As the Canadian Council on Animal Care does not regulate tests on avian embryos until hatching, an official animal care protocol is not required for this test (Desforges 2021). In spite of the prevailing interest in the avian Early-Life Stage (ELS) method, its application has, to date, been confined to a relatively limited selection of chemicals. To foster a more comprehensive understanding of the method's applicability, it is imperative to conduct additional research encompassing a broader spectrum of chemicals, each characterized by diverse physico-chemical properties, potencies, and mechanisms of action. Within this context, the primary objective of the current study was to examine the correlation between avian ELS toxicity testing and traditional avian toxicity (AT) testing. Specifically, I utilized the avian ELS method to evaluate the toxicity of six pesticides with a wide range of LD50 values determined using traditional avian testing. I then compared the toxicity rankings of these pesticides obtained via the ELS method with their LD50

rankings derived from traditional AT testing. By conducting this analysis, I aimed to determine the extent to which the results of the two testing methods corresponded with one another.

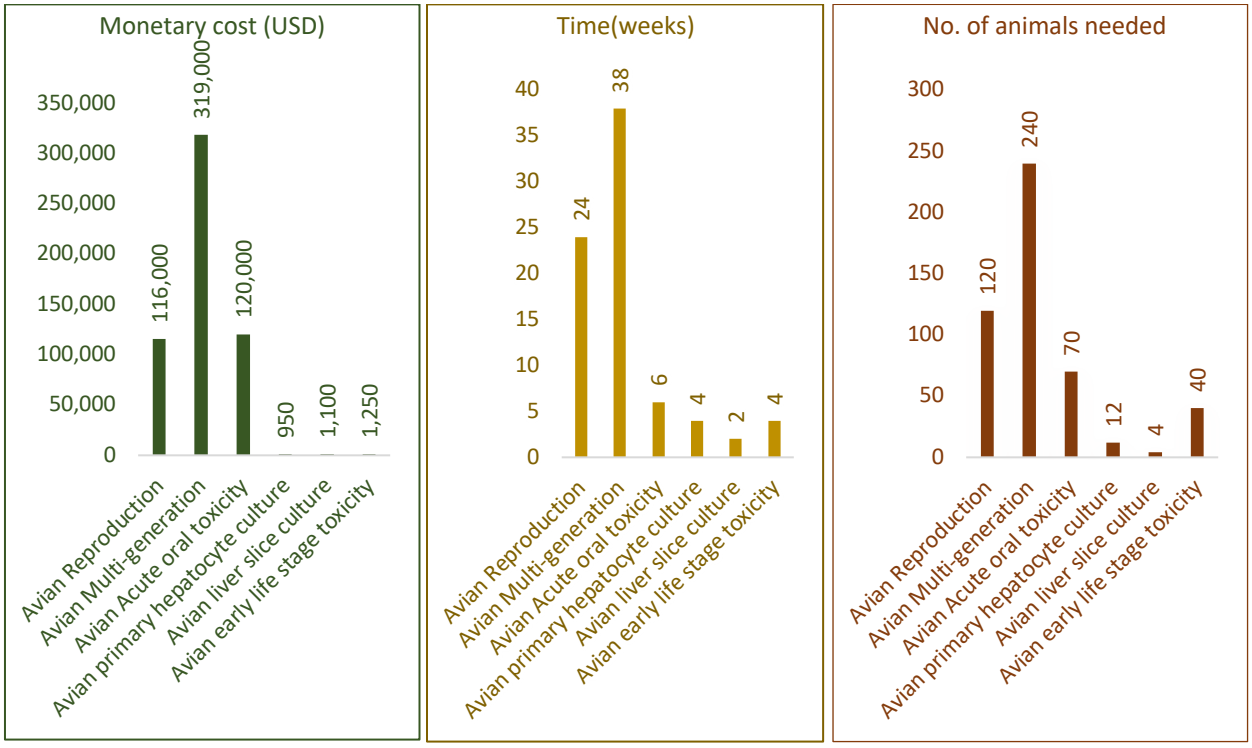

Figure 1. Monetary cost, test duration and number of animals needed of traditional (whole animal) tests versus alternative tests

3.3 Materials and methods

Chemicals and solutions

The pesticides selected for study were endrin, ethoprophos, carbofuran, trichlorfon, permethrin, and glufosinate-ammonium (Table 2). These pesticides were selected based on their wide range of LD50 values determined through traditional Japanese quail toxicity testing. The pesticides were ranked in order of toxicity based on their LD50 values from the traditional testing, with the

following order: endrin > ethoprophos > carbofuran > trichlorfon > permethrin, glufosinate-ammonium.

Chemical testing solutions were prepared using either dimethyl sulfoxide (DMSO) (Farhat et al. 2020) or pure water (Heinz et al. 2011; Heinz et al. 2012) and stored at -20°C until needed for egg injections. Details of the test solutions preparation can be found in the Supplemental Data, table S1-S7.

Japanese quail egg collection and egg injection

Fertilized, unincubated Japanese quail eggs were purchased from Ferme Patrick Brodeur (481 Rang St Jean Baptiste, Saint-François-du-Lac, QC J0G 1M0) and stored at room temperature for 4 to 6 days prior to incubation initiation (Franci et al. 2018).

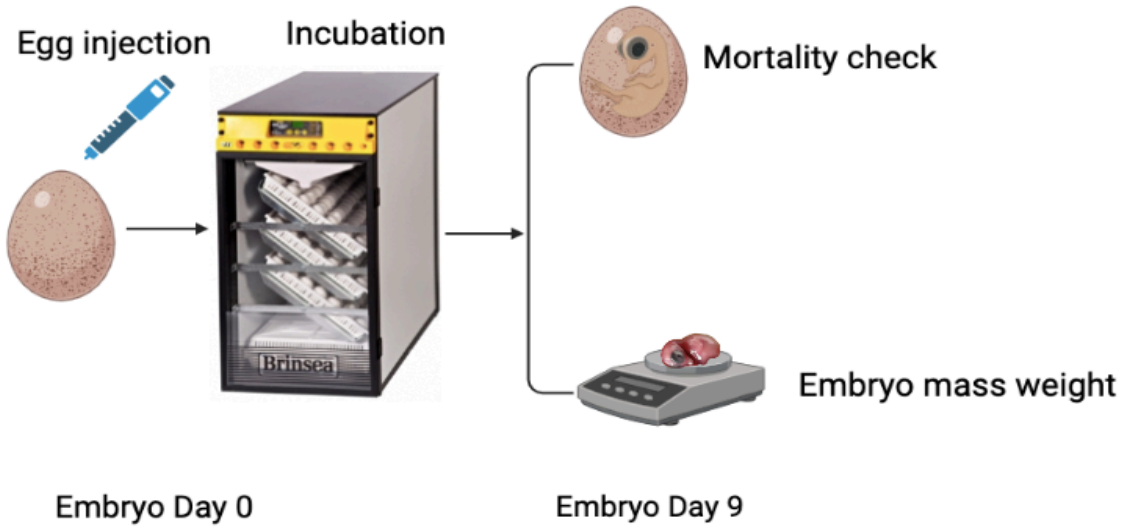

Figure 2. Schematic overview of the early-life stage exposure study

Table 2. The pesticides selected for study

Pesticide	CAS No.	Purity (%)	Vendor	Classification	Mode of action	Age	Exposure type	Observed period	LD50 (ppm)	Reference
endrin	72-20-8			organchlorines	non-competitive inhibition of γ-aminobutyric acid (GABA)	14days	food	8 days	18	Heath, R.G. et al., 1972
						2-3weeks	food	5 days	17	Hill, E.F. et al., 1986
ethoprop	13194-48-4	96.8	Sigma-aldrich	organophosphates	acetylcholinesterase (AChE) inhibitors, Nerve action	14 days	food	8 days	132	Hill,E.F. et al., 1981
						14 days	food	5 days	89	Hill,E.F. et al,. 1986
						7 days	food	8 days	71	U.S. EPA, 1992
						7 days	food	8 days	74	Hill,E.F. et al., 1981
						21 days	food	8 days	155	Hill,E.F. et al., 1981
						14 days	Oral via capsule	8 days	100	Hill,E.F. et al., 1975
carbofuran	1563-66-2	99.9	Sigma-aldrich	carbamate	acetylcholinesterase (AChE) inhibitors, Nerve action	14 days	food	8 days	746	Hill,E.F. et al., 1981
						21 days	food	8 days	1001	Hill,E.F. et al., 1981
						7 days	Food	8 days	592	Hill,E.F. et al., 1981
						14 days	Oral via capsule	8 days	438	Hill,E.F. et al., 1975
trichlorfon	52-68-6	99.9	Sigma-aldrich	phosphonate	acetylcholinesterase (AChE) inhibitors, Nerve action	12 days	food	8 days	1901	U.S. EP A, 1992
						12 days	food	5 days	1899	Hill,E.F. et al., 1986
						2-3weeks	Food	8 days	1901	Heath,R.G. et al., 1972
						12 days	Oral via capsule	8 days	1901	Hill,E.F. et al., 1975
permethrin	52645-53-1	99.5	Canadian life science	pyrethroid	sodium channel modulators, Nerve action	14 days	food	5 days	>5000	Hill,E.F. et ai., 1986
						-	food	8 days	>23000	U.S. EP A, 1992
						-	Oral via capsule	14 days	>20000	U.S. EP A, 1992
glufosinate-ammonium	77182-82-2	99.5	Sigma-aldrich	phosphinic acid	inhibition of glutamine synthetase	-	food	8 days	>5000	U.S. EPA, 1992
						-	Oral via capsule	14 days	>2000	U.S. EP A, 1992

On ED 0, eggs were candled to detect cracks, vascularization/development, and the air cell was visualized with a fiber optic lamp and marked at the same time. Cracked or vascularized eggs were not used. Next, eggs were weighed and randomly divided into nominal dose groups, including a solvent control (Table 3). An alcohol sterilized hand-held Dremel tool (with a 3/32" diamond tip bit) was used to make a small hole in the shell above the center of the air cell. The injection volume was 10 µl, assuming a 15-g average egg weight. The chemical solution was injected into the hole using an Eppendorf positive displacement repeater pipette, as described by Farhat et al. (2020). The air cell was checked to ensure that it was not punctured. If this occurred, the egg was replaced with another of equivalent size, and the substitution was recorded (Karen et al. 2016). The hole was then sealed with Air Pore Tape (~1.5 cm^2; Qiagen), and eggs were left upright at room temperature for approximately 1 h before being placed horizontally into an egg incubator (Brinsea Ova-Easy 190 Series II) set at 37.5±0.5ºC and 60 ± 5% humidity. The egg trays were automatically rotated (approximately 90º) every 2 h. The test was terminated at ED9 (Embryonic Day 9) (Figure 2).

Observation and tissue collection

At ED9, an evaluation of all embryos from every dose group was performed to identify infertile eggs or dead embryos. Any dead embryos were opened, staged, and evaluated for deformities, following the methodology described by Franci et al. (2018). Additionally, the weight of all viable embryos was measured.

Table 3. Test period, number of eggs used, dose levels, and solvent used in the study

Pesticide	Test period	Total No. of eggs	No. of eggs/dose	Dose level (µg/g egg)	solvent
endrin	13 Feb -Jul 6, 2023	96	10-13	0.1, 0.32, 1.0, 3.2, 10, 32, 100	DMSO
ethoprophos	27 Jun-Jul 6, 2022	85	10-11	0.1, 0.32, 1.0, 3.2, 10, 32, 100	DMSO
carbofuran	11 Jul-Jul 20, 2022	82	10-11	0.1, 0.32, 1.0, 3.2, 10, 32, 100	DMSO
trichlorfon	Jul 18-Jul 27, 2022	81	10-11	0.1, 0.32, 1.0, 3.2, 10, 32, 100	DMSO
permethrin	Oct 24-Nov 2, 2022	98	10-12	0.1, 0.32, 1.0, 3.2, 10, 32, 100, 320	DMSO
glufosinate-ammonium	Aug 1 -Aug 10, 2022	85	10-12	0.1, 0.32, 1.0, 3.2, 10, 32, 100	autoclave pure water
glufosinate-ammonium	Oct 24-Nov 2, 2022	109	9-12	0.01, 0.032, 0.1, 0.32, 1.0, 3.2, 10, 32, 100	autoclave pure water

Data analysis

In the study, embryo mortality rate was calculated as the number of dead embryos at the end of the test divided by the number of fertile, injected eggs. An egg was classified as infertile if no visible development was observed after incubation (Crump et al. 2021). To perform the calculation of the Media lethal dose (LD50), where possible, the statistical software SPSS Statistics version 29.0.0.0 was utilized. Furthermore, the lowest observed effect level (LOEL) was determined using a one-way ANOVA with a significance level of $p < 0.05$, followed by Dunnett's test with a significance level of $p < 0.05$, as described in Franci et al. (2018) utilizing SAS ® Proprietary Software 9.4. Finally, the Benchmark Dose (BMD), based on growth, was calculated using the U.S. EPA BMDS (Benchmark Dose Software) 3.3.

3.4 Results

Mortality

To gauge the quality of the study, the test would be considered unacceptable or invalid if the mortality in the solvent control exceeded 10% during the test (US EPA 2012b; US EPA 2012c). Here, across all six chemicals exposure studies, the mortality in the solvent control group at ED9 was less than 10% (see Figure 3). No abnormal mortality was observed in embryos exposed to endrin, ethoprophos, carbofuran, trichlorfon, or permethrin up to the highest test concentration. Thus, I concluded that the ED9 LD50s for endrin, ethoprophos, carbofuran, and trichlorfon were >100 µg/g egg, and that the LD50 for permethrin was >320 µg/g egg (see Table 4). I did not proceed with testing higher doses of these chemicals given the relatively high price of the pesticides along with a recognition that the upper values were already several orders of magnitude higher than environmentally relevant levels (Table 5).

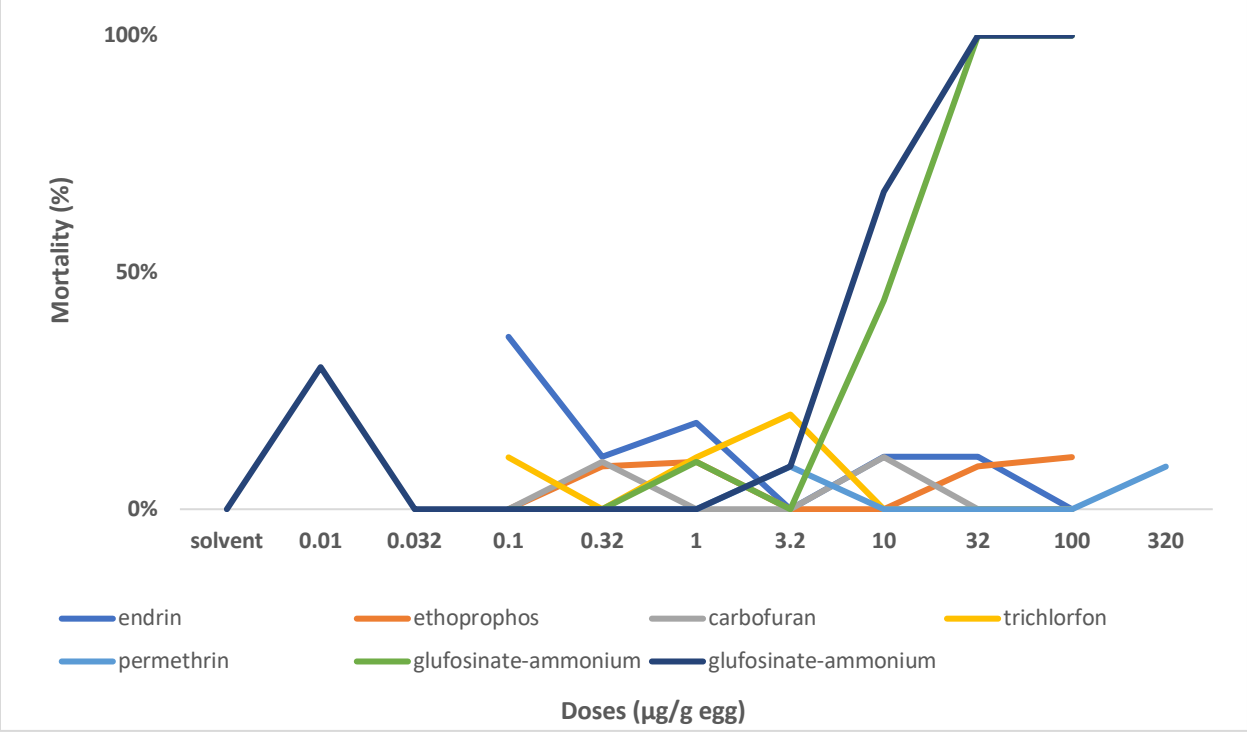

Figure 3. Mortality rate curves for the pesticides at different doses of exposure. See Supplemental data, Table S7-S12

Table 4. Mortality rate for the pesticides at different doses of exposure. See Supplemental data, Table S7-S12

pesticide	Mortality rate (%)											LD50 (µg/g egg)	95% confidence limits (µg/g egg)
	solvent	0.01	0.032	0.1	0.32	1	3.2	10	32	100	320		
					(µg/g egg)								
endrin	8%	-	-	36%	11%	18%	0%	11%	11%	0%	-	> 100	-
ethoprophos	9%	-	-	0%	9%	10%	0%	0%	9%	11%	-	> 100	-
carbofuran	0%	-	-	0%	10%	0%	0%	11%	0%	0%	-	> 100	-
trichlorfon	0%	-	-	11%	0%	11%	20%	0%	0%	0%	-	> 320	-
permethrin	0%	-	-	0%	0%	0%	9%	0%	0%	0%	9%	> 100	-
Glufosinate-ammonium	9%	-	-	0%	0%	10%	0%	44%	100%	100%	-	8.76	5.03-16.30
Glufosinate-ammonium	0%	30%	0%	0%	0%	0%	9%	67%	100%	100%	-	7.38	4.74- 12.31

Table 5. Environmental relevant concentrations of the pesticides

pesticide	Environmental related concentrations			Reference
	In eggs of bird (µg/g)	In bird (mg/kg)	In soil (mg/kg dry weight)	
Endrin	0.06-1.60 0.05-1.31	0.10-0.86 0.13-1.42	0.02-1.00 0.016-0.629	IPCS 1992
ethoprophos	-	0.28-5.191	Below the detection limit-0.162	1. EFSA 2013 2. Al-Mughrabi et al. 2002
carbofuran	Not detected1	0.641	0.010-1.0092 Below the detection limit-1.53	1. Blumton etal. 1990 2. Otieno et al. 2010 3. Miles et al. 1979
trichlorfon	0.01-0.05 0.27-0.48	0.01-1.5	0.24-0.48 0.49-1.03 0.002-0.02 0.002-0.17	IPCS 1992
Permethrin	0.003-0.3281	-	0.062-1.1782 0.011-4223	1. Parente et al. 2017 2. Thapinta et al. 2000 3. NCBI 2023
Glufosinate-ammonium	Not detected	Not detected	-	EFSA 2005

In this study, glufosinate-ammonium was the only pesticide for which LD50 was determined. To confirm the initial findings, this pesticide underwent two independent tests (Aug 2022 and Oct 2022). Both tests demonstrated a significant increase in mortality of bird embryos exposed to the dose of 10 μg/g egg, and all bird embryos died at doses of 32 and 100 μg/g egg (Figure 3). These results indicated that glufosinate-ammonium had a high level of toxicity in Japanese quail embryos. In consideration of the observed dose-response relationship concerning embryo mortality, statistical analysis was conducted using SPSS Statistics version 29.0.0.0. The LD50 was calculated to be 8.76 μg/g egg, with 95% confidence limits ranging from 5.03-16.30 μg/g egg in study 1. In study 2, the LD50 was computed as 7.38 μg/g egg, with 95% confidence limits spanning from 4.74-12.31μg/g egg. Notably, the LD50 values derived from the two testing instances exhibited a remarkable similarity. The mean LD50 across both studies was determined to be 8.07 μg/g egg. Furthermore, the investigation showcased the reproducibility of the avian ELS test, confirming the consistent and reliable outcomes in both study contexts. A comprehensive presentation of bird embryo mortality resulting from pesticide exposure is available in Table S8-S14 of the Supplemental Data. From the aggregate data, pesticides were ranked in order of toxicity based on LD50 values as follows: Glufosinate-ammonium (8.07 μg/g egg) > Ethoprophos, Carbofuran, Trichlorfon, Endrin, Permethrin (>100 or 320 μg/g egg).

Growth

In addition to evaluating embryo mortality, I also assessed the adverse effects of the pesticides on the growth of Japanese quail embryos by measuring the embryo mass at ED9. The lowest observed effect level (LOEL) for each pesticide was determined by comparing the embryo mass between the control group and each treatment group. The LOEL value was defined as the lowest dose level of

the pesticide that resulted in a statistically significant reduction in embryo mass compared to the control group. The test results revealed that the LOEL values varied among the pesticides.

SAS software version 9.4 was employed for conducting significance testing. The findings indicated that Ethoprophos significantly decreased embryo mass only at the highest dose group of 100µg/g egg compared to the solvent control ($p < 0.05$), with a LOEL of 100 µg/g egg. Endrin, carbofuran, and trichlorfon did not cause any statistically significant decrease in embryo mass when compared to the controls ($p > 0.05$), and their corresponding LOEL values were greater than the highest test concentration of 100 µg/g egg. In the case of Permethrin exposure, a significant decrease in embryo weight was observed only at the 100 µg/g egg dose group in comparison to the control ($p<0.05$). However, no dose-response relationship was observed, leading to the calculation of a LOEL greater than the maximum test dose (>320 µg/g egg). Conversely, Glufosinate-ammonium demonstrated a significant decrease in embryo mass at the dose of 3.2 µg/g egg in two repeated test. Notably, a dose-response relationship was observed in both tests, with the LOEL value consistently determined as 3.2 µg/g egg. The embryo mass data at ED9 for each pesticide and their respective control groups is presented in Figure 4. Additional details regarding embryo mass in each testing can be found in Supplemental data S15-S21. For an in-depth understanding of the significance testing procedures, refer to Supplemental data S22-S28.

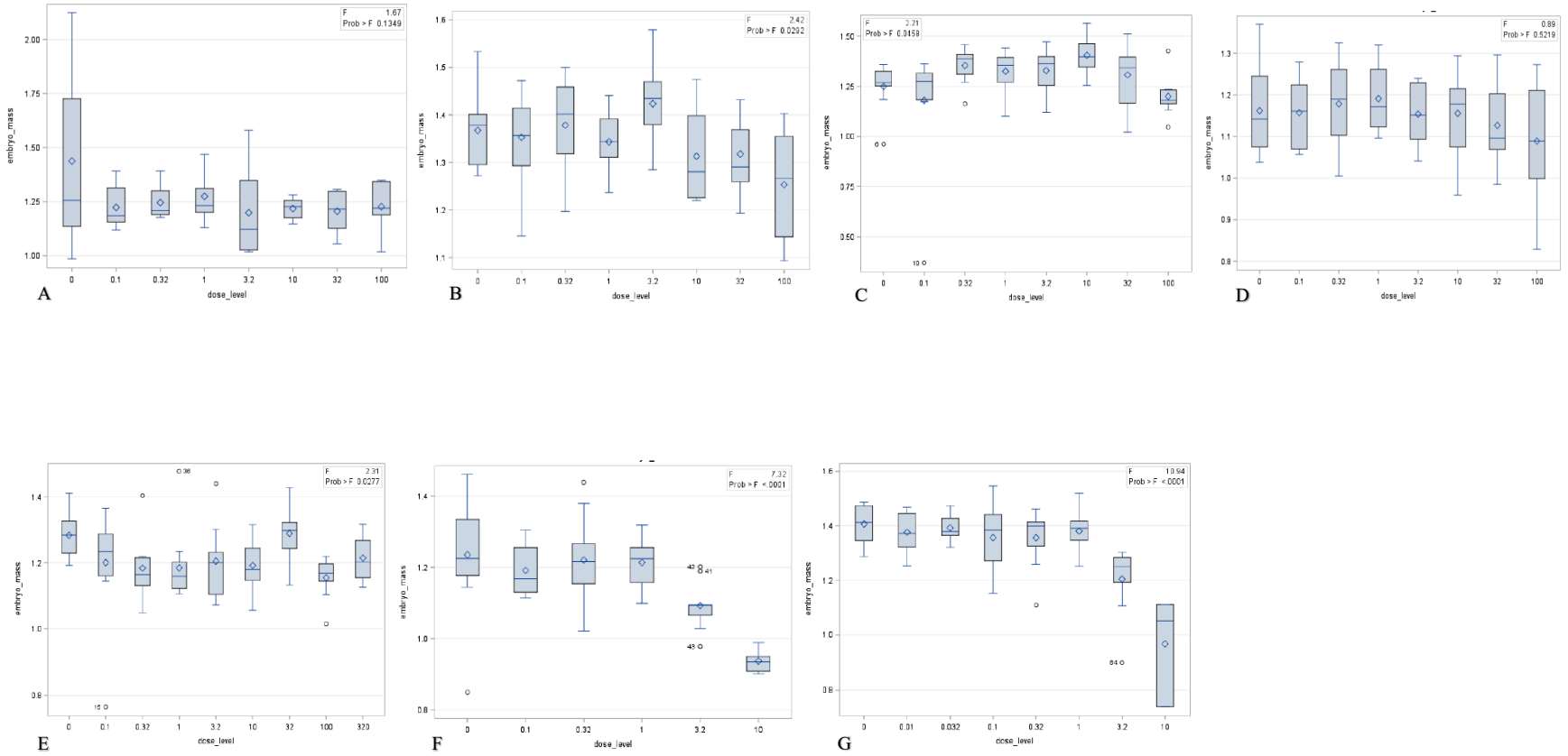

Figure 4. Japanese quail embryos mass at ED9 for each pesticide exposure. A: endrin; B: ethoprophos; C: carbofuran; D: trichlorfon; E: permethrin; F: glufosinate-ammonium test 1; G: glufosinate-ammonium test 2 (See Supplemental data, Table S15-S21)

Based on the LOEL values concerning embryonic weight, the toxicity of the pesticides was ranked as glufosinate-ammonium (3.2 µg/g egg) > ethoprophos (100 µg/g egg) > carbofuran, trichlorfon, endrin, permethrin (>100 or 320 µg/g egg).

For each of the test chemicals benchmark dose modelling was applied to calculate the concentration at which embryonic growth deviated from background measures. In doing so, the toxicity rank order based on BMD values was glufosinate-ammonium (averaged 2.8 µg/g egg) > Ethoprophos (63.0 µg/g egg) > carbofuran (98.1 µg/g egg) > trichlorfon （171.9 µg/g egg） > endrin (458.9 µg/g egg) > permethrin (2640.9 µg/g egg).

3.5 Discussion

The purpose of this study was to objective of Aim #1 was to evaluate the suitability of avian ELS testing as an alternative to traditional AT testing for acute toxicity evaluation. This was addressed by exposing Japanese quail embryos to six pesticides with diverse potencies and comparing the results from this ELS test with from traditional AT testing. The findings help increase understanding of the relationship between ELS and traditional AT testing thus contributing to ongoing discussions concerning the applicability domain of ELS testing as an alternative approach to traditional avian acute toxicity assessment, as well as its technical performance.

Increasing scientific confidence in a NAM requires technical characterization in order to demonstrate that the method is of high quality (van der Zalm et al 2022). In the current study I demonstrate that the method is reproducible. Specifically, glufosinate-ammonium was tested on

two different occasions and yielded the same LD50 value (8.76µg/g and 7.38µg/g). Another measure of technical performance may consider the negative or solvent controls. The avian acute oral toxicity method (US EPA 2100) and the avian dietary toxicity test method (US EPA 2200) indicate that the mortality in the controls should not exceed 10%. In the current study, in which 6 different pesticides were evaluated, the mortality in the control never exceeded this threshold. In 4 of the studies there was no mortality, and in the remaining studies the mortality ranged from 8 to 9 %.

Another technical performance criterion is accuracy, or how closely do the results from the method align with reference values. In this case, how do the apical results from the ELS test compare with traditional avian toxicity (AT) testing. In my study there was no concordance in apical measures between the two test methods. First off, for five of the pesticides tested in the ELS, a LD50 value was not determined up to a maximal concentration of 100 or 320ug/g. Higher concentrations were not tested due to concerns over environmental relevancy and cost, as discussed earlier. Second, I found diametrically different toxicity values for glufosinate-ammonium and endrin between the two test methods. Specifically, in the avian ELS test, glufosinate-ammonium was found to be the most toxic pesticide among the six tested, while it exhibited the lowest toxicity in the traditional AT test. On the other hand, endrin was found to be the most toxic pesticide in the traditional AT test, but not toxic in the ELS test up to the dose level of 100 µg/g egg. Third, to move beyond LD50 values, I compared the LOEL and BMD values from avian ELS testing with the LD50 values from traditional AT testing. Although this comparison was not direct, similar differences between the two testing methods were observed, including glufosinate-ammonium and endrin. Beyond these two exceptions, the toxicity rankings of ethoprophos,

carbofuran, trichlorfon, and permethrin were generally consistent across the two testing methods by comparing the BMD from avian ELS testing and LD50 values from traditional AT testing.

The difference in toxicity of endrin observed between the two methods may be attributed to differences in metabolic pathways between adult birds and bird embryos. Adult birds have fully developed organs and metabolic pathways, which increase their susceptibility to the toxic effects of endrin as compared to developing embryos, which have not yet fully established these processes. Endrin undergoes oxidation of the methylene bridge to anti- and syn-12-hydroxyendrin, followed by dehydrogenation to 12-ketoendrin, which is a more toxic metabolite than the parent compound. This metabolic pathway is mediated by liver microsomal mono-oxygenases found across all vertebrates (US EPA 1987; Benn et al. 2005). According to the toxicological profile for endrin (Agency for Toxic Substances and Disease Registry (US) 2021), 12-ketoendrin is likely the primary toxic entity of endrin. The efficient oxidation pathways in adult birds result in rapid conversion of endrin to 12-ketoendrin, leading to a more severe toxic response. In contrast, the less developed metabolic processes in developing embryos may result in less efficient metabolism of endrin in embryonic tissues and a less severe toxic response.

It is also possible that the adverse effects of endrin to avian embryos may have been realized if the embryos were allowed to develop beyond ED9. As embryonic tissues become more differentiated and their metabolic processes become more active, there may be an enhanced metabolism of endrin, which could generate metabolites such as 12-ketoendrin. Therefore, it is important to consider the developmental stage of the embryos when assessing the toxicity of endrin and other pesticides to avian species.

The mode of action underlying the toxicity of glufosinate-ammonium to avian embryos is likely mediated by the inhibition of glutamine synthetase activity. Glutamine synthetase plays a crucial role in the metabolism of glutamine, an essential amino acid involved in cellular processes such as protein and aminoacid synthesis (Castegna et al. 2018). Injection of glufosinate-ammonium into fertilized bird eggs may lead to the inhibition of glutamine synthetase activity, resulting in a depletion of glutamine within the cells. This depletion can interfere with normal cellular processes that are essential for embryo growth and development, including cell division and differentiation, potentially resulting in abnormal development or embryo death (Donthi 2022; Schulte-Hermann et al. 2006). It is important to note that the toxic effects of glufosinate-ammonium on developing embryos may involve additional pathways beyond glutamine synthetase inhibition. Nonetheless, this inhibition is a significant factor to consider when evaluating the potential harm of glufosinate-ammonium to developing embryos (Watanabe et al. 1996; EFSA, 2005). However, our findings from the egg injection test contradict the results of traditional avian toxicity tests, which have generally found glufosinate-ammonium to be of low toxicity to birds when administered orally or through dietary routes. For example, an acute oral toxicity test on Japanese quail showed that both males and females had median lethal doses (LD50) greater than 2000 mg/kg body weight, and the 8-day dietary LC50 for bobwhite quail chicks was approximately 5000 mg/kg diet. A long-term reproduction toxicity test conducted on bobwhite quail did not demonstrate any adverse effects of glufosinate-ammonium at 400 ppm (40 mg/kg bw/day) on reproduction, according to experimental data from the European Chemicals Agency (ECHA 2017) website for chemical REACH registration. This variance in toxicokinetics may explain why glufosinate-ammonium displayed low toxicity in traditional bird testing but high toxicity in egg injection testing.

One significant distinction between our study and those mentioned above concerns life stage. In our study, glufosinate-ammonium was injected into embryonic eggs, whereas traditional studies exposed young or adult birds to glufosinate-ammonium orally or through dietary means. Previous studies have investigated the toxicokinetics of glufosinate-ammonium in rats, dogs, livestock (goats and hens), and found that the pesticide is rapidly excreted in all test species regardless of the route of administration (FAO/WHO, 1991; USEPA, 1999). For instance, in the aforementioned species, within 48 hours of oral exposure, approximately 80-90% of the administered dose of glufosinate-ammonium was excreted unchanged in the faces, while about 10-15% was eliminated via urine (FAO/WHO, 1991). Consequently, the low toxicity observed in traditional bird testing may be attributed to the primary mode of excretion of glufosinate-ammonium via the kidneys of birds, resulting in elimination through feces and urine. Nevertheless, when glufosinate-ammonium is administered via embryonic egg injection, the pesticide is unable to be effectively eliminated from the embryo due to its metabolic system and lack of a functional excretory system, possibly resulting in higher toxicity. Thus, the results of our study emphasize the importance of considering the timing of exposure or life stage of exposure when determining the toxicity of pesticides in avian species.

Besides accounting for the variations in metabolic and elimination processes between embryos and adult birds, the disparity in toxicity observed between the two methods may also be attributed to the impact of physical-chemical properties of pesticides on their uptake through the air sac. In our study, I separately injected solutions of six pesticides at a concentration of 100 μg/g egg and a DMSO solvent into the air sac of the Japanese quail embryo. The injection volume in each egg was 10 μl. I found that ethoprophos, trichlorfon, permethrin, and glufosinate-ammonium were

effectively absorbed by the eggshell membrane, while endrin and carbofuran showed incomplete absorption (Figure 5). When a pesticide remains in the air sac after injection, it indicates inadequate uptake, leading to an incomplete manifestation of its toxicity. This issue of incomplete uptake is not present in traditional acute toxicity (AT) testing, which typically involves oral or dietary administration of pesticides. As a result, the disparity in exposure routes contributes to variations in pesticide uptake, ultimately leading to differences in toxicity between avian ELS testing and traditional AT testing. Along these lines, and coupled with some arguments earlier, one limitation of our study was that I did not measure the resulting levels of these pesticides in the embryo. Multiple laboratories in academia and the private sector were contracted, but unfortunately none were equipped with the methods or resources to accommodate this request.

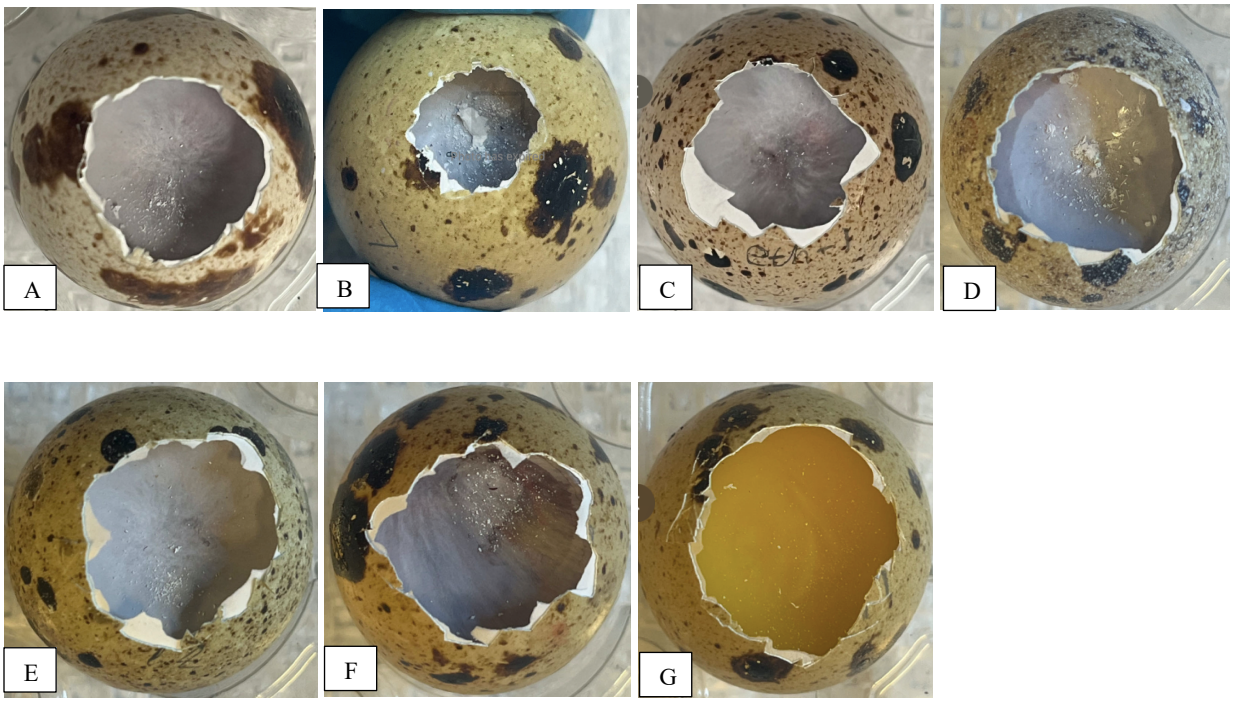

Figure 5. Pesticides (100 µg/g egg dose solution) uptake at ED9. A: DMSO control; B: endrin; C: ethoprophos; D: carbofuran; E: trichlorfon; F: permethrin; G: glufosinate-ammonium

3.6 Conclusion

Our study highlights significant differences in toxicity rankings for certain pesticides between avian ELS testing and traditional AT testing. Notably, glufosinate-ammonium and endrin exhibited the most substantial discrepancies in toxicity between the two methods. Although the toxicity rankings of ethoprophos, carbofuran, trichlorfon, and permethrin were generally consistent between the two testing methods, it is essential to acknowledge that the comparison between them was not direct due to the lack of exact LD50 values for some of the pesticides tested. This limitation could potentially affect the accuracy of our conclusions and the ability to broadly use the avian ELS method to study chemicals if indeed the goal is to replicate data one may expect from traditional tests.

Our findings suggest that a strong correlation may not exist between avian ELS and traditional AT testing methods. The primary reason for the observed differences in toxicity between these methods is the disparity in the life stage and the route of exposure. When a pesticide's toxicity is highly dependent on the metabolic and elimination processes of the test organism, the toxicity observed in the two methods may vary due to the significant differences in metabolite and elimination capacity of embryos versus more developed avian life stages. Furthermore, the physical-chemical properties of pesticides, which affect their uptake through the avian air sac, significantly contribute to the differences in toxicity between avian ELS and traditional AT testing methods.

While our study provides initial insights into the concordance between the avian ELS testing and traditional AT testing, further research could focus on expanding the number of pesticides tested

to increase understanding of the application domains for the avian ELS method (in terms of it potentially serving as a replacement test). In the current study there was some agreement for 4 of the chemicals, though complete opposite results for two chemicals. In the two exceptions, it is essential to conduct further investigations to verify the toxicity mechanisms of glufosinate-ammonium and endrin in both the avian ELS test and traditional AT test. This will provide a better understanding of the comparability of the two methods and help identify any factors that may influence the results. Moreover, more extensive research is needed to explore the uptake of chemicals through the avian air sac and the various factors that affect this uptake, including measuring the chemical. These are some areas that require further research and exploration without which I cannot gain a complete understanding of the applicability domain of avian ELS testing, along with biological relevance and technical performance.

There needs to be consideration of the ELS method, and if it is intended to replace traditional AT testing or serve in some other complementary manner. Notably, since oral/dietary exposure is more representative of the way birds are exposed to pesticides under actual use conditions than egg injection, the risk assessment for endrin and glufosinate-ammonium to birds should still favour data from oral/dietary exposure studies as these are more relevant. Egg injection experiments, in contrast, serve as a new method that does not use “animals”, require much fewer resources (including time and consumables), and focus on a sensitive life stage. It is relevant to mention that our study had some limitations. For example, I was not able to definitively calculate LD50 values of five pesticides using the avian ELS toxicity test due to the prohibitive cost of the pesticides. Also, I did not measure the concentrations of pesticides in the test solutions or bird tissues, which limits the completeness of our study. However, I believe that these limitations do not affect the scientific

validity of the study or our ability to draw relevant conclusions. I followed a detailed study plan, and all test operations were carried out according to strict standard operating procedures. Moreover, the low mortality rate of bird embryos in the control group in all seven batches of tests and the consistent results obtained from repeated tests with glufosinate-ammonium support the technical performance of our study. Nonetheless, future studies may seek to address these limitations to enhance the completeness and comprehensiveness of the findings.

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Preface to Chapter 4

In this chapter, the focus is on advancing avian early-life stage (ELS) testing methodology by exploring real-time, simultaneous assessment of embryonic survival, specifically through the utilization of piezoelectric sensors to monitor embryonic heartbeat.

Whithin this exploration, two types of piezoelectric sensors were meticulously selected and tested for accuracy and suitability in detecting embryonic heartbeat. However, the findings of this study revealed that the chosen piezoelectric sensors did not yield the anticipated signals from the embryos.

This chapter provided a detailed exploration of the motivations, methods, and outcomes of the study. It emphasized the significance of continuous research and innovation to improve the reliability and effectiveness of heartbeat detection in avian ELS toxicity testing. This research laid a foundation for future studies in this critical domain.

This chapter is authored by Yanan Zhang and coauthored by Niladri Basu.

Yanan Zhang contributed to the study by designing experiments, performing experimental tasks, crafting the results and discussion, and writing the chapter.

Dr. Niladri Basu made significant contributions to experimental design and edited the chapter.

Chapter 4

4. Advancing Embryonic Survival Detection Method for Avian Early-Life Stage Toxicity Testing

4.1 Abstract

The avian early-life stage (ELS) testing method shows promise as a New Approach Method (NAM) to assess the potential toxicity of substances on avian species. However, the manual candling process used for mortality assessment has limitations in terms of time required, accuracy in measurement, and risks associated with handling fragile eggshells. To address these limitations, this study aimed to advance the avian ELS testing method by evaluating different options for real-time simultaneous assessment of embryonic survival. The focus was on utilizing piezoelectric sensors to monitor embryonic heartbeat. Two types of piezoelectric sensors were selected and tested for their accuracy and suitability in detecting embryonic heartbeat. The results revealed that the selected piezoelectric sensors used did not provide the expected signals from the embryos, indicating the need for more research in this area. This study highlighted the importance of continuous research and innovation in improving the reliability and effectiveness of heartbeat detection in avian ELS toxicity testing.

4.2 Introduction

The avian early-life stage (ELS) testing method shows promise as an alternative to traditional avian toxicity tests. It plays a crucial role in assessing the potential toxicity of substances at both the molecular level and apical level. However, the current approach has notable limitations. The manual candling process, which involves visually inspecting the mortality or survival of each

individual egg, is time-consuming and raises concerns regarding prolonged working hours impacting human health (Vargas, 2018). Additionally, eggs with dark shell pigmentation pose challenges for accurate readings, and subjective judgements of different researchers may further complicate the assessment process. Furthermore, the candling process itself may elevate the risk of dropping or cracking eggshells. Identifying and removing dead eggs from the incubator is essential to eliminate possible sources of contamination of other healthy eggs and to maintain optimal incubation conditions (Abd El-Hack et al 2022).

To address these limitations, I proposed to investigate whether advanced equipment capable of assessing embryonic survival or mortality in real-time and simultaneously evaluating multiple embryos. This approach drawed inspiration from methods employed in medicine and physiology, where researchers have developed various techniques to assess embryonic heart rate (HR) using equipment such as electrocardiograms (ECG), impedance-cardiograms (ICG), ballistocardiograms (BCG), acoustocardiograms (ACG), photoplethysmography (PPG), pulse oximetry or detecting blood pressure of the allantoic artery (Akiyama et al., 1999; Suzuki et al., 1989; Akiyama et al., 1997; Rahn et al., 1990; Wang et al., 1990; Youssef et al., 2020; Cain et al., 1967; Tazawa et al., 1989). However, to my knowledge, these methods have not been thoroughly tested in the context of avian toxicity testing.

In this study, I aimed to improve the method of detecting embryonic mortality or survival in avian ELS testing. The specific objective was to assess various equipment options and determine the most efficient and accurate device for long-term, continuous, and routine evaluation of embryonic

survival status. By improving the detection method, it becomes possible to achieve real-time and simultaneous assessment of multiple embryos, thereby advancing avian ELS toxicity testing.

I hypothesized that the equipment employed for detecting embryonic survival or mortality will exhibit an accuracy rate exceeding 95%. This hypothesis drives the evaluation and selection of the most suitable equipment for avian ELS toxicity testing.

4.3 Materials and Methods

Equipment Selection

Based on literature review, three primary types of technologies for embryo heartbeat checking were identified: invasive, semi-invasive and non-invasive methods. Invasive and semi-invasive methods involve needle penetration of the eggshell or placement of the electrode on the shell membrane after a partial removal of the eggshell (Suzuki et al. 1989; Akiyama et al. 1997; Akiyama et al. 1999). Since our avian ELS toxicity testing requires long-term, continuous measurement of survival or mortality of the avian embryo, I aimed to minimize interruptions during the testing, except for injecting the test solution into the air cell. Therefore, the selected method should be low risk and easy to perform. As such, my priority was to select a non-invasive method. From my research, there are three non-invasive detection technologies for checking embryonic heartbeats : ballistocardiogram (BCG), acoustocardiogram (ACG), and photoplethysmography (PPG). The acoustocardiogram (ACG) utilizes the effect of pulsatile air movement (acoustic pressure changes) across the eggshell, which is detected by a microphone (Rahn et al. 1990) or a differential pressure transducer (Wang et al. 1990) attached hermetically to the eggshell. To minimize the ambient

acoustic noises, the egg sample must be placed in a tightly sealed vibration-free container. Therefore, the ACG method does not meet my requirement for real-time and continuous assessment of embryos. Similarly, PPG is based on the reflection of light and the detection of blood volume changes in the microvascular bed of tissue through red and infrared lights (Youssef et al. 2020; Tamura et al. 2014; Khaliduzzaman et al. 2019; Phuphanin et al. 2019) as an indicator of embryonic survival. Commercially available equipment to achieve this includes small devices designed to detect one egg at a time (Lierz et al. 2006; Pollard et al. 2016), or large-scale equipment used in vaccine production or poultry farming industries (Yu et al. 2019), neither of which does not meet my requirement for simultaneously evaluating multiple embryos in real time.

Finally, the BCG method was selected. It is based on detecting slight movements or vibrations of the eggshell caused by contraction of the embryonic heart (Youssef et al. 2020). Various techniques can be employed to detect these movement, such as using a piezoelectric sensor (Cain et al. 1967), an optical method using laser interference (Tazawa et al. 1989), or a phonograph cartridge (Suzuki et al. 1989; Pearson et al. 1999; Pearson et al. 1998). However, the optical method using laser interference and the phonograph cartridge are not suitable for detecting avian embryo heartbeat within the incubator in real time, which is a requirement for our testing purposes. Therefore, a piezoelectric sensor was chosen and tested to evaluate its accuracy and suitability for detecting embryo heartbeat in avian ELS toxicity testing. The sensor provides a convenient method for detecting avian embryo heartbeat by simply attaching it to the eggshell, capturing the signal, amplifying it using an amplifier charger, and then outputting the signal through an oscilloscope (Pearson et al. 1999, Tazawa et al. 1993). In our study, two different piezoelectric sensors were tested. The first sensor was a piezoelectric PVDF sensor with an active

film thickness 45µm and an active film area of 10mm x 50mm), which was purchased from PolyK Technologies, LLC. The second sensor was an SDT1-028K shielded piezo sensor with an active film thickness of 130µm and an active film area of 11.2mm x 28.6mm, which was purchased from TE Connectivity company.

Test Procedure

Ten Japanese quail eggs were horizontally placed in a poultry incubator set at a temperature of 37.5±0.5ºC and a humidity of 60±5% on embryonic day (ED) 0. The egg trays were automatically rotated approximately 90º every 2 hours to ensure uniform incubation conditions (Farhat et al., 2020). The two different piezoelectric sensors were attached to the eggshells, respectively, and their signals were amplified using a charge amplifier (model PK-QA, purchased from PolyK Technologies, LLC). An oscilloscope (model DSO2512G, purchased from PolyK Technologies, LLC) was used to monitor the output. From embryonic day 6 (ED6) to ED16, embryonic heartbeat was detected.

Observation

Throughout the test period, the movement of embryos was diligently monitored using the selected piezoelectric sensors, and the heartbeat rate was recorded.

4.4 Results

No signals were detected from either piezoelectric sensor throughout the entire incubation period (Figure 6). It suggests that the weak embryonic heartbeat signal and resulting movements or vibrations of the eggshell may pose challenges in accurately detecting and capturing the signal

using the particular sensors. Despite careful placement and monitoring, the sensors failed to provide the desired signals throughout the entire incubation period.

To verify the functionality of the sensors, a human pulse was monitored using the SDT1-028K shielded piezo sensor, and a clear and distinct signal was obtained (Figure 7). This confirmed that the sensors themselves were capable of detecting strong vibrations or movements, but they failed to capture the embryonic heartbeat signals in this experimental setup.

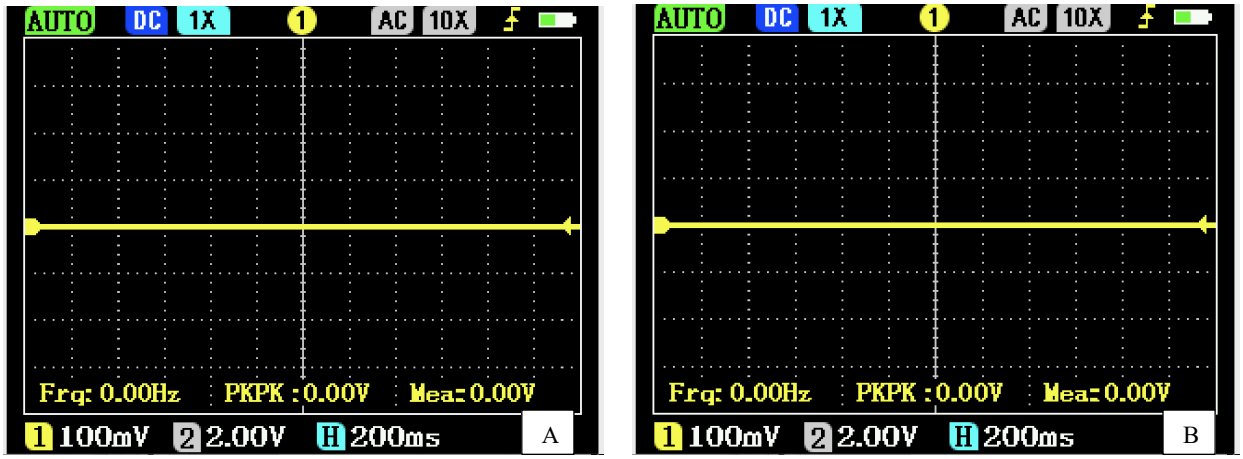

Figure 6.Output from piezoelectric sensors for Japanese quail heartbeat detection. (A)Output from Piezoelectric PVDF; (B) output from SDT1-028K shielded piezo sensor

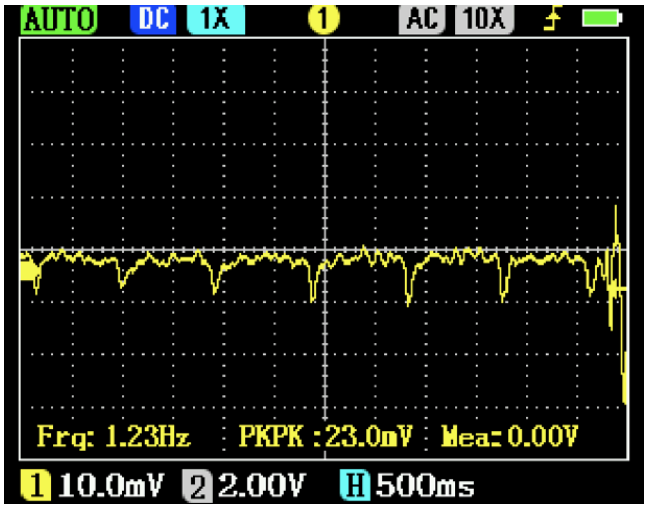

Figure 7. Output from SDT1-028K shielded piezo sensor for human pulse monitor

4.5 Discussion

The lack of a discernible signal indicating embryonic heartbeat raised concerns regarding the sensitivity and effectiveness of the chosen piezoelectric sensors for avian ELS toxicity testing. It is possible that the weak nature of embryonic heart contractions, coupled with the minimal movements or vibrations of the eggshell caused by these contractions, made it difficult for the sensors to capture and amplify the signals effectively.

Alternative approaches need to be explored to accurately and reliably monitor the heartbeat of Japanese quail embryos during the incubation period. One possible avenue for improvement is to explore different types or configurations of sensors and/or using an amplifier with higher sensitivity and better signal capture capabilities. Additionally, advanced imaging or lighting techniques can be considered to enhance the detection process.

4.6 Conclusion

In conclusion, the utilization of the selected piezoelectric sensors for detecting quail embryonic heartbeats in avian ELS toxicity testing proved unfeasible in this study. The absence of detectable signals raised concerns about the sensitivity and effectiveness of the chosen sensor configurations to address this issue and ensure accurate monitoring of embryonic heart rates, alternative approaches should be explored. These may include utilizing different sensor types, exploring alternative configurations or amplifiers, and considering advanced imaging or lighting techniques. By pursuing these alternatives, the challenges associated with accurately monitoring embryonic heart rates may be overcome.

4.7 References

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Chapter 5

5. General discussion and conslucion

5.1 Discussion

In this thesis, I aimed to explore the relationship between avian early-life stage (ELS) toxicity testing and traditional avian toxicity (AT) testing through a comparative study, while also advancing our ability to measure embryonic survival non-invasively. This work is motivated by regulatory change which is calling for New Approach Methods (NAMs) that are more cost-effective, quicker, and less reliant on animal models. While the avian ELS test has started to gain favour, there is a need for additional research to build scientific confidnce in the method. In particular, a need to further assess its technical performance (e.g., through careful study of responses in controls, and repeated studies) and ability to yield data that is comparable to data from whole animal studies using traditional avian test methods.

The major study within my thesis found that there was no concordance in apical measures between the ELS tests I conducted and information from traditional avian tests extracted from databases for certain chemicals. These differences in toxicity rankings between avian ELS and traditional AT testing can be attributed to several factors. First, the variations in metabolic pathways between developing embryos and adult birds play a crucial role. For example, the rapid metabolism of endrin to a more toxic metabolite, 12-ketoendrin, in adult birds may explain its higher toxicity in the traditional AT test compared to avian ELS testing, where the less developed metabolic processes in embryos result in a less severe toxic response. Second, the sensitivity, and the efficiency of elimination mechanisms differ between developing embryos and adult birds. For

instance, the embryonic stage may be more sensitive to the toxic effects of glufosinate-ammonium compared to young or adult birds due to the crucial role of glutamine in embryo growth and development, including its involvement in cell division and differentiation. Furthermore, young or adult birds have more effective elimination mechanisms to clear the pesticide from their bodies, whereas avian embryos are unable to eliminate it efficiently (likely due to immature metabolism processes), resulting in more severe toxic effect in avian ELS testing compared to traditional AT testing. Third, the physical-chemical properties of pesticides influence their uptake in avian embryos, which is not a concern in traditional AT testing. The route of exposure can influence the uptake and subsequent toxicity of pesticides in avian species. These distinctions are crucial to highlight when advocating for a NAM, as they underscore fundamental differences between the ELS method and traditional animal testing methods.

In summary, some of the differences in toxicity rankings between avian ELS testing and traditional AT testing may be attributed to variations in the life stages of avian exposure and the routes of exposure. These disparities may subsequently affect pesticide uptake, metabolic pathways and elimination processes, ultimately leading to varying toxicity effects between the two testing methods. Currently, it remains uncertain which method is more sensitive for chemical toxicity assessment. Analyzing data collected from USEPA ECOTOX (2023)(Table 6), I have found that avian ELS testing is more sensitive for certain chemicals, while traditional AT testing yields more toxic results for other chemicals, as evidenced by the comparision of similar or identical endpoints derived from the two testing methods(Figure 8). However, the utilization of egg injection to evaluate chemical toxicity in early-age avian tests encounters criticism from various perspectives. Detractors posit that this method may inadequately represent real-world scenarios, particularly

concerning ingestion exposure. Moreover, the developmental stages of embryos may not precisely align with the phases at which birds are most susceptible to certain toxins in the wild, as exemplified by endrin, which is toxic to adult birds but not to quail embryos. Additionally, critical behavioral aspects, essential for understanding ecological impact, may be overlooked in embryonic tests. Wild birds exhibit a diverse range of behaviors that could be impacted by their exposure to environmental contaminants, and capturing these aspects in embryonic tests presents a formidable challenge. It is imperative to acknowledge, however, that the principal objective of bird egg injection experiments, as a NAM, lies in the analysis of transcriptomics, proteomics, and metabolomics to gain a comprehensive understanding of the pathways leading to toxicity (Desforges et al. 2021; Basu 2019). While avian ELS testing provides valuable insights into pesticide toxicity mechanisms, further research is necessary to validate its comparability with traditional avian toxicity testing for risk assessment purposes. Significantly, our research has uncovered some consistency between traditional acute toxicity (AT) testing and avian ELS testing. Consequently, additional investigations are warranted to thoroughly delineate the distinctions in toxicity between the two testing methodologies and to ascertain the applicability and scope of avian ELS testing as an alternative approach to conventional avian toxicity assessment.

While some alternative testing methods, such as the Fish Cell line Acute Toxicity test or the 3 T3 Neutral Red Uptake (3 T3 NRU) phototoxicity tests, have gained regulatory acceptance, it is essential to recognize that NAMs face a substantial journey before they can completely replace traditional animal models in chemical toxicity testing (Fischer & Wallace 2020). Presently, NAMs are often integrated as supplementary steps, providing additional insights for toxicological risk assessments (Bruner et al. 1996) or serving as preliminary chemical screening and prioritization

(Luijten et al. 2022). The Toxicology in the 21st Century Consortium (Tox21) has been at the forefront, issuing a pivotal report in 2007 titled 'Toxicity Testing in the 21st Century – A Vision and a Strategy' (Tox21c), aiming to catalyze a paradigm shift in toxicity testing towards NAMs grounded in mechanistic endpoints models (NRC2007). However, it is evident that more strides need to be taken in this direction.

Complications arise from disparities in perspectives between toxicologists and regulators. Toxicologists strive to unravel truths and expound natural mechanisms through hypothesis-driven investigations, while regulators seek solutions based on available data, even when its quality may be suboptimal. This disjunction hampers progress, where scientific advancements outpace the gradual changes in regulations (Fischer & Wallace 2020).

The gap between research and regulatory acceptance currently impedes the full abandonment of traditional testing methods (Ritskes-Hoitinga 2022; Schmeisser et al. 2023), such as those applied in the case of chemicals. This challenge is exacerbated by the considerable time and numerous prerequisites involved in validating NAMs for comprehensive regulatory acceptance (Abarkan et al. 2022). A potential solution to this issue may involve a thorough overhaul of toxicity test regulations, ensuring they evolve in harmony with ongoing scientific innovations (Schmeisser et al. 2023; Fischer & Wallace 2020) and generate scientific confidence in NAMs for the regulatory evaluation of chemical impacts, both domestically and globally (van der Zalm et al. 2020; Abarkan et al. 2022)

In October 2018, Tox21 expanded its focus, not only centering on the development and application of High Throughput Screening (HTS) methods but also striving to refine alternative test systems

and address limitations in existing NAMs over the next 5 years (Fischer & Wallace 2020). This strategic shift underscores the critical need for a transition from traditional testing to NAMs, positioning them as valuable tools in advancing toxicological research and regulatory practices.

The evolving landscape in the scientific community and advancements in scientific methodologies are evidently reshaping traditional toxicity testing for the better. Remarkable technological and intellectual innovations are instrumental in bridging existing gaps between toxicology and regulations (Fischer & Wallace 2020). While a complete paradigm shift has not materialized, the growing adoption of NAMs in chemical assessment, the full transition of the cosmetics industry towards NAMs, and persistent efforts in other domains to attain the desired toxicity-testing approaches collectively reinforce the vision of both industries and regulatory toxicology. The prospect of NAMs taking on a leading role in toxicity testing seems significant, shaping the future trajectory of toxicity assessment (van Vliet 2011; Fischer & Wallace, 2020).

The thesis also discussed the challenges encountered in accurately monitoring the heart rates of avian embryos during ELS testing. The weak nature of embryonic heart contractions, coupled with minimal movements or vibrations of the eggshell caused by these contractions, made it difficult for the selected sensors to capture and amplify the signals effectively. Unfortunately, no heartbeat signal of the avian embryo was detected in our experiment.

The method of attaching a transducer or sensor to the eggshell to measure embryo heartbeat, such as using a microphone to detect the pulsatile air movement (acoustic pressure changes) across the eggshell or using piezoelectric sensors, audio cartridge to measure slight movements or vibrations of the eggshell caused by the contraction of the embryonic heart, was a technique

used in the 1990s (Wang et al. 1990; Rahn et al. 1990; Akiyama et al. 1997; Akiyama et al. 1999; Tazawa et al. 1989; Suzuki et al. 1989; Tazawa et al. 1993; Tazawa et al. 1989; Pearson et al. 1998; Pawlak et al. 2004, Szymanski et al. 2002). This technology did not undergo further advancements and instead shifted towards new techniques, specifically the use of advanced imaging or lighting techniques (Youssef et al. 2020; Tamura et al. 2014; Yu et al. 2019; Khaliduzzaman et al. 2019; Phuphanin et al. 2019; Lierz et al. 2006; Pollard et al. 2016). The instruments available on the market that utilize advanced imaging or lighting techniques for detecting heartbeat of avian embryos are either small devices designed to monitor one egg at a time (such as the Buddy-digital egg monitor), or large-scale equipment used in vaccine production and poultry farming industries (Yu et al. 2019). Neither of these options meets our requirements, which involve real-time and simultaneous monitoring of the heart rates of multiple bird eggs. Therefore, it is necessary for us to develop a specialized avian embryo heart rate detector using advanced imaging or lighting techniques that caters to our experimental needs.

Table 6. Comparison of chemical toxicity between Japanese quai egg and traditional avian toxicity testing

Chemical name	CAS No.	Test organism	Age	Route	Observed duration	LD50/LOEC/NOEC	source
(17beta)Estra-1,3,5(10)triene-3,17-diol	50-28-2	egg	3 days	Injection/ unspecified	12 days	LOEC (Development): 0.1 µg/g egg NOEC (Growth): 0.1 µg /g egg NOEC (Development): 0.01µg/g egg	Razia,S., Y. Maegawa, S. Tamotsu, and T. Oishi.
		immature	4 weeks	diet	14 days	LOEL (morphology) : 100ppm diet NOEL (morphology, feeding behavior, growth): 100 ppm diet	Wilhelms,K.W., S.A. Cutler, J.A. Proudman, R.V. Carsia, L.L. Anderson, and C.G. Scanes.
(17beta)Estra-1,3,5(10)triene-3,17-diol-3-benzoate	50-50-0	egg	17 days	Injection/ unspecified	NA	LOEL (reproduction): 20 µg/org	Brain Res. Bull.65(3): 199-209
		immature	4 weeks	diet	14 days	NOEL (morphology, feeding behavior, growth): 1 ppm diet	Wilhelms,K.W., S.A. Cutler, J.A. Proudman, R.V. Carsia, L.L. Anderson, and C.G. Scanes
4,4'-[(1E)-1,2-Diethyl-1,2-ethenediyl]bisphenol	56-53-1	egg	3 days	Yolk	12 days	LOEL (development): 0.02 µg/g egg NOEL (mortality): 0.02 µg/g egg NOEL (development): 0.002 µg/g egg	Berg,C., K. Halldin, and B. Brunstrom
					58 days	LOEL (morphology): 57 ng/g egg	Halldin,K., C. Berg, I. Brandt, and B. Brunstrom
					72 days	NOEL (growth, morphology): 57 ng/g egg	Halldin,K., C. Berg, I. Brandt, and B. Brunstrom
		immature	3 weeks	Intraperitoneal	3 days	NOEL (genetics): 1µg/g bdwt	Hanafy,A.M., T. Sasanami, and M. Mori
					4 days	NOEL (genetics): 1µg/g bdwt	
(17alpha)-19-Norpregna-1,3,5(10)-trien-20-yne-3,17-diol (17alpha)-19-Norpregna-1,3,5(10)-trien-20-yne-3,17-diol	57-63-6	egg	0 days	injection/ Air sac	16 days	LOEL (growth): 54.2 µg/g egg NOEL (morphology, development): 54.2 µg/g egg	Farhat,A., D. Crump, L. Bidinosti, E. Boulanger, N. Basu, M. Hecker, and J.A. Head
			0 days	injection/ Air sac	9 days	NOEL (mortality): 54.2 µg/g egg	Farhat,A., D. Crump, L. Bidinosti, E. Boulanger, N. Basu, M. Hecker, and J.A. Head
			3 days	Yolk	72 days	LOEL (morphology): 6ng/g egg NOEL (growth, morphology)):6ng/g egg	Halldin,K., C. Berg, I. Brandt, and B. Brunstrom
		Immature	3 weeks	Intraperitoneal	1 days	LOEL (genetics): 1 µg/g bdwt	Hanafy,A.M., T. Sasanami, and M. Mori
					4 days		
		mature	NA	Intraperitoneal	NA	LOEL (genetics): 1 µg/g bdwt LOEL (genetics): 0.5 µg/g bdwt NOEL (genetics): 1 µg/g bdwt	Hanafy,A.M., T. Sasanami, and M. Mori
4,4'-(1-Methylethylidene)bisphenol	80-05-7	egg	3 days	Yolk	12 days	LOEL (development): 200 µg/g egg NOEL (mortality): 200 µg/g egg NOEL (development): 67 µg/g egg NOEL (development): 200 µg/g egg	Berg,C., K. Halldin, and B. Brunstrom

					75 days	NOEL (morphology, reproduction, morphology): 200 µg/g egg	Halldin,K., C. Berg, A. Bergman, I. Brandt, and B. Brunstrom
		immature	3 weeks	Intraperitoneal	3 days 2 days 4 days 1 days	NOEL (genetics): 1µg/g bdwt	Hanafy,A.M., T. Sasanami, and M. Mori
		mature	NA		NA	NOEL (genetics): 1 µg/g bdwt	Hanafy,A.M., T. Sasanami, and M. Mori
6,7-Dihydrodipyrido[1,2-a:2',1'-c]pyrazinediium bromide (1:2)	85-00-7	egg	NA	Albumin injection	15 days	LOEL (weight): 1 µg/g egg	Zeman,M., I. Herichova, J. Navarova, S. Gressnerova, and P. Skrobanek
		juvenile	NA	diet	8 days	LC50 (mortality): 1346 ppm diet	Heath,R.G., J.W. Spann, E.F. Hill, and J.F. Kreitzer
3a,4,7,7a-Tetrahydro-2-[(trichloromethyl)thio]-1H-isoindole-1,3-(2H)-dione	133-06-2	egg	9 days	Air sac injection	5 days	LOEL (mortality): 18mg/kg egg LOEL (mortality): 24mg/kg egg NOEL (mortality): 18mg/kg egg NOEL (weight): 24mg/kg egg NOEL (mortality): 12mg/kg egg	Varnagy,L., R. Imre, T. Fancsi, and A. Hadhazy
		juvenile	14 days	diet	8 days	LC50 (mortality) > 5000 ppm diet	Hill,E.F., and M.B. Camardese
O,O-Dimethyl O-(4-nitrophenyl) ester phosphorothioic acid	298-00-0	egg	NA	Air sac injection	6 ays	LOEL (mortality): 225 mg/kg LOEL (development): 280 mg/kg NOEL (growth): 280 mg/kg NOEL (growth): 225 mg/kg NOEL (mortality): 280 mg/kg NOEL (mortality): 22.5 mg/kg	Varnagy,L., R. Imre, T. Fancsi, and A. Hadhazy
		juvenile	20 days	diet	5 days	LC50(mortality): 69 ppm diet	Hill,E.F., and M.B. Camardese
			14 days	Oral via capsule	8 days	LC50(mortality): 79ppm	Hill,E.F., R.G. Heath, J.W. Spann, and J.D. Williams
		mature	9 weeks	diet	42 days	LOEL (reproduction): 48 ppm diet NOEL (reproduction): 12 ppm diet	Solecki,R., A.S. Faqi, R. Pfeil, and V. Hilbig
6-Chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine	1912-24-9	egg	N.A.	Injection/ unspecified	33 days	LOEL (growth, morphology): 0.504 µg/g egg NOEL (morphology, growth): 0.246 µg/g egg NOEL (mortality): 0.504 µg/g egg	Arch. Environ. Contam. Toxicol.51(1): 117-122
		Juvenile	20 days	food	5 days	LC50 (mortality)>5000 ppm food	Fish and Wildlife Technical Report 2, Fish and Wildlife Service, U.S. Department of the Interior:147 p.

		mature	4 weeks	food	14 days	LOEL/NOEL (morphology, growth, hormones, feeding behaviour): 1000 ppm food NOEL (morphology, growth, feeding behaviour, hormones): 10 ppm food	Wilhelms,K.W., S.A. Cutler, J.A. Proudman, L.L. Anderson, and C.G. Scanes 85511: Wilhelms,K.W., S.A. Cutler, J.A. Proudman, R.V. Carsia, L.L. Anderson, and C.G. Scanes
Phosphorothioic acid, O,O-Diethyl O-(3,5,6-trichloro-2-pyridinyl) ester	2921-88-2	egg	Embryo day 0	injection/ Air sac	9 days	NOEL (mortality): 41.1 µg/g egg	Farhat,A., D. Crump, L. Bidinosti, E. Boulanger, N. Basu, M. Hecker, and J.A. Head
					16 days	LOEL (morphology, growth, development): 41.1 µg/g egg NOEL (mortality): 41.1 µg/g egg NOEL (growth, morphology, development): 4.9 µg/g egg	Farhat,A., D. Crump, L. Bidinosti, E. Boulanger, N. Basu, M. Hecker, and J.A. Head
		Mature	2 months	Oral/capsule	14days	LD50=17.8 mg/kg	Hudson,R.H., R.K. Tucker, and M.A. Haegele
			2.5 months	Oral/capsule	14days	LD50=15.9 mg/kg	U.S. Environmental Protection Agency
		juvenile	14 days	diet	5 days	LC50 (mortality): 293 ppm diet	Hill,E.F., and M.B. Camardese
			14 days	Oral via capsule	8 days	LC50 (mortality): 299 ppm diet	Hill,E.F., R.G. Heath, J.W. Spann, and J.D. Williams
			20 days	diet	5 days	LC50 (mortality): 492 ppm diet	Hill,E.F., and M.B. Camardese
(2S)-2-Amino-4-(methylseleno)butanoic acid	3211-76-5	egg	Embryo day 0	injection/ Air sac	9 days	NOEL (mortality): 0.03 µg/g egg	Farhat,A., D. Crump, L. Bidinosti, E. Boulanger, N. Basu, M. Hecker, and J.A. Head
					16 days	NOEL (morphology, development, growth): 0.03 µg/g egg	Farhat,A., D. Crump, L. Bidinosti, E. Boulanger, N. Basu, M. Hecker, and J.A. Head
		mature	NA	diet	28 days	LOEL (morphology, growth): 22 µg/g diet NOEL (feeding behavior): 22 µg/g diet NOEL (growth, morphology): 12 µg/g diet	Santolo,G.M., J.T. Yamamoto, P.S. Neiberg, and B.W. Wilson
Nitric acid, Lead (2+) salt (2:1)	10099-74-8	egg	Embryo day 0	injection/ Air sac	9 days	NOEL (mortality): 6.7 µg/g egg	Environ. Toxicol. Chem.39(1): 141-154
					16 days	NOEL (morphology, development, growth): 6.7 µg/g egg	Environ. Toxicol. Chem.39(1): 141-154
		juvenile	14 days	diet	5 days	LC50 (mortality): >5000 ppm diet	Hill,E.F., and M.B. Camardese
Nonylphenol	25154-52-3	egg	NA	Injection, unspecified	7 days	NOEL (reproduction): 200µg/egg	Oshima,A., R. Yamashita, K. Nakamura, M. Wada, and K. Shibuya
					16 days	LOEL (development): 0.2µg/egg NOEL (development, reproduction, population): 200µg/egg	Oshima,A., R. Yamashita, K. Nakamura, M. Wada, and K. Shibuya
		immature	3 weeks	Intraperitoneal	1 day	NOEL (genetics): 1µg/g bdwt	Hanafy,A.M., T. Sasanami, and M. Mori
					4 days	NOEL (genetics): 1µg/g bdwt	Hanafy,A.M., T. Sasanami, and M. Mori
3-(3,5-Dichlorophenyl)-5-	50471-44-8	egg	NA	Air sac injection	42 days	LOEL(morphology): 100 µg/g bdwt	McGary,S., P.F.P. Henry, and M.A. Ottinger

ethenyl-5-methyl-2,4-oxazolidinedione					NOEL (morphology, reproduction): 100 µg/g bdwt	
	immature	NA	diet	42 days	LOEL (reproduction): 124.5ppm LOEL (population, reproduction): 482.65ppm NOEL (population, reproduction): 124.5ppm NOEL (growth, hormones): 482.65ppm	Niemann,L., B. Selzsam, W. Haider, C. Gericke, and I. Chahoud

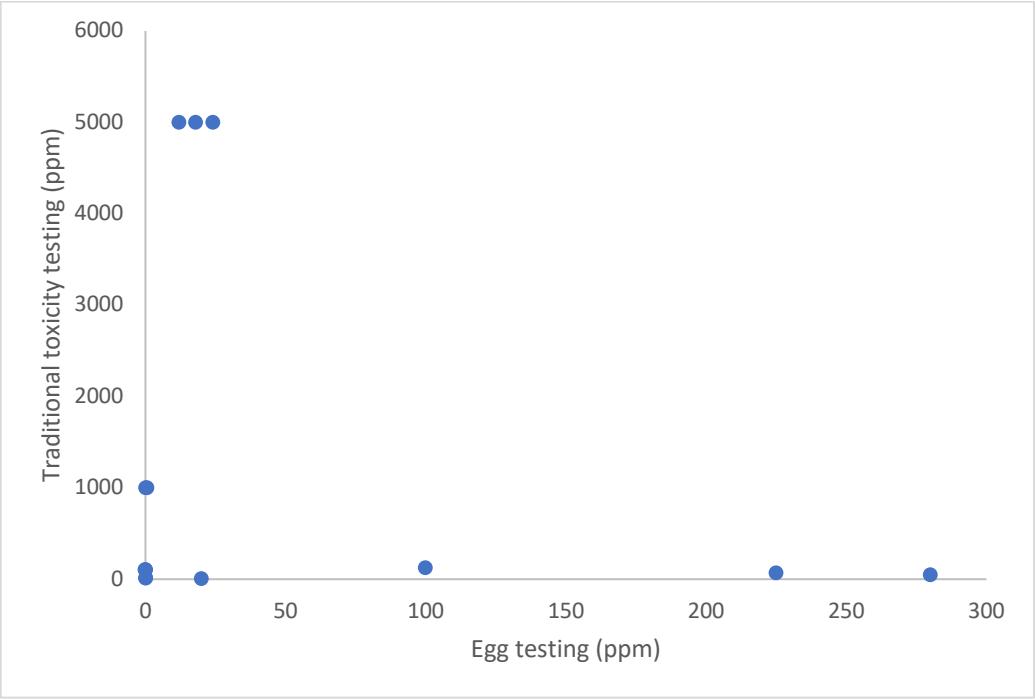

Figure 8. Comparison of chemical toxicity between Japanese quail egg and traditional avian toxicity testing

5.2 Conclusions and future directions

The thesis included two projects. Project 1 explored the correlation between avian ELS testing and traditional AT testing methods, while Project 2 focused on advancing the embryonic survival detection method. Together, these projects aim to enhance the field of avian toxicity testing, providing valuable insights and contributing to the development of more efficient and ethically sound approaches in chemical hazard assessment.

Based on the findings of project 1, it was observed that the toxicity levels in avian ELS testing and traditional AT testing differ for certain chemicals. In order to fully comprehend and define the applicability domain where avian ELS testing can effectively replace alternative AT testing for acute toxicity assessment, it is imperative to understand the underlying reasons for these differences. Therefore, further research is required to test additional chemicals and investigate the factors contributing to the variations in toxicity between the two methods.

In project 2, although the selected piezoelectric sensors did not provide the expected results, this study emphasizes the need for future exploration of alternative methods to improve the detection and assessment of embryonic survival. Future research should focus on development of a specialized avian embryo heart rate detector using advanced imaging or lighting techniques that caters to our experimental needs.

Continued research and innovation in both these areas are necessary given regulatory and scientific interest in New Approach Methods (NAMs) to help innovate and advance the field of avian toxicity testing into one that is more ethical, cost-effective, and faster.

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Supplemental Data

Table S1. Stock solution and working solutions preparation for endrin

Dose level (µg/g egg)	Test solution (mg/ml)	Stock solution*		DMSO added (µl)	Final volume (µl)
		Concentration (mg/ml)	Volume (µl)		
100	150	-	-	-	603
100	150	150	200	-	200
32	48	150	64	136	200
10	15	150	20	180	200
3.2	4.8	48	20	180	200
1	1.5	15	20	180	200
0.32	0.48	4.8	20	180	200
0.1	0.15	1.5	20	180	200

* Endrin stock solution was prepared by dissolving 90.52mg of test substance (purity: 96.8%) in 0.603ml of DMSO at a concentration of 150mg/L.

Table S2. Stock solution and working solutions preparation for ethoprophos

Dose level (µg/g egg)	Test solution (mg/ml)	Stock solution*		DMSO added (µl)	Final volume (µl)
		Concentration(mg/ml)	Volume (µl)		
100	150	150	200	-	200
32	48	150	64	136	200
10	15	150	20	180	200
3.2	4.8	48	20	180	200
1	1.5	15	20	180	200
0.32	0.48	4.8	20	180	200
0.1	0.15	1.5	20	180l	200

* Ethoprophos stock solution was prepared by dissolving 93µl of test substance (purity: 96.8%; density: 1106 mg/ml) in 0.571ml of DMSO at a concentration of 150mg/L.

Table S3. Stock solution and working solutions preparation for carbofuran

Dose level (µg/g egg)	testsolution (mg/ml)	Stock solution*		DMSO added (µl)	Final volume (µl)
		Concentration(mg/ml)	volume		
100	150	150	200 µl	-	200
32	48	150	64 µl	136µl	200
10	15	150	20 µl	180µl	200
3.2	4.8	48	20 µl	180µl	200
1	1.5	15	20µl	180µl	200
0.32	0.48	4.8	20µl	180µl	200
0.1	0.15	1.5	20µl	180µl	200

*Carbofuran stock solution was prepared by dissolving 150mg of the test substance in 1ml of DMSO at a concentration of 150mg/L.

Table S4. Stock solution and working solutions preparation for trichlorfon

Dose level (µg/g egg)	test solution (mg/ml)	Stock solution*		DMSO added (µl)	Final volume (µl)
		Concentration(mg/ml)	volume		
100	150	150	200 µl	-	200
32	48	150	64 µl	136µl	200
10	15	150	20 µl	180µl	200
3.2	4.8	48	20 µl	180µl	200
1	1.5	15	20µl	180µl	200
0.32	0.48	4.8	20µl	180µl	200
0.1	0.15	1.5	20µl	180µl	200

* Trichlorfon stock solution was prepared by dissolving 48.88mg of the test substance in 326µl of DMSO at a concentration of 150mg/L.

Table S5. Stock solution and working solutions preparation for permethrin

Dose level (µg/g egg)	Test solution (mg/ml)	Stock solution*		DMSO added	Final volume (µl)
		Concentration (mg/ml)	volume		
320	480	480	200 µl	-	200
100	150	480	62.5 µl	137.5	200
32	48	480	20 µl	180µl	200
10	15	150	20 µl	180µl	200
3.2	4.8	48	20 µl	180µl	200
1	1.5	15	20µl	180µl	200
0.32	0.48	4.8	20µl	180µl	200
0.1	0.15	1.5	20µl	180µl	200

*Permethrin stock solution was prepared by dissolving 338.80mg of the test substance in 706 µl of DMSO at a concentration of 480mg/L.

Table S6. Stock solution and working solutions preparation for glufosinate-ammonium study 1

Dose level (µg/g egg)	work solution (mg/ml)	Stock solution* Concentration(mg/ml)	volume	Pure water added (µl)	Final volume (µl)
100	150	150	200 µl	-	200
32	48	150	64 µl	136µl	200
10	15	150	20 µl	180µl	200
3.2	4.8	48	20 µl	180µl	200
1	1.5	15	20µl	180µl	200
0.32	0.48	4.8	20µl	180µl	200
0.1	0.15	1.5	20µl	180µl	200

*Glufosinate ammonium stock solution was prepared by dissolving 78.95mg of the test substance in 526µl autoclave pure water at a concentration of 150mg/L.

Table S7. Stock solution and working solutions preparation for glufosinate-ammonium study 2

Dose level (µg/g egg)	Test solution (mg/ml)	Stock solution*		Pure water added	Final volume (µl)
		Concentration (mg/ml)	volume		
100	150	150	200 µl	-	200
32	48	150	64 µl	136µl	200
10	15	150	20 µl	180µl	200
3.2	4.8	48	20 µl	180µl	200
1	1.5	15	20µl	180µl	200
0.32	0.48	4.8	20µl	180µl	200
0.1	0.15	1.5	20µl	180µl	200
0.032	0.048	0.48	20µl	180µl	200
0.01	0.015	0.15	20µl	180µl	200

*Glufosinate ammonium stock solution was prepared by dissolving 98.34mg of the test substance in 655µl pure water at a concentration of 150mg/L.

Table S8. Mortality result of Japanese quail embryos for expusure in Endrin

Dose level (µg/g egg)	Total No. of eggs	Infertile egg	Total No. of fertile eggs	Mortality on day 9	Mortality rate
DMSO	13	1	12	1	8%
0.1	11	0	11	4	36%
0.32	11	2	9	1	11%
1	11	0	11	2	18%
3.2	10	2	8	0	0%
10	10	1	9	1	11%
32	10	1	9	1	11%
100	10	3	7	0	0%

Table S9. Mortality result of Japanese quail embryos for expusure in Ethoprophos

Dose level (μg/g egg)	Total No. of eggs	Infertile egg	Total No. of fertile egg	Mortality on day 9	Mortality rate
DMSO	11	0	11	1	9%
0.1	11	0	11	0	0%
0.32	11	0	11	1	9%
1	10	0	10	1	10%
3.2	10	1	9	0	0%
10	10	2	8	0	0%
32	11	0	11	1	9%
100	11	2	9	1	11%

Table S10. Mortality result of Japanese quail embryos for expusure in Carbofuran

Dose level (μg/g egg)	Total No. of eggs	Infertile egg	Total No. of fertile egg	Mortality on day 9	Mortality rate
DMSO	10	1	9	0	0%
0.1	11	1	10	0	0%
0.32	11	1	10	1	10%
1	10	2	8	0	0%
3.2	10	1	9	0	0%
10	10	1	9	1	11%
32	10	2	8	0	0%
100	10	1	9	0	0%

Table S11. Mortality result of Japanese quail embryos for expusure in Trichlorfon

Dose level (μg/g egg)	Total No. of eggs	Infertile egg	Total No. of fertile egg	Mortality on day 9	Mortality rate
DMSO	10	0	10	0	0%
0.1	10	1	9	1	11%
0.32	10	0	10	0	0%
1	10	1	9	1	11%
3.2	10	0	10	2	20%
10	10	0	10	0	0%
32	10	0	10	0	0%
100	11	1	10	0	0%

Table S12. Mortality result of Japanese quail embryos for expusure in Permethrin

Dose level (μg/g egg)	Total No. of eggs	Infertile egg	Total No. of fertile egg	Mortality	Mortality rate
DMSO	12	0	12	0	0%
0.1	11	0	11	0	0%
0.32	10	2	8	0	0%
1	11	0	11	0	0%
3.2	11	0	11	1	9%
10	11	1	10	0	0%
32	10	0	10	0	0%
100	10	0	10	0	0%
320	12	1	11	1	9%

Table S13. Mortality result of Japanese quail embryos for expusure in Glufosinate- ammonium

Dose level (μg/g egg)	Total No. of eggs	Infertile egg	Total No. of fertile egg	Mortality on day 9	Mortality rate
Pure water	12	1	11	1	9%
0.1	10	0	10	0	0%
0.32	10	1	9	0	0%
1	10	0	10	1	10%
3.2	10	1	9	0	0%
10	11	2	9	4	44%
32	11	2	9	9	100%
100	11	1	10	10	100%

Table S14. Mortality result of Japanese quail embryos for exposure in Glufosinate- ammonium

Dose level (μg/g egg)	Total No. of eggs	Infertile egg	Total No. of fertile egg	Mortality	Mortality rate
Pure water	11	0	11	0	0%
0.01	11	1	10	3	30%
0.032	11	1	10	0	0%
0.1	11	0	11	0	0%
0.32	9	1	8	0	0%
1	11	1	10	0	0%
3.2	11	0	11	1	9%
10	11	2	9	6	67%
32	11	1	10	10	100%
100	12	1	11	11	100%

Table S15. Growth of Japanese quail embryos for exposure in Endrin

Doses (µg/g egg)	Embryo mass (g)										
solvent	1.106	2.001	1.219	1.184	1.726	1.508	1.256	0.986	2.124	1.572	1.136
0.1	1.156	1.179	1.119	1.185	1.392	1.219	1.314	-	-	-	-
0.32	1.228	1.202	1.372	1.177	1.216	1.392	1.190	1.191	-	-	-
1	1.199	1.232	1.130	1.469	1.292	1.214	1.424	1.201	1.311	-	-
3.2	1.018	1.033	1.580	1.517	1.082	1.162	1.179	1.021	-	-	-
10	1.179	1.240	1.147	1.253	1.172	1.281	1.214	1.258	-	-	-
32	1.307	1.293	1.303	1.156	1.271	1.055	1.161	1.098	-	-	-
100	1.189	1.220	1.349	1.018	1.215	1.253	1.343	-	-	-	-

Table S16. Growth of Japanese quail embryos for exposure in Ethoprophos

Doses (µg/g egg)	Embryo mass (g)										
solvent	1.272	1.401	1.295	1.533	1.282	1.391	1.429	1.327	1.378	-	-
0.1	1.397	1.329	1.269	1.412	1.357	1.472	1.322	1.472	1.415	1.293	1.145
0.32	1.459	1.444	1.408	1.318	1.197	1.395	1.367	1.500	1.486	1.211	-
1	1.352	1.237	1.423	1.344	1.326	1.391	1.311	1.441	1.267	-	-
3.2	1.513	1.579	1.399	1.380	1.447	1.435	1.305	1.285	1.470	-	-
10	1.223	1.325	1.475	1.472	1.305	1.229	1.256	1.220	-	-	-
32	1.273	1.260	1.423	1.291	1.360	1.193	1.369	1.259	1.432	-	-
100	1.219	1.124	1.328	1.403	1.382	1.163	1.315	1.093	-	-	-

Table S17. Growth of Japanese quail embryos for exposure in Carbofuran

Doses (µg/g egg)	Embryo mass (g)									
solvent	1.268	1.356	1.257	1.275	1.253	0.962	1.326	1.358	1.185	-
0.1	0.374	1.214	1.172	1.264	1.315	1.361	1.299	1.184	1.288	1.329
0.32	1.386	1.402	1.272	1.348	1.415	1.387	1.459	1.162	-	-
1	1.355	1.395	1.442	1.334	1.272	1.100	1.377	-	-	-
3.2	1.236	1.12	1.406	1.362	1.376	1.255	1.332	1.472	1.399	-
10	1.453	1.475	1.345	1.352	1.565	1.349	1.444	1.255	-	-
32	1.378	1.513	1.397	1.022	1.335	1.166	1.341	-	-	-
100	1.133	1.427	1.164	1.181	1.209	1.233	1.17	1.047	1.235	-

Table S18. Growth of Japanese quail embryos for exposure in Trichlorfon

Doses (µg/g egg)	Embryo mass (g)									
solvent	1.260	1.370	1.089	1.215	1.044	1.245	1.106	1.178	1.075	1.038
0.1	1.215	1.057	1.224	1.070	1.279	1.161	1.097	-	-	-
0.32	1.150	1.325	1.261	1.251	1.090	1.263	1.230	1.103	1.108	1.005
1	1.172	1.250	1.320	1.171	1.096	1.273	1.097	1.150	-	-
3.2	1.041	1.225	1.240	1.093	1.156	1.233	1.094	1.147	-	-
10	1.294	1.215	1.075	1.071	0.959	1.167	1.189	1.157	1.219	1.213
32	1.296	1.118	1.216	1.203	1.069	1.065	1.096	1.092	0.985	-
100	1.273	1.073	1.156	1.211	0.829	1.105	1.265	0.908	0.999	1.072

Table S19. Growth of Japanese quail embryos for exposure in Permethrin

Doses (µg/g egg)	Embryo mass (g)											
solvent	1.411	1.32	1.329	1.343	1.324	1.256	1.291	1.215	1.209	1.193	1.243	1.277
0.1	1.288	1.366	0.765	1.254	1.25	1.228	1.353	1.16	1.144	1.171	1.235	-
0.32	1.134	1.21	1.22	1.404	1.161	1.127	1.049	1.169	-	-	-	-
1	1.202	1.181	1.159	1.134	1.478	1.123	1.159	1.12	1.235	1.139	1.106	-
3.2	1.104	1.072	1.1	1.441	1.302	1.192	1.19	1.209	1.21	1.232	-	-
10	1.147	1.27	1.317	1.215	1.165	1.181	1.244	1.056	1.147	1.18	-	-
32	1.243	1.323	1.429	1.396	1.3	1.288	1.182	1.132	1.296	1.307	-	-
100	1.197	1.144	1.015	1.22	1.159	1.197	1.182	1.178	1.154	1.103	-	-
320	1.25	1.318	1.146	1.181	1.302	1.126	1.155	1.201	1.268	1.203	-	-

Table S20. Growth of Japanese quail embryos for exposure in Glufosinate-ammonium 1

Doses (µg/g egg)	Embryo mass (g)									
solvent	1.329	1.334	1.218	1.206	0.849	1.231	1.177	1.144	1.399	1.461
0.1	1.305	1.163	1.157	1.255	1.121	1.293	1.199	1.114	1.173	1.130
0.32	1.438	1.379	1.127	1.157	1.216	1.021	1.266	1.222	1.154	-
1	1.248	1.318	1.203	1.224	1.255	1.271	1.157	1.143	1.099	-
3.2	1.028	1.089	1.189	1.202	0.978	1.093	1.094	1.093	1.066	-
10	0.950	0.989	0.935	0.908	0.901	-	-	-	-	-
32	-	-	-	-	-	-	-	-	-	-
100	-	-	-	-	-	-	-	-	-	-

Table S21. Growth of Japanese quail embryos for exposure in Glufosinate-ammonium 2

Doses (µg/g egg)	Embryo mass (g)										
solvent	1.382	1.486	1.474	1.413	1.388	1.421	1.345	1.346	1.288	1.482	1.450
0.01	1.340	1.373	1.433	1.323	1.468	1.446	1.254	-	-	-	-
0.032	1.374	1.384	1.428	1.474	1.375	1.362	1.365	1.389	1.458	1.322	-
0.1	1.443	1.384	1.273	1.442	1.267	1.311	1.153	1.288	1.393	1.547	1.423
0.32	1.259	1.396	1.412	1.402	1.110	1.416	1.462	1.394	-	-	-
1	1.385	1.404	1.399	1.347	1.348	1.519	1.417	1.430	1.310	1.253	-
3.2	1.249	1.270	1.283	1.302	1.202	1.293	0.898	1.193	1.107	1.251	-
10	0.739	1.111	1.052	-	-	-	-	-	-	-	-
32	-	-	-	-	-	-	-	-	-	-	-
100	-	-	-	-	-	-	-	-	-	-	-

Table S22. Least Squares Means and Differences (Dunnett adjusted) for Endrin

Least Squares Means						
Effect	dose_level	Estimate	Standard Error	DF	t Value	Pr > |t|
dose_level	0	1.4380	0.1146	58	12.55	<.0001
dose_level	0.1	1.2234	0.03637	58	33.64	<.0001
dose_level	0.32	1.2460	0.03026	58	41.18	<.0001
dose_level	1	1.2747	0.03713	58	34.33	<.0001
dose_level	3.2	1.1990	0.07948	58	15.09	<.0001
dose_level	10	1.2180	0.01689	58	72.12	<.0001
dose_level	32	1.2055	0.03545	58	34.00	<.0001
dose_level	100	1.2267	0.04207	58	29.16	<.0001

Differences of Least Squares Means									
Effect	dose_level	_dose_level	Estimate	Standard Error	DF	t Value	Pr > t	Adjustment	Adj P
dose_level	0	0.1	0.2146	0.1202	58	1.78	0.0398	Dunnett	0.0887
dose_level	0	0.32	0.1920	0.1185	58	1.62	0.0553	Dunnett	0.1183
dose_level	0	1	0.1633	0.1205	58	1.36	0.0902	Dunnett	0.1799
dose_level	0	3.2	0.2390	0.1395	58	1.71	0.0460	Dunnett	0.1007
dose_level	0	10	0.2200	0.1158	58	1.90	0.0313	Dunnett	0.0717
dose_level	0	32	0.2325	0.1200	58	1.94	0.0287	Dunnett	0.0666
dose_level	0	100	0.2113	0.1221	58	1.73	0.0444	Dunnett	0.0977

Table S23 Least Squares Means and Differences (Dunnett adjusted) for Ethoprophos

Least Squares Means						
Effect	dose_level	Estimate	Standard Error	DF	t Value	Pr > |t|
dose_level	0	1.3676	0.03182	65	42.98	<.0001
dose_level	0.1	1.3530	0.02878	65	47.01	<.0001
dose_level	0.32	1.3785	0.03019	65	45.66	<.0001
dose_level	1	1.3436	0.03182	65	42.22	<.0001
dose_level	3.2	1.4237	0.03182	65	44.74	<.0001
dose_level	10	1.3131	0.03375	65	38.91	<.0001
dose_level	32	1.3178	0.03182	65	41.41	<.0001
dose_level	100	1.2534	0.03375	65	37.14	<.0001

Differences of Least Squares Means									
Effect	dose_level	_dose_level	Estimate	Standard Error	DF	t Value	Pr > t	Adjustment	Adj P
dose_level	0	0.1	0.01456	0.04291	65	0.34	0.3678	Dunnett	0.7688
dose_level	0	0.32	-0.01094	0.04386	65	-0.25	0.5981	Dunnett	0.9266
dose_level	0	1	0.02400	0.04500	65	0.53	0.2978	Dunnett	0.6916
dose_level	0	3.2	-0.05611	0.04500	65	-1.25	0.8915	Dunnett	0.9960
dose_level	0	10	0.05443	0.04639	65	1.17	0.1225	Dunnett	0.3960
dose_level	0	32	0.04978	0.04500	65	1.11	0.1364	Dunnett	0.4268
dose_level	0	100	0.1142	0.04639	65	2.46	0.0082	Dunnett	0.0425

Table S24 Least Squares Means and Differences (Dunnett adjusted) for Carbofuran

Least Squares Means						
Effect	dose_level	Estimate	Standard Error	DF	t Value	Pr > |t|
dose_level	0	1.2489	0.05160	59	24.20	<.0001
dose_level	0.1	1.1800	0.04896	59	24.10	<.0001
dose_level	0.32	1.3539	0.05473	59	24.74	<.0001
dose_level	1	1.3250	0.05851	59	22.64	<.0001
dose_level	3.2	1.3287	0.05160	59	25.75	<.0001
dose_level	10	1.4048	0.05473	59	25.66	<.0001
dose_level	32	1.3074	0.05851	59	22.34	<.0001
dose_level	100	1.1999	0.05160	59	23.25	<.0001

Differences of Least Squares Means									
Effect	dose_level	_dose_level	Estimate	Standard Error	DF	t Value	Pr > t	Adjustment	Adj P
dose_level	0	0.1	0.06889	0.07113	59	0.97	0.1684	Dunnett	0.5028
dose_level	0	0.32	-0.1050	0.07523	59	-1.40	0.9160	Dunnett	0.9981
dose_level	0	1	-0.07611	0.07802	59	-0.98	0.8334	Dunnett	0.9915
dose_level	0	3.2	-0.07978	0.07298	59	-1.09	0.8606	Dunnett	0.9943
dose_level	0	10	-0.1559	0.07523	59	-2.07	0.9787	Dunnett	0.9999
dose_level	0	32	-0.05854	0.07802	59	-0.75	0.7720	Dunnett	0.9827
dose_level	0	100	0.04900	0.07298	59	0.67	0.2523	Dunnett	0.6429

Table S25 Least Squares Means and Differences (Dunnett adjusted) for Trichlorfon

Least Squares Means						
Effect	dose_level	Estimate	Standard Error	DF	t Value	Pr > |t|
dose_level	0	1.1620	0.03259	64	35.66	<.0001
dose_level	0.1	1.1576	0.03895	64	29.72	<.0001
dose_level	0.32	1.1786	0.03259	64	36.17	<.0001
dose_level	1	1.1911	0.03644	64	32.69	<.0001
dose_level	3.2	1.1536	0.03644	64	31.66	<.0001
dose_level	10	1.1559	0.03259	64	35.47	<.0001
dose_level	32	1.1267	0.03435	64	32.80	<.0001
dose_level	100	1.0891	0.03259	64	33.42	<.0001

Differences of Least Squares Means									
Effect	dose_level	_dose_level	Estimate	Standard Error	DF	t Value	Pr > t	Adjustment	Adj P
dose_level	0	0.1	0.004429	0.05079	64	0.09	0.4654	Dunnett	0.8642
dose_level	0	0.32	-0.01660	0.04609	64	-0.36	0.6401	Dunnett	0.9510
dose_level	0	1	-0.02912	0.04888	64	-0.60	0.7233	Dunnett	0.9742
dose_level	0	3.2	0.008375	0.04888	64	0.17	0.4323	Dunnett	0.8401
dose_level	0	10	0.006100	0.04609	64	0.13	0.4476	Dunnett	0.8516
dose_level	0	32	0.03533	0.04735	64	0.75	0.2291	Dunnett	0.6131
dose_level	0	100	0.07290	0.04609	64	1.58	0.0593	Dunnett	0.2382

Table S26 Least Squares Means and Differences (Dunnett adjusted) for Permethrin

Least Squares Means						
Effect	dose_level	Estimate	Standard Error	DF	t Value	Pr > |t|
dose_level	0	1.2843	0.02811	83	45.69	<.0001
dose_level	0.1	1.2013	0.02936	83	40.92	<.0001
dose_level	0.32	1.1843	0.03442	83	34.40	<.0001
dose_level	1	1.1851	0.02936	83	40.37	<.0001
dose_level	3.2	1.2052	0.03079	83	39.14	<.0001
dose_level	10	1.1922	0.03079	83	38.72	<.0001
dose_level	32	1.2896	0.03079	83	41.88	<.0001
dose_level	100	1.1549	0.03079	83	37.51	<.0001
dose_level	320	1.2150	0.03079	83	39.46	<.0001

Differences of Least Squares Means									
Effect	dose_level	_dose_level	Estimate	Standard Error	DF	t Value	Pr > t	Adjustment	Adj P
dose_level	0	0.1	0.08298	0.04064	83	2.04	0.0222	Dunnett	0.1174
dose_level	0	0.32	0.1000	0.04444	83	2.25	0.0135	Dunnett	0.0767
dose_level	0	1	0.09916	0.04064	83	2.44	0.0084	Dunnett	0.0503
dose_level	0	3.2	0.07905	0.04169	83	1.90	0.0307	Dunnett	0.1542
dose_level	0	10	0.09205	0.04169	83	2.21	0.0150	Dunnett	0.0839
dose_level	0	32	-0.00535	0.04169	83	-0.13	0.5509	Dunnett	0.9293
dose_level	0	100	0.1294	0.04169	83	3.10	0.0013	Dunnett	0.0090
dose_level	0	320	0.06925	0.04169	83	1.66	0.0502	Dunnett	0.2291

Table S27 Least Squares Means and Differences (Dunnett adjusted) for Glufosinate-ammonium_1

Least Squares Means						
Effect	dose_level	Estimate	Standard Error	DF	t Value	Pr > |t|
dose_level	0	1.2348	0.03353	46	36.82	<.0001
dose_level	0.1	1.1910	0.03353	46	35.52	<.0001
dose_level	0.32	1.2200	0.03535	46	34.52	<.0001
dose_level	1	1.2131	0.03535	46	34.32	<.0001
dose_level	3.2	1.0924	0.03535	46	30.91	<.0001
dose_level	10	0.9366	0.04742	46	19.75	<.0001

Differences of Least Squares Means									
Effect	dose_level	_dose_level	Estimate	Standard Error	DF	t Value	Pr > t	Adjustment	Adj P
dose_level	0	0.1	0.04380	0.04742	46	0.92	0.1802	Dunnett	0.4697
dose_level	0	0.32	0.01480	0.04872	46	0.30	0.3813	Dunnett	0.7483
dose_level	0	1	0.02169	0.04872	46	0.45	0.3291	Dunnett	0.6907
dose_level	0	3.2	0.1424	0.04872	46	2.92	0.0027	Dunnett	0.0118
dose_level	0	10	0.2982	0.05808	46	5.13	<.0001	Dunnett	<.0001

Table S28 Least Squares Means and Differences (Dunnett adjusted) for Glufosinate-ammonium_2

Least Squares Means						
Effect	dose_level	Estimate	Standard Error	DF	t Value	Pr > |t|
dose_level	0	1.4068	0.02898	62	48.54	<.0001
dose_level	0.01	1.3767	0.03633	62	37.89	<.0001
dose_level	0.032	1.3931	0.03040	62	45.83	<.0001
dose_level	0.1	1.3567	0.02898	62	46.81	<.0001
dose_level	0.32	1.3564	0.03399	62	39.91	<.0001
dose_level	1	1.3812	0.03040	62	45.44	<.0001
dose_level	3.2	1.2048	0.03040	62	39.63	<.0001
dose_level	10	0.9673	0.05550	62	17.43	<.0001

Differences of Least Squares Means									
Effect	dose_level	_dose_level	Estimate	Standard Error	DF	t Value	Pr > t	Adjustment	Adj P
dose_level	0	0.01	0.03010	0.04648	62	0.65	0.2598	Dunnett	0.6836
dose_level	0	0.032	0.01372	0.04200	62	0.33	0.3725	Dunnett	0.8123
dose_level	0	0.1	0.05009	0.04099	62	1.22	0.1131	Dunnett	0.4057
dose_level	0	0.32	0.05044	0.04467	62	1.13	0.1315	Dunnett	0.4505
dose_level	0	1	0.02562	0.04200	62	0.61	0.2721	Dunnett	0.7004
dose_level	0	3.2	0.2020	0.04200	62	4.81	<.0001	Dunnett	<.0001
dose_level	0	10	0.4395	0.06261	62	7.02	<.0001	Dunnett	<.0001