2026 S.T. Yau High School Science Award (Asia)

Research Report

The Team

Registration Number: Bio-101

Name of team member: Connie Shien Jin
School: UWC Thailand
Country: Thailand

Name of supervising teacher: Adam Dorsey
Job Title: High School Biology Teacher
School: UWC Thailand
Country: Thailand

Title of Research Report:

Evaluating the toxicity of 1-Nitronaphthalene (1-NN) on chicken embryos using egg shell-less incubation system

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Evaluating the toxicity of 1-Nitronaphthalene (1-NN) on chicken embryos using egg shell-less incubation system

[Connie Shien Jin]

Abstract

1-Nitronaphthalene (1-NN), a nitro-polycyclic aromatic hydrocarbon (nitro-PAH) with a nitro group attached to the 1-position of the naphthalene ring, accounts for approximately 30–50% of the total concentration of gas-phase nitro-PAHs. 1-NN induces developmental, haematological, hepatic, and genotoxic effects in mammals or aquatic animals. However, the toxicity of 1-NN to avian embryonic development remains unclear.

This study utilized the *Ex ovo* shell-less incubation system, which enables real-time developmental observation, to evaluate the developmental toxicity of environmentally relevant concentrations of 1-NN in chicken embryos. Embryos were microinjected at embryonic day 0 (E0) with 0.1% DMSO serving as the solvent control or three environmentally relevant concentrations of 1-NN (100, 1000, and 10000 ng/g). After 52 h of pre-incubation, embryos were transferred to artificial vessels and cultured at 37.8 °C with 60% humidity. Dynamic morphological monitoring was performed from E3 to E9, including vascular parameters (E3–E4), heart rate (E3–E5), and growth parameters. All embryos were dissected at E9 to measure body weight and organ weights. Cardiac tissues were collected for subsequent molecular analyses via RT-qPCR and western blot assays.

In this study, we found no significant differences in the survival rates of chicken embryos on E9. Compared to the control group, heart weight and heart / body weight ratio were both significantly increased (p<0.05) in 1-NN-H group at E9. There is a trend of vessel junctions and vessel length decreasing from control group to 1-NN-H group in E3 and E4 (no significant difference). There is no significant difference in body length, heart rate, forelimb length, hindlimb length, and eye diameter across all exposure groups from E3 to E9. Similarly, no significant differences were observed in body weight, liver weight, liver/body weight ratio, left eye weight, left eye/body weight ratio, right eye weight, right eye/body weight ratio, brain weight, and brain/body weight ratio compared to the control group at E9. The fold change of both *CYP1A5* relative to *18S* (RNA) and AhR relative to GAPDH (protein) showed no significant difference across exposure groups or a clear dose-dependent trend.

In summary, our findings provide statistical evidence for the adverse effects of environmentally relevant 1-NN exposure. Using egg shell-less incubation systems, we demonstrated that 1-NN possesses potential cardiovascular toxicity to the development of chicken embryos. Given the widespread occurrence of 1-NN in the environment, this model can serve as a reference for studying nitro-PAH-induced embryonic toxicity and conducting ecological risk assessments.

Keywords: Environmental Pollutant; 1-Nitronapthalene (1-NN); Shell-less Incubation System; Toxicity; Cardiovascular

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1. Background

1.1 Polycyclic Aromatic Hydrocarbons

The combustion of fossil fuels, ever-increasing to meet transportation demand, industrial expansion, and population growth, have led to the expedited production of hazardous air pollutants, posing significant health risks to humans.

Among such molecules are polycyclic aromatic hydrocarbons (PAHs), a class of ubiquitous and highly lipophilic environmental pollutants. PAHs consist of only hydrogen and carbon atoms, and are composed of two or more bonded benzene rings [9]. They are mostly derived from anthropogenic sources through incomplete combustion of organic materials, though natural sources contribute minimally [10]. PAHs are commonly detected in air (<5-200000 ng/m³), water (25-4427 ng/L), soil (29-1940 ng/g), and sediment (0.01-21329 ng/g,) but concentrations vary vastly based on sampling location [11-16]. The molecule can enter the human body via various routes [17], as shown in Figure 1.

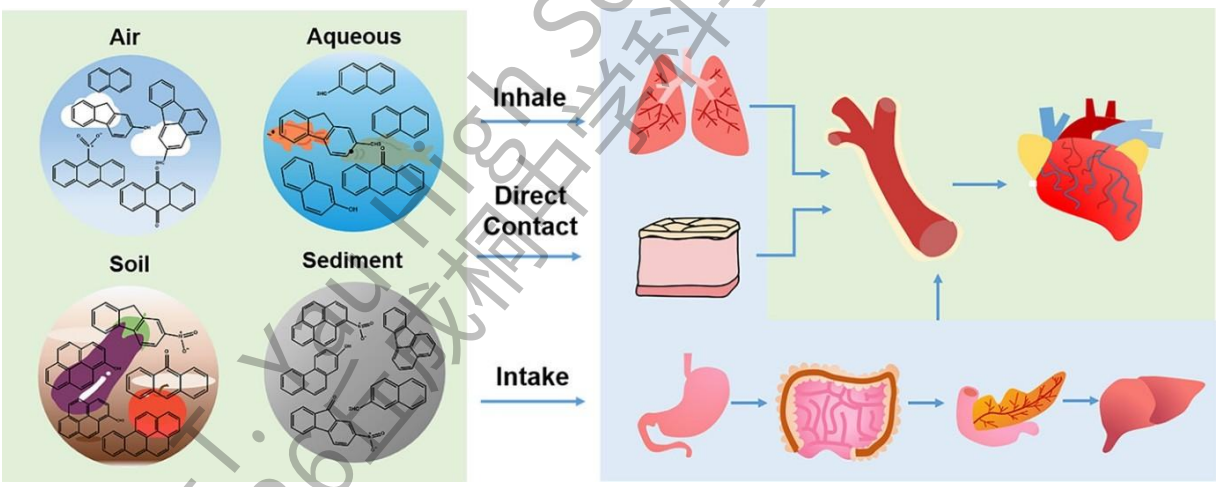


Figure 1. Diagram of main pathways of the exposure of PAHs to human body. Left shows PAHs presence in the environment (air, aqueous, soil sediment). Right shows systems affected by PAHs exposure (lung, skin, bloodstream, heart, digestive system) [18].

Widely recognised as carcinogens, mutagens, and teratogens, PAHs have been proven to pose a significant threat to human health. PAH toxicity has been linked with their ability to activate aryl hydrocarbon receptors (AhR), highly expressed in the liver and adipose tissue. AhR significantly upregulates the transcription of cytochrome P450 (CYP)1 isoforms (CYP1A1, CYP1B1). PAH hepatic bioactivation, through major pathways including CYP-mediated epoxidation, peroxidase activation,

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and aldo-keto reductase-mediated o-quinone formation, collectively generates reactive or redox-active intermediates (epoxides and diol-epoxides), capable of inducing oxidative stress or forming DNA adducts [19]. The formation of DNA adducts may interfere with DNA replication and transcription, and induce mutations [20], while epoxides, with electrophilic reactivity, can damage essential cellular components [21]. In humans, prenatal exposure to carcinogenic PAHs at an environmentally relevant range have been linked to fetal chromosomal abnormalities. Exposure to PAHs during pregnancy has also led to intrauterine growth restriction and premature birth, highlighting their developmental toxicity potential [22].

1.2 Nitrogenated polycyclic aromatic hydrocarbons

Nitrogenated polycyclic aromatic hydrocarbons (nitro-PAHs) are derivatives of PAHs, with at least one nitro group attached to the aromatic ring. Although concentrations of nitro-PAHs vary as much as 5 times from month to month, urban areas have concentrations 2-3 times higher than suburban areas, and their concentrations are one or two orders of magnitude lower than their parent PAHs [23-24]. Over half of nitro-PAHs are formed through secondary atmospheric reactions, but nitro-PAHs are also directly produced by the pyrolysis of fossil fuels. Its primary formation is exacerbated by urbanisation, a primary driver of climate change; its secondary formation is favoured by increasing NO2 concentrations, higher temperatures, and stagnant air, conditions increasingly associated with climate-change-driven atmospheric changes [25]. Additionally, nitro-PAHs are persistent pollutants that are highly resistant to degradation [26]. Hence, nitro-PAHs play an increasingly relevant role in modern-day atmospheric pollution and climate change, especially in urban areas, emphasising the necessity of investigating their toxicological effects and health concerns.

Nitro-PAHs can be 100000 times more mutagenic and 10 times more carcinogenic compared to unsubstituted PAHs [24]; some nitro-PAHs have been assigned higher toxicity equivalence factors than their parent PAHs, due to additional nitroreduction and conjugation reactions creating reactive nitrogen species, on top of PAH-typical CYP oxidation to reactive epoxides and DNA adducts [27-28]. Other

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effects include acute and chronic cytotoxicity, observed in mouse cells [28]. This distinction indicates that toxicity data for PAHs may not accurately represent or may underestimate the effects of nitro-PAHs, highlighting the necessity to evaluate members independently.

1.3 1-Nitronaphthalene

1-nitronaphthalene (1-NN) is a nitro-PAH consisting of a naphthalene ring system substituted with a nitro group at the 1-position. Although this chemical is predominantly used in manufacturing, with relatively low environmental concentrations (up to 10.46 ng/m³) [3], its presence in diesel exhaust [29], occupational settings, and environmental persistence collectively warrants toxicological investigation.

The potential routes of exposure of 1-NN are showcased in Figure 1. It can undergo metabolic reduction to form 1-naphthylamine (1-NA), a potential carcinogen [30]. Electrophilic metabolic precursors of 1-NN and 1-NA form aminonaphthylcysteine (ANC) adducts, which bind to haemoglobin, hindering its oxygen carrying capacity. As a nitro-PAH, the nitroreduction of 1-NN may produce N-oxy intermediates, which have shown to be carcinogenic and/or mutagenic across several studies, both in vitro and in vivo [30]. Additionally, like many PAHs, its oxidation by CYP forms epoxides, eventually leading to cytotoxicity. According to Baldwin et al. [31], CYP2F bioactivation of 1-NN results in its metabolism to both 5,6- and 7,8-epoxides, differing from the single epoxide formed by its parent PAH, naphthalene. This shows that the addition of the nitro group may increase genotoxicity potential due to the formation of dual epoxide species with independent DNA adduct-forming potential. Epoxide carcinogenicity is largely dependent on structure, hence the toxicity of 1-NN cannot be reliably extrapolated [21].

Literature demonstrates organ-specific and systemic toxicity of 1-NN. Mouse liver and lung microsomes have metabolised 1-NN into products which bind macromolecules. Lung slices show binding in the epithelial cells of the bronchioles and the smaller airways [32]; at 25000 ng/g, cells were swollen and necrotic [33]. Implantation of 1-NN into pregnant mummichog (*Fundulus heteroclitus*), an aquatic vertebrate, has affected several parameters, the most significant being hatchability, with others

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including embryo normality, and embryo growth and survival. The lowest observed effect concentration (LOEC) of 1-NN was 447 ng/g [34]. Notably, our experimental concentrations (100 ng/g - 10000 ng/g) span and exceed this LOEC, supporting their environmental and toxicological relevance.

Although its bioactivation pathways and organ-specific toxicity have been well-documented, primarily in adult rodent models, the effect of 1-NN on embryonic development, a period of heightened xenobiotic vulnerability, remains relatively uncharacterised. In particular, its developmental toxicology in avian species remains understudied. Given that 1-NN generates reactive metabolites, binds to essential macromolecules, and induces oxidative stress, all mechanisms known to interfere with embryonic development and organogenesis, 1-NN may plausibly interfere with avian growth. Additionally, though there is no documentation of 1-NN's cardiac toxicity specifically, AhR activation, inhibition, and knockdown have been shown to significantly inhibit the formation of contractile cardiomyocyte nodes, and disrupt multiple cardiac morphogenesis and differentiation signaling pathways [35]. This suggests potential for 1-NN cardiac toxicity, beyond the much-investigated lungs and liver. Thus, this study investigates the potential teratogenic and cardiotoxic effects of 1-NN on avian embryos using an *Ex ovo* chicken model.

1.4 Chicken ex ovo model

Chicken embryos (*Gallus gallus domesticus*) are one of the most well-studied and documented biological organisms, particularly for developmental research, embryology, and teratology. Their oviparity enables a wider range of experiments, without external influence from maternal factors. In particular, their chorioallantoic membrane (CAM) allows for non-invasive administration of drugs. The complete sequencing of the chicken genome allows for genetic analysis and broad comparison to humans [36]. Chicken embryos adhere to the 3R principles (replacement, reduction, refinement), reducing ethical concerns present in mammalian models, and present a simple, quick (21-day development), low-cost model [37].

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Ex ovo culture of chicken embryos, compared with the traditional *in ovo* method, allows for greater precision in manipulation at specific stages, makes the developmental process transparent, and showcases embryonic development without a protective eggshell layer [38]. Researchers have already developed such systems for environmental toxicology studies, which this study follows [8].

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2. Methodology

2.1 Chemicals and reagents

1-nitronaphthalene (1-NN) was acquired from Fudan University. A stock solution of 50000 ng/L was made, then diluted to 10000 ng/L, 1000 ng/L, and 100 ng/L. Ultrapure water was acquired from MILL-Q Direct 8 (Millipore). The 1-NN-L (100 ng/g), 1-NN-M (1000 ng/g), and 1-NN-H (10000 ng/g) groups are collectively referred to as "exposure groups".

2.2 *Ex ovo* culture system

2.2.1 1-NN exposure and incubation

Fertilised Hy-Line white chicken (*Gallus gallus domesticus*) eggs were acquired from Zhejiang Lihua Agricultural Development (Group) Co., Ltd.), ranging from 52.27-56.48 g. Eggs were assigned randomly to four groups: control group (CO), low concentration (1-NN-L), medium concentration (1-NN-M), high concentration (1-NN-H), with n=20. On embryonic day 0 (E0), a 1mm hole was drilled in the air sac of the egg. Exposure groups were injected with 1-NN (1-NN-L, 1-NN-M, 1-NN-H), while CO group was injected with 0.1% DMSO. The holes were covered using parafilm. Eggs were incubated at 37.8°C, 60% humidity, rotated 180° every hour, for 52 hours (E0-E2) before being transferred into artificial vessels.

2.2.2 Preparation of Ex ovo incubation system

The experiment was conducted in a fume hood, sanitised with ultraviolet (UV) radiation for 30 minutes prior to the experiment. The artificial incubation vessel was made using food-grade polyethene wrap (PE cling wrap, MiaoJie, China), formed into a 8 cm deep concave using a template, then secured on top of a 450 mL polystyrene (PS) cup. The finished vessel was sterilised under an hour of UV radiation. Incubated eggs were cleaned with 70% ethanol, and transferred inside the PE mould. The vessel was covered with PE wrap. Embryos were incubated by *Ex ovo* from E3-E9. Procedures are described in Figure 2.

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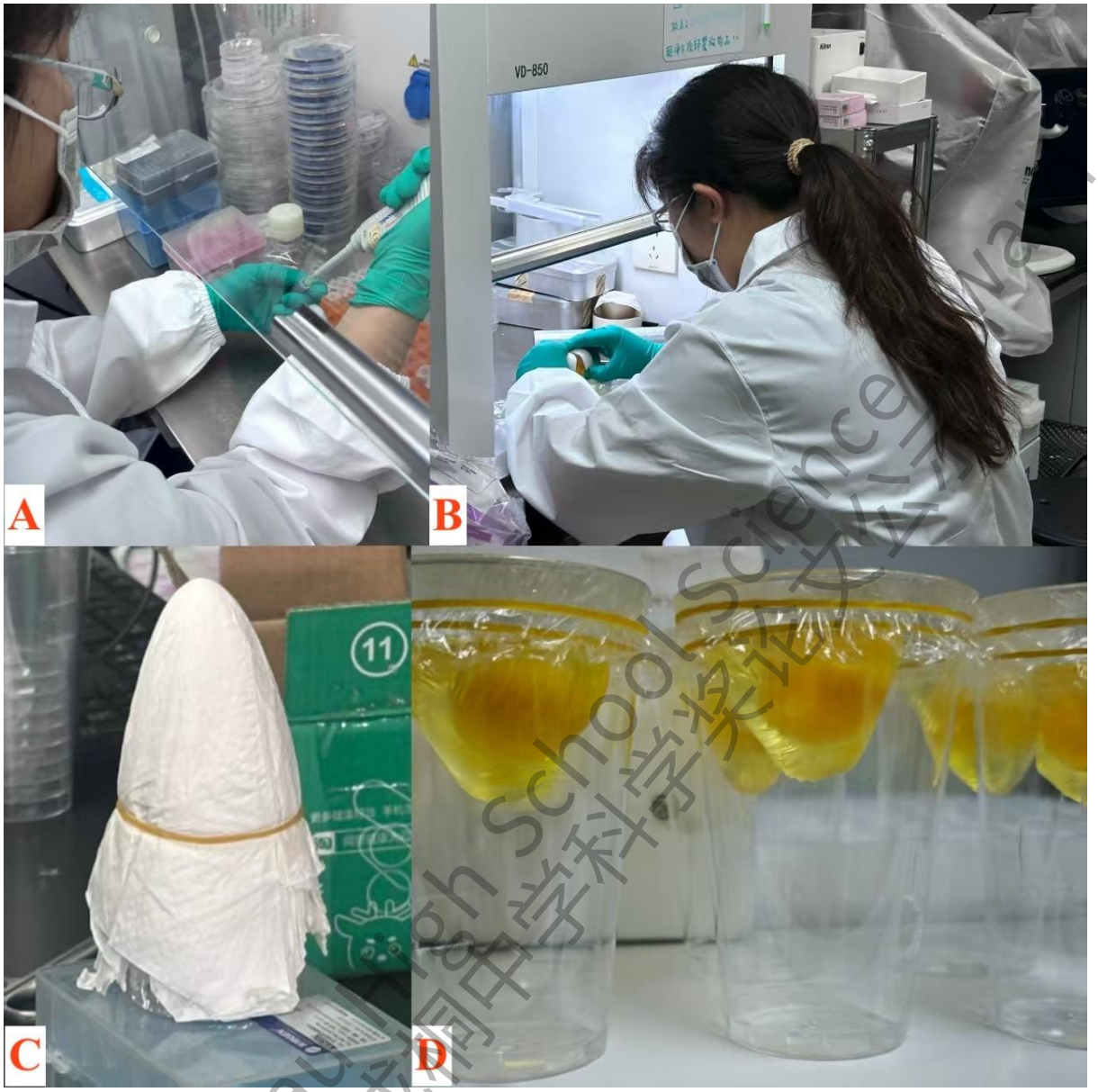


Figure 2. Process of establishing *Ex ovo* system. A. The dilution of 1-NN stock solution. B. The production of the *Ex ovo* system, by cracking an egg into the PE wrap. C. The template used to form the incubation vessel. D. Image of incubation vessels containing cracked eggs, illustrating the complete experimental set-up. It is for demonstrative purposes only, and was not taken during the experimental trials.

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2.3 Dissection

Embryos were dissected at E9. The body, heart, eyes, liver, and brain were weighed. The chorioallantoic membrane (CAM), heart, eyes, liver, and brain were collected and stored at −80°C for further testing.

2.4 Embryo monitoring

2.4.1 Morphometric measurements

Body and head length were recorded from E3 to E9, while eye diameter, forelimb, and hindlimb length were recorded from E4 to E9, when they were first visible. Photos were taken using a Canon EOS 700D, with a ruler placed in-frame as scale reference, calibrating lengths to measure parameters in ImageJ. Each measurement was taken thrice, and averaged. Organ/weight ratios were acquired by dividing the weight of respective organs by the total weight of the embryo. Throughout this study, "organ/weight" refers to the ratio between a given organ and total embryo weight. Method for quantification of morphometric measurements is reflected in Figure 3.

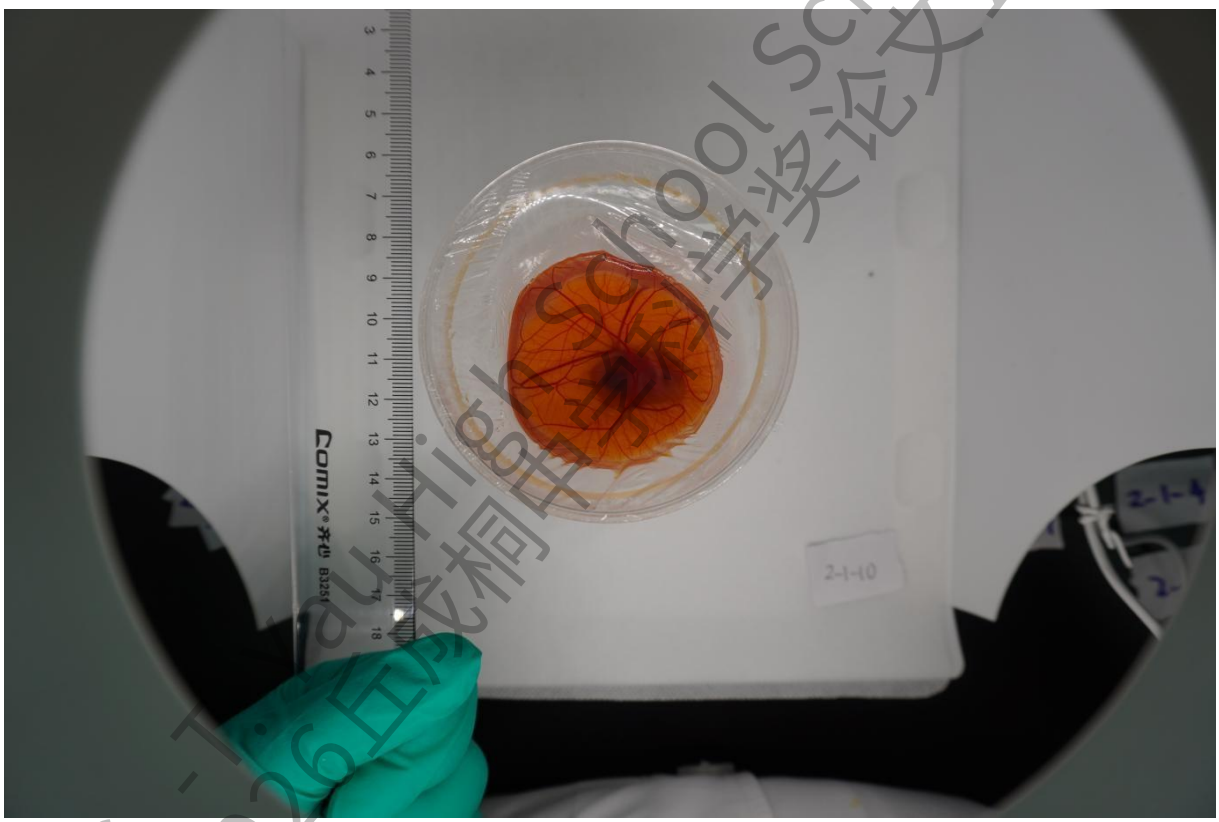


Figure 3. Representative image of the *Ex oxo* embryo (E9, 1-NN-H) used to acquire morphometric measurements. Images were taken under standardised conditions, and a ruler (left) is included to calibrate measurements in ImageJ.

2.4.2 Vascular parameters

Vascular parameters (vessel junction and length) were recorded from E3-E4. Photos were cropped to leave only the CAM, and then converted to 8-bit in ImageJ. Vascular parameters were counted using AngioTool (vessel diameter: 0-3, intensity: 3, remove small particles: 24, fill holes:0). (Figure 4).

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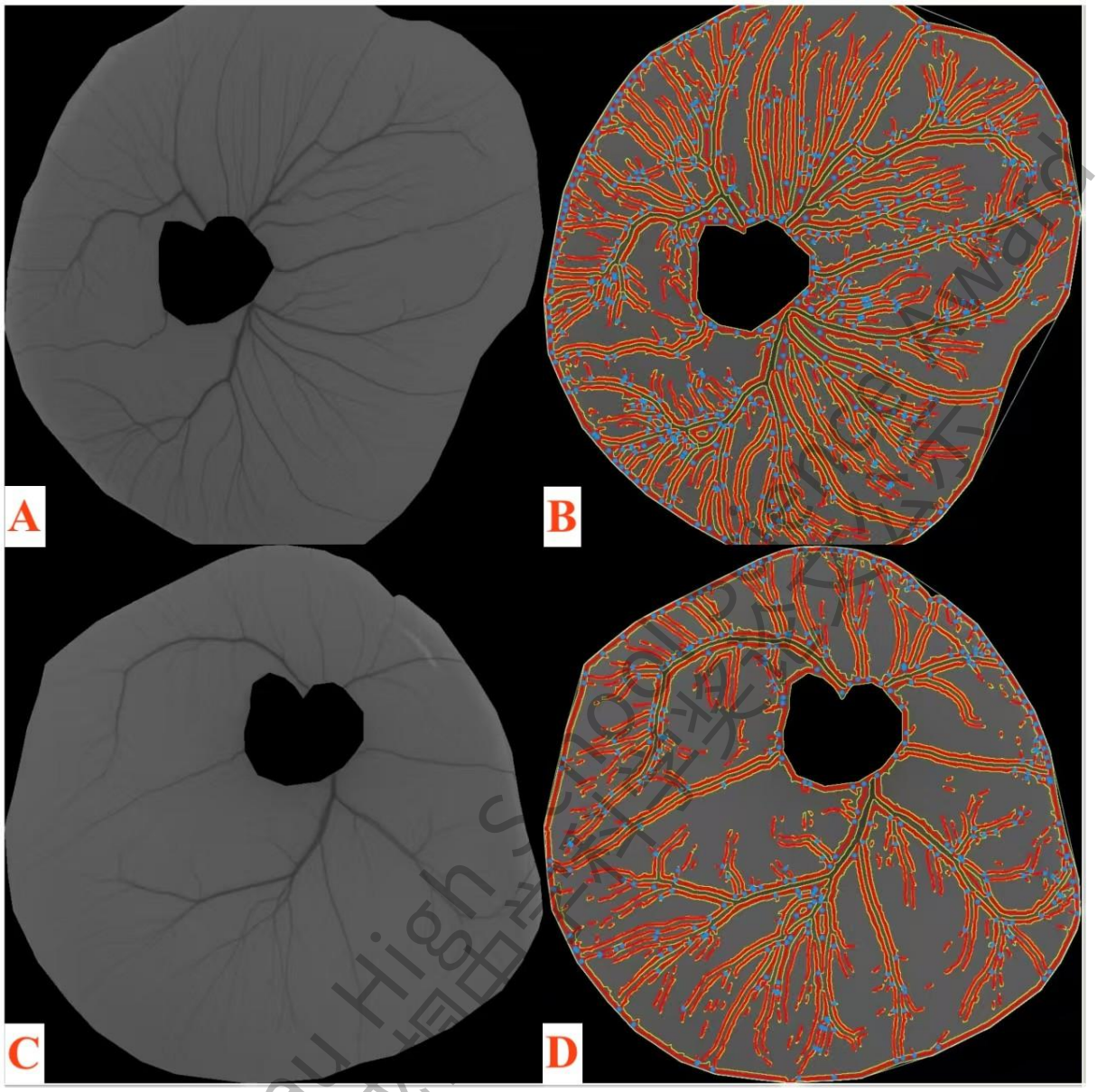


Figure 4. Quantifying the vascular parameters of the CAM membrane. Image of the CAM: A (E4, CO), C (E4, 1-NN-H), prior to processing in ImageJ; measuring of vascular parameters in AngioTool: B (E4, CO), D (E4, 1-NN-H); red lines indicate vessel length, blue dots highlight junctions.

2.4.3 Heart rate

Heart rate was recorded from E3-E5. 30-second videos were taken. Beats per minute (bpm) were manually counted, with the help of a counting app (Click Counter). Each video was counted independently twice by the same observer.

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2.5 Gene expression analysis

2.5.1 Tissue selection and sampling

Based on morphometric analyses, the organ exhibiting the most apparent exposure-associated differences, the heart, was selected for further molecular analysis. From the samples collected within each exposure group, three samples were randomly chosen for RNA quantification and protein extraction.

2.5.2 RNA extraction and cDNA

Total RNA from the heart was extracted using RNA Easy Fast Tissue/Cell Kit (TIANGEN, Ref. No. GDP451), according to the manufacturer's instructions. Tissue samples were homogenised by repeated pipetting in lysis buffer, until a homogeneous lysate was acquired. RNA was eluted in 50 µL of RNase-free water. Complementary DNA (cDNA) was synthesised from 1 µg of RNA, using PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Cat. No. RR047A). Reverse transcription was performed using a mixture of oligo-dT primers and random hexamers. Synthesised cDNA and primers were then prepared for Real Time quantitative PCR (RT-qPCR) analysis.

The target gene is *CYP1A5*, with *18S* used as the housekeeping gene. RT-qPCR primers targeting *CYP1A5* and *18S* were ordered from Sangon Biotech, whose length and sequence are described in Table 1. The reaction was performed using THUNDERBIRD™ SYBR™ qPCR Mix (TOYOBO, Cat. No. QPS-201), containing 2.5 µL SYBR Green I qPCR Mix, 1 µL cDNA, 0.25 µL of each forward and reverse primer, and 0.95 µL of RNase-free water in a total reaction volume of 5 µL. Reactions were run on a 96-well plate on a Quantagene 900 (Kubotechnology). Cycling conditions were: precycling (95°C for 30 seconds), followed by 40 cycles of denaturing (95°C for 5 seconds) and annealing (60°C for 30 seconds), followed by a melt curve analysis from 60-95°C to confirm amplicon specificity. Relative expression of genes were quantified using the ΔΔCt method, normalised to CO.

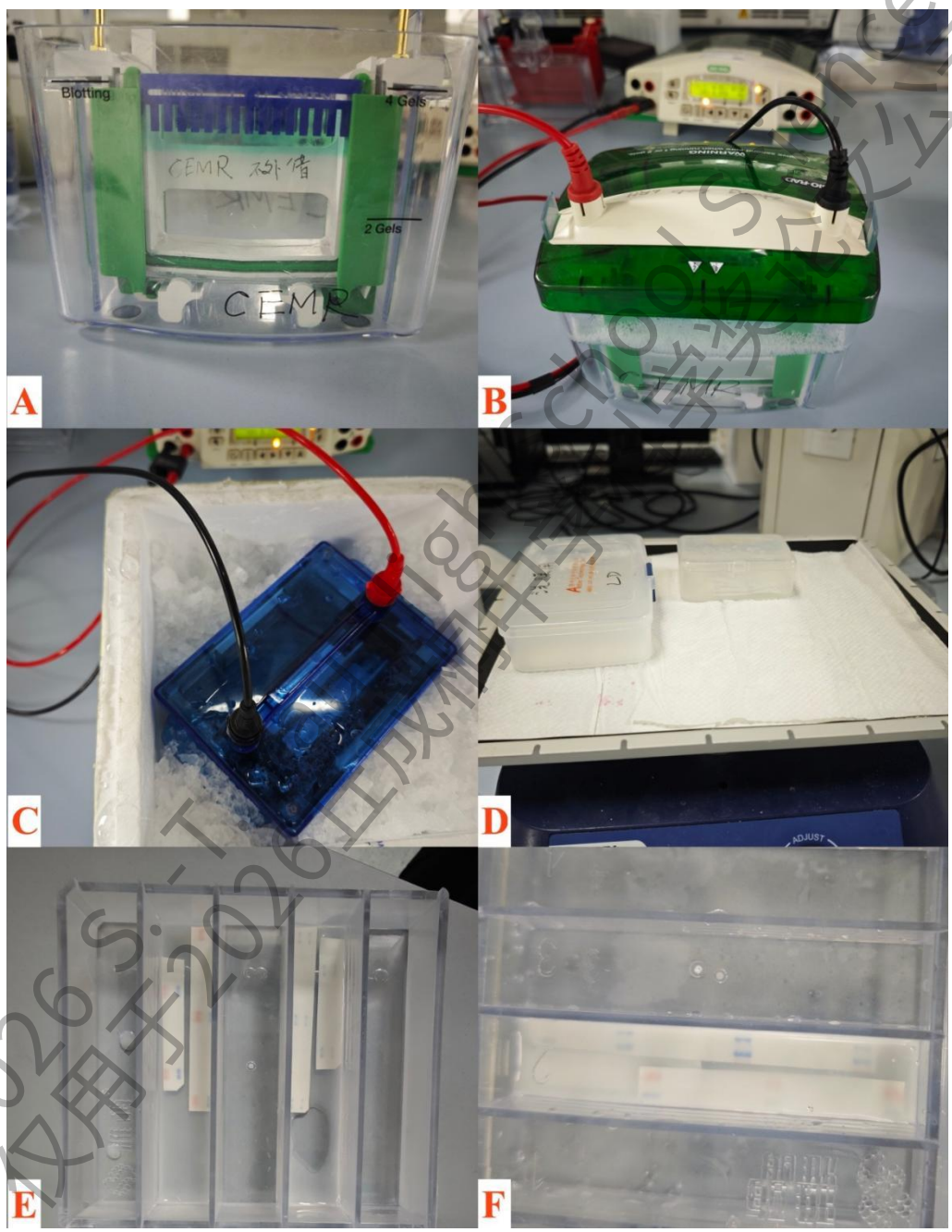
	Forward primer (5'-3')	Primer length	Reverse primer (3'-5')	Primer length

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		(nt)		(nt)
CYP1A5	CACCACGCTTCCCCTAGGTT	20	CAGGAGCAGGCAGAAAGTGG	20
18S	GTAACCCGTTGAACCCCATT	20	CCATCCAATCGGTAGTAGCG	20

Table 1. Sequence and length of gene *CYP1A5* and *18S* primers for RT-qPCR.

2.6 Western blot



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Figure 5. Workflow of the Western blot procedure. A. Preparation of gel. B. Gel electrophoresis, with the proteins and marker loaded. C. Transfer of proteins from gel to membrane, over ice. D. Blocking, on the rotary shaker. E. Incubation of primary antibody: anti-GAPDH (left), anti-*AhR* (right). F. Incubation of secondary antibody.

2.6.1 Protein extraction and quantification

Heart tissue was lysed in 300 µL of T-PER™ Tissue Protein Extraction Reagent (Thermo Scientific, Cat. No. 78510), supplemented with 6 µL of protease inhibitor (Thermo Scientific, Cat. No 87786) and 6 µL of phosphatase inhibitor (Thermo Scientific, Cat. No 78420, diluted 1:100), together with 2 steel beads per sample, and homogenised at 60 Hz for 1 minute. Lysates were centrifuged at 12000 rpm at 4°C for 10 minutes. 250 µL of supernatant was collected per sample. Protein concentration was determined using a BCA Protein Assay Kit (Novoprotein Scientific Inc., Cat. No. PA002). A bovine serum albumin (BSA) standard curve was created, combining 0, 1, 2, 4, 8, 12, 16, and 20 µL of protein standard with standard diluent, made up to a total volume of 20 µL per well, corresponding to final concentrations of 0, 0.025, 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 mg/mL. Absorbance was measured at 562 nm, within the recommended range of 540-595 nm, using a BioTek Synergy Microplate Reader. Protein concentrations of samples were calculated from the standard curve, accounting for dilution factor.

2.6.2 Gel electrophoresis

Protein concentrations were normalised with standard diluent, according to the standard curve. Loading buffer (Omni-Easy™ Rapid-Dissolving Protein Loading Buffer, Epizyme, Cat. No. LT101s) was added at ⅕ of the total volume to increase sample density. Proteins were boiled at 100°C for 10 minutes. 5µL of denatured proteins and PageRuler™ Plus Prestained Protein Ladder (Thermo Scientific, Cat. No. 26619) were loaded onto a 10% one-step polyacrylamide gel, prepared according to manufacturer's instructions. Electrophoresis was carried out in Tris-Glycine SDS-PAGE Running Buffer (Beyotime, Cat. No. P0014B), initially at 80 V for 30 minutes, then at 120 V for 1 hour.

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2.6.3 Membrane transfer

Proteins were transferred onto PVDF membranes (Immobilon® -P PVDF Membrane, Merck Millipore, Ref. No. IPVH00010), first activated for 5 minutes in 100% methanol. Wet transfer was conducted in wet-transfer buffer (Western Rapid Transfer Buffer (Powder), Beyotime, Cat. No. P0575-1L), mixed with 800 mL of ultrapure water and 200 mL of 100% methanol, at 250 mA for 1 hour 20 minutes on ice.

2.6.4 Blocking and antibody probing

Membranes were blocked in 5% non-fat milk (NON-Fat Powdered Milk, Sangon Biotech, No. A600669-0250) in TBST (8 g Tris-base, 16 g NaCl, 0.4 g KCl, 1 mL of TWEEN-20, 1 L distilled water), at room temperature for an hour, on a rotary shaker at 40 rpm. Membranes were then incubated with primary antibodies against AhR (Rabbit Polyclonal, Beyotime, Cat. No. AF6165) and GAPDH (Rabbit Monoclonal, Beyotime, Cat. No. AF1186), both diluted 1:1000 in 5% non-fat milk, overnight at 4°C. Membranes were washed with TBST for 8 minutes for three times at 60 rpm. Afterwards, membranes were incubated with HRP-conjugated Goat Anti-Rabbit IgG (H+L) secondary antibody (Beyotime, Cat. No. A0208) at room temperature for 1 hour. Finally, membranes were washed with TBST for 5 minutes for three times at 80 rpm.

2.6.5 Detection and quantification

Protein bands were visualised using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Cat. No. WBKLS0100), and imaged on Bio-Rad Image Lab. Band intensities were quantified using ImageJ, and normalised to GAPDH. Normalised values were expressed as fold change relative to CO.

2.7 Statistical analyses

Unless otherwise indicated, all data is represented as mean ± standard error of the mean (SEM). Differences between survival rates were calculated using Kaplan-Meier Method on the GraphPad Prism software. Similarly, GraphPad was used for one-way analysis of significance (ANOVA) followed by Tukey's test to analyse the significance between treatment groups. Results with $p<0.05$ were considered to be statistically significant.

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3. Results

3.1 General morphological development

Representative images of CO group embryo are shown from E3-E9 in Figure 6, indicating the development of the CAM membrane, including vessel length, density and thickness, and corresponding growth in the embryo. No overt morphological disfigurations/abnormalities were observed across treatment groups throughout development.

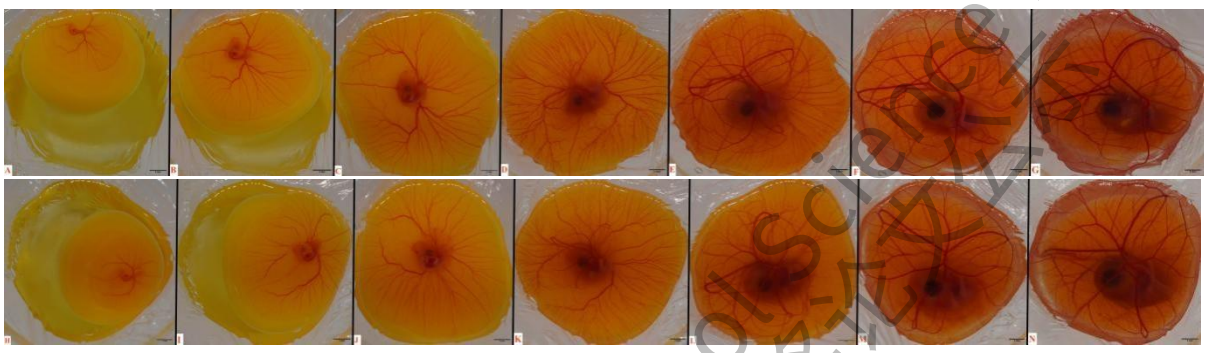


Figure 6. Representative images of embryo from E3-E9. A. to G. is CO, H. to N. is 1-NN-H, indicating the development of the CAM membrane and growth of embryo. Scale bar = 5 mm.

Chicken embryos were incubated for a total of 7 days, from E3 to E9. The survival rate of CO (35.7%) was higher than 1-NN-M (22.2%) and 1-NN-H (30.8%), though differences between all treatment groups were not statistically significant, and showed no dose-dependent relationship (Figure 7).

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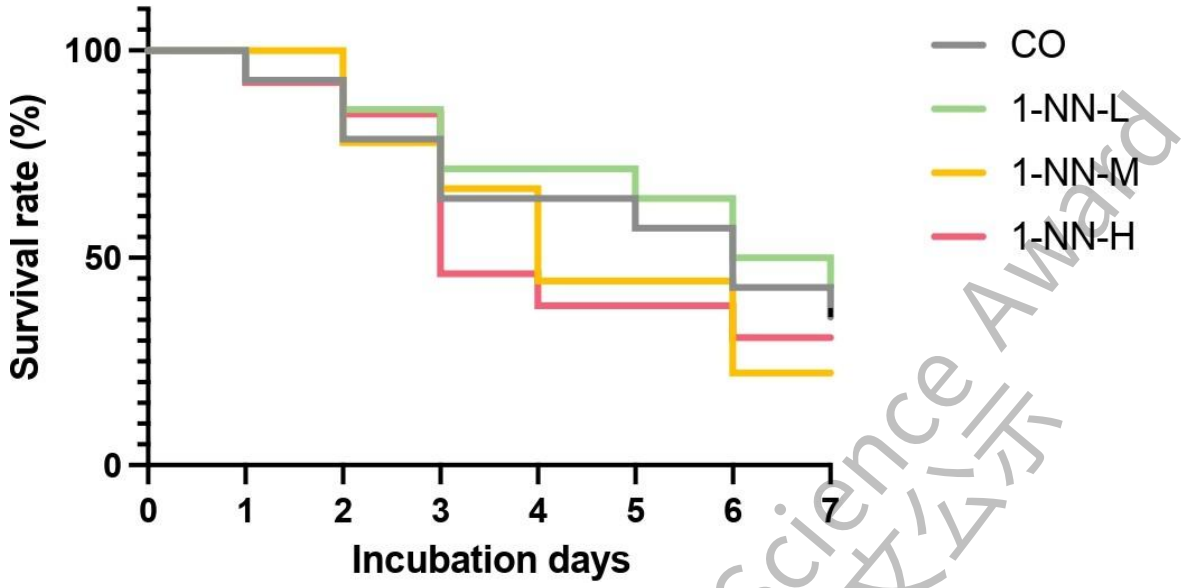


Figure 7. Effect of 1-NN on survival rate of chicken embryo, from E3-E9. CO: control; 1-NN-L: low concentration; 1-NN-M: medium concentration; 1-NN-H: high concentration.

3.2 Morphometric measurements

No significant differences were observed between treatment groups for body, head length and eye diameter across all embryonic days. Furthermore, there are no notable, consistent trends between all morphometric measurements across several days.

Mean body length (mm) increased progressively across the observation period in all treatment groups. On E3, the mean body length was relatively comparable. CO had a length of 9.693 ± 0.189, 1-NN-L presented longer length of 9.735 ± 0.245, 1-NN-M was shortest at 9.655 ± 0.556, and 1-NN-H was longest at 9.816 ± 0.179, during this time point. E4 showed larger divergence. CO had a length of 12.786 ± 0.528, 1-NN-L had the shortest length at 12.302 ± 0.491, 1-NN-M had a length of 13.376 ± 0.439, and 1-NN-H had the longest length at 13.671 ± 0.541. E5 showed greater difference. CO had a length of 15.604 ± 0.677, 1-NN-L had the shortest length at 14.564 ± 0.560, 1-NN-M had a longest length at 17.707 ± 1.980, and 1-NN-H had a length of 15.398 ± 1.337. E6 showed comparable length. CO had a length of 20.176 ± 0.641, 1-NN-L had the longest length at 20.386 ± 1.022, 1-NN-M had a length of 19.666 ± 1.640, and 1-NN-H had the shortest length at 18.968 ± 1.314. E7 showed similar difference. CO had a length of 28.358 ± 1.522, 1-NN-L had a comparable length of 28.058 ± 0.371, 1-NN-M had the shortest length at 27.678 ±

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1.579, and 1-NN-H had the longest length of 29.313 ± 0.857. E8 showed minimal divergence. CO had a length of 29.595 ± 2.220, 1-NN-L had a comparable length of 29.853 ± 1.477, 1-NN-M had the shortest length at 28.212 ± 1.612, and 1-NN-H had the longest length at 29.989 ± 0.441 (Figure 8).

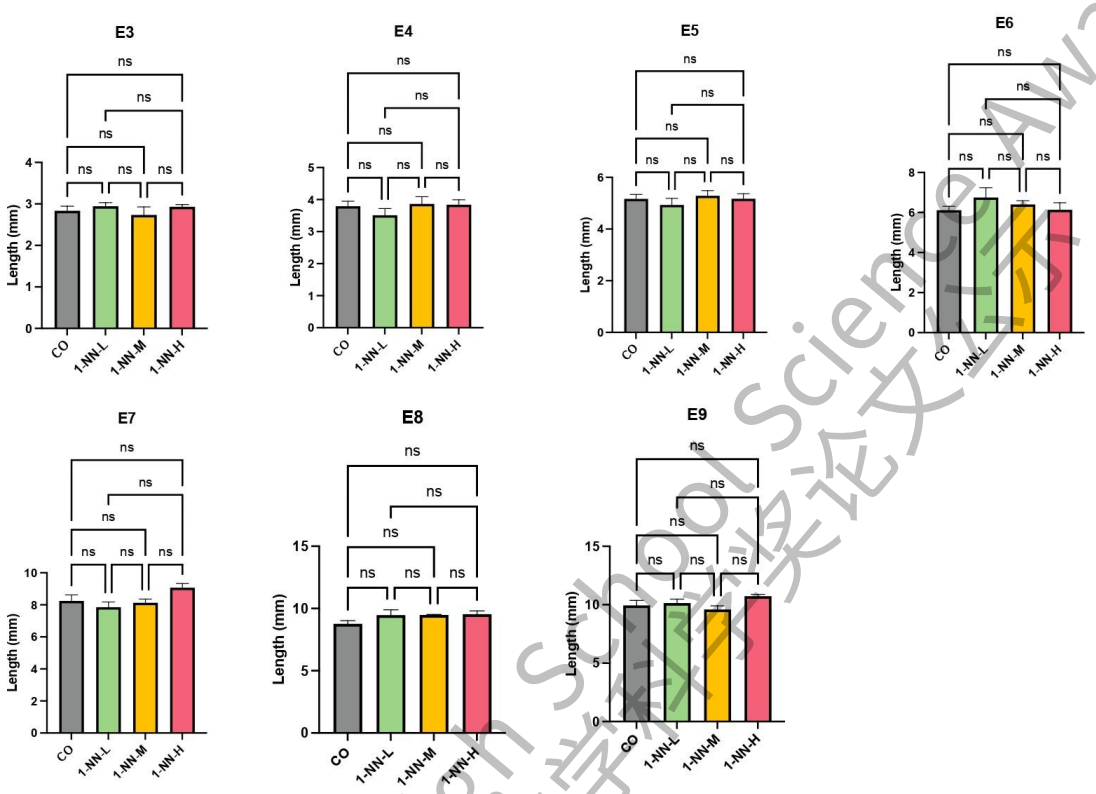


Figure 8. Effect of 1-NN on body length of chicken embryo, from E3-E9. CO: control; 1-NN-L: low concentration; 1-NN-M: medium concentration; 1-NN-H: high concentration.

Mean head length (mm) increased progressively across the observation period in all treatment groups. At E3, the mean head length was comparable. CO had a length of 2.835 ± 0.111, 1-NN-L had the longest length of 2.945 ± 0.086, 1-NN-M had the shortest length of2.736 ± 0.192, and 1-NN-H had a length of 2.932 ± 0.054. E4 showed similar difference. CO had a length of 3.793 ± 0.156, 1-NN-L had the shortest length of 3.510 ± 0.210, 1-NN-M had the longest length of 3.863 ± 0.227, and 1-NN-H had a comparable length of 3.840 ± 0.153. E5 showed similar divergence. CO had a length of 5.169 ± 0.167, 1-NN-L had the shortest length of 4.934 ± 0.256, 1-NN-M had the longest length of 5.288 ± 0.198, and 1-NN-H had a comparable length of 5.171 ± 0.197. E6 showed larger, but still little difference. CO had the shortest length of 6.117 ± 0.194, 1-NN-L had the longest length of 6.749 ± 0.490, 1-NN-M had a comparable length of 6.404 ± 0.189, and 1-NN-H had a length of 6.137 ± 0.355. E7 showed greater difference. CO had

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a length of 8.243 ± 0.381, 1-NN-L had the shortest length of 7.856 ± 0.326, 1-NN-M had a length of 8.126 ± 0.240, and 1-NN-H had the longest length of 9.068 ± 0.264. E8 showed lesser divergence. CO had the shortest length of 8.765 ± 0.259, 1-NN-L had a length of 9.465 ± 0.428, 1-NN-M had a comparable length of 9.475 ± 0.043, and 1-NN-H had the longest length of 9.544 ± 0.257. E9 showed similar difference. CO had a length of 9.947 ± 0.418, 1-NN-L had a length of 10.140 ± 0.344, 1-NN-M had the shortest length of 9.590 ± 0.316, and 1-NN-H had the longest length of 10.730 ± 0.163 (Figure 9).

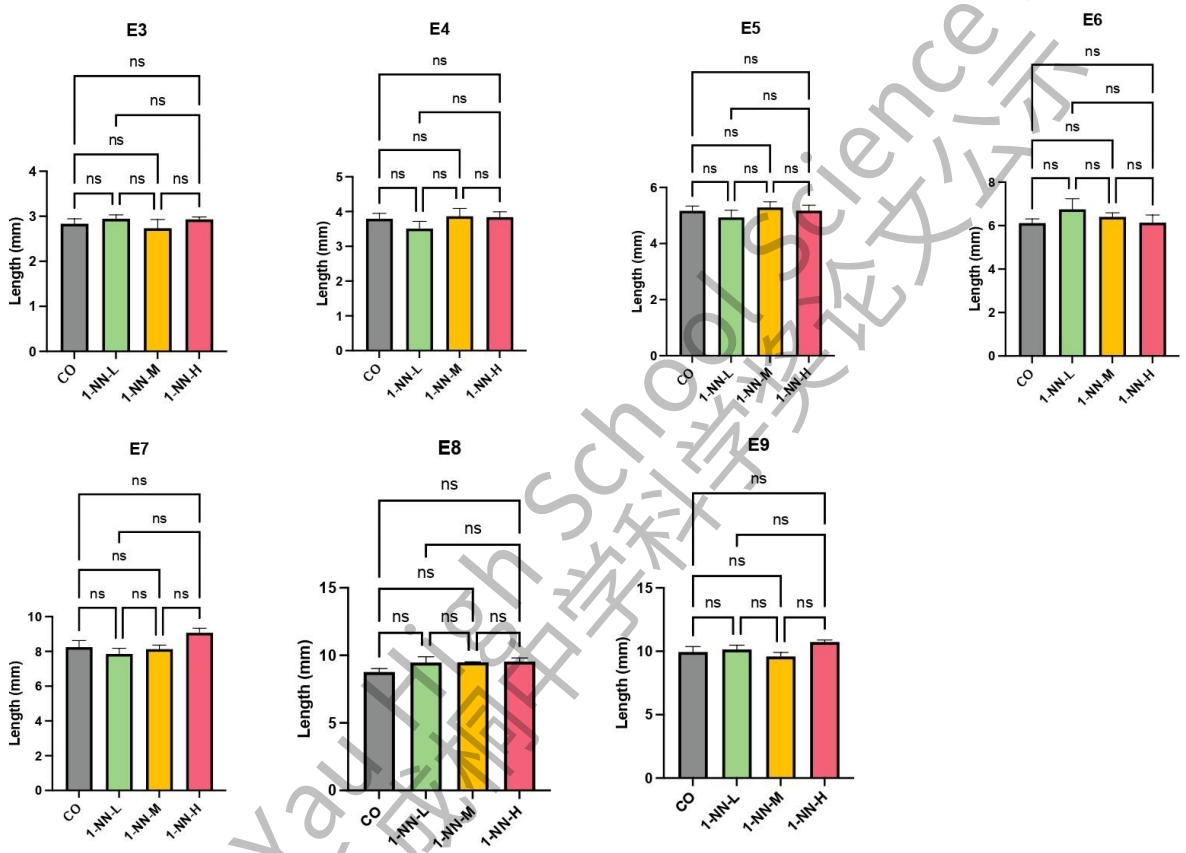


Figure 9. Effect of 1-NN on head length of chicken embryo, from E3-E9. CO: control; 1-NN-L: low concentration; 1-NN-M: medium concentration; 1-NN-H: high concentration.

Mean eye diameter (mm) increased progressively across the observation period in all treatment groups. On E4, mean eye diameter was comparable. CO had the longest length of 1.129 ± 0.090, 1-NN-L had the shortest length of 1.087 ± 0.083, 1-NN-M had a similar length of 1.101 ± 0.066, and 1-NN-H had a length of 1.125 ± 0.070. E5 showed similar difference. CO had a length of 2.081 ± 0.086, 1-NN-L had the shortest length of 1.915 ± 0.038, 1-NN-M had a length of 2.076 ± 0.021, and 1-NN-H had the longest length of 2.123 ± 0.090. E6 showed larger divergence. CO had the shortest length of 2.947 ± 0.145,

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1-NN-L had a length of 3.056 ± 0.092, 1-NN-M had a similar length of 3.120 ± 0.137, and 1-NN-H had the longest length of 3.210 ± 0.277. E7 showed increasing difference. CO had a length of 4.357 ± 0.257, 1-NN-L had the longest length of 4.414 ± 0.094, 1-NN-M had a comparable length of 4.216 ± 0.256, and 1-NN-H had the shortest length of 4.099 ± 0.265. E8 showed smaller divergence. CO had a length of 5.164 ± 0.098, 1-NN-L had the longest length of 5.236 ± 0.157, 1-NN-M had the shortest length of 5.158 ± 0.045, and 1-NN-H had a comparable length of 5.171 ± 0.364. E9 showed more significant difference. CO had a length of 5.661 ± 0.095, 1-NN-L had a similar length of 5.545 ± 0.221, 1-NN-M had the shortest length of 5.520 ± 0.007, and 1-NN-H had the longest length of 5.856 ± 0.412 (Figure 10).

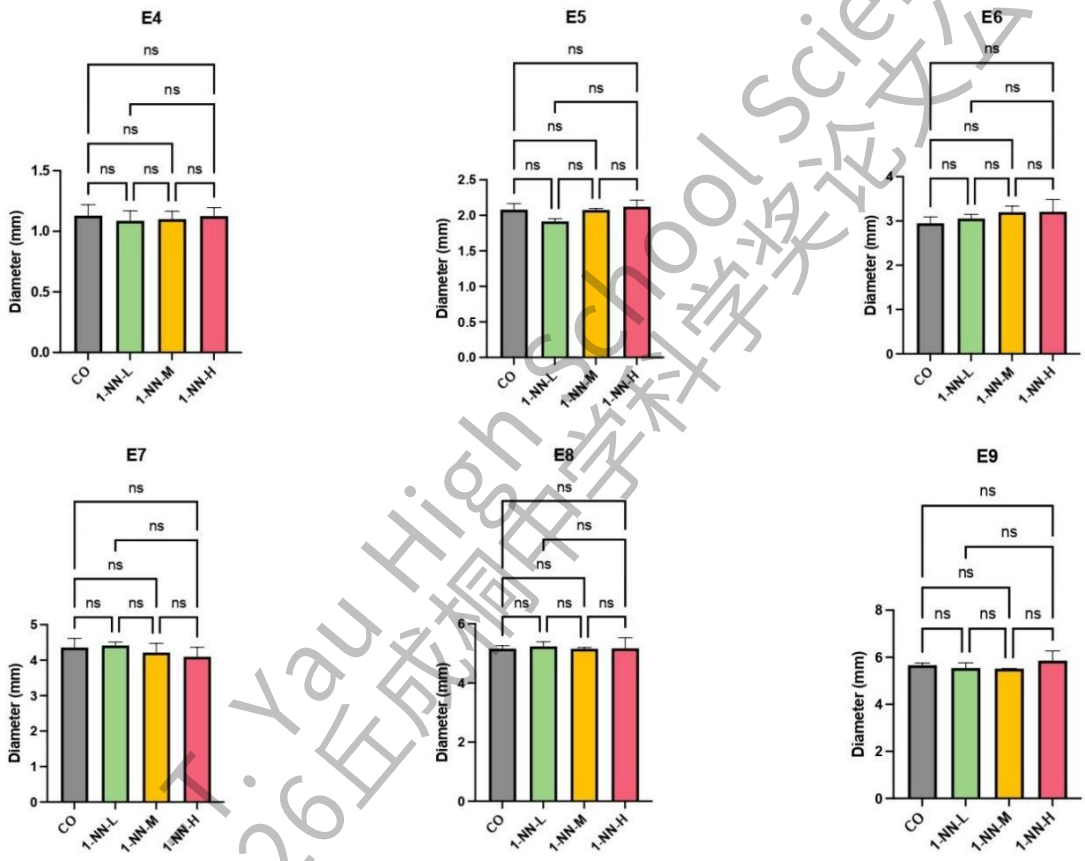


Figure 10. Effect of 1-NN on eye diameter of chicken embryo, from E4-E9. CO: control; 1-NN-L: low concentration; 1-NN-M: medium concentration; 1-NN-H: high concentration.

There is no observable trend between 1-NN and forelimb length across treatment groups from E4 to E9. However, on E5, two isolated pairings differed significantly: CO and 1-NN-L ($p<0.01$), and 1-NN-L and 1-NN-H ($p<0.05$). In both instances, however, this trend was not reflected in adjacent days, or seen

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consistently across treatment groups. Hence, the significant pairings are unlikely to reflect a genuine concentration-related effect of 1-NN on forelimb length.

Mean forelimb length (mm) increased progressively across the observation period in all treatment groups. At E4, mean forelimb length showed notable difference. CO had a length of 1.344 ± 0.050, 1-NN-L had a length of 1.259 ± 0.063, 1-NN-M had the shortest length of 1.039 ± 0.060, and 1-NN-H had the longest length of 1.500 ± 0.191. E5 showed similar divergence. CO had a length of 2.025 ± 0.048, 1-NN-L had the shortest length of 1.675 ± 0.049, 1-NN-M had a length of 1.963 ± 0.108, and 1-NN-H had the longest length of 2.083 ± 0.119. E6 showed decreasing difference. CO had the longest length of 3.163 ± 0.205, 1-NN-L had a length of 2.986 ± 0.237, 1-NN-M had a comparable length of 2.872 ± 0.029, and 1-NN-H had the shortest length of 2.848 ± 0.181. E7 showed similar difference. CO had a length of 4.061 ± 1.136, 1-NN-L had a length of 4.236 ± 0.243, 1-NN-M had the longest length of 4.466 ± 0.000, and 1-NN-H had the shortest length of 4.057 ± 0.487. E8 showed decreased divergence. CO had a length of 5.049 ± 0.140, 1-NN-L had a length of 4.884 ± 0.259, 1-NN-M had the lowest length of 4.939 ± 0.064, and 1-NN-H had a similar length of 5.092 ± 0.742. E9 showed largest difference. CO had a length of 6.552 ± 0.595, 1-NN-L had a length of 7.109 ± 0.244, 1-NN-M had the shortest length of 6.010 ± 0.322, and 1-NN-H had the longest length of 7.780 ± 1.067 (Figure 11).

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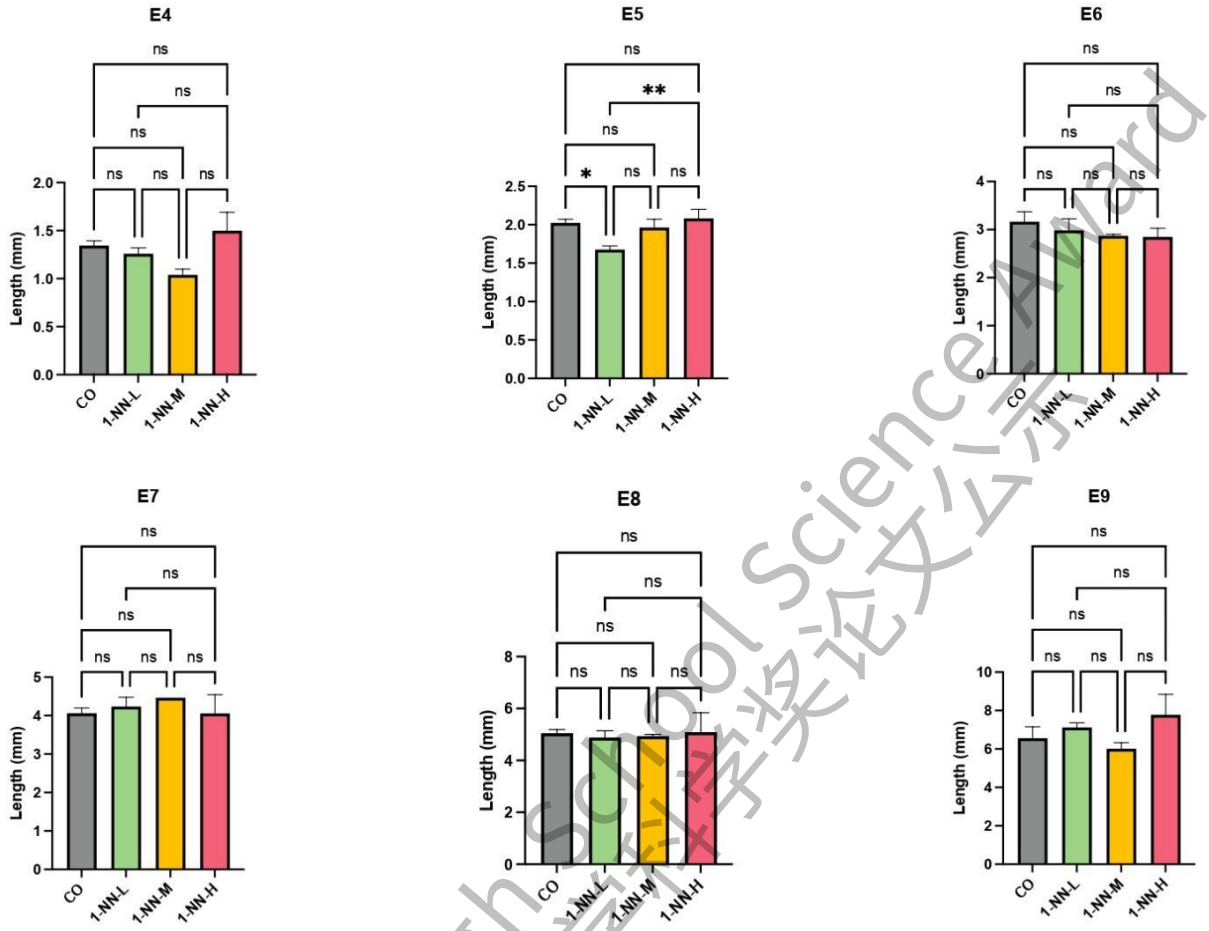


Figure 11. Effect of 1-NN on forelimb length of chicken embryo, from E4-E9. CO: control; 1-NN-L: low concentration; 1-NN-M: medium concentration; 1-NN-H: high concentration. **p<0.01, *p<0.05.

Similarly, there is no observable trend between 1-NN and hindlimb length across treatment groups. E6, E7, E8, and E9 showed no significant differences across all treatment groups. On E4 and E5, two isolated pairings showed significant differences: 1-NN-M and 1-NN-H on E4 ($p<0.05$), and 1-NN-L and 1-NN-M on E5 ($p<0.05$). Neither day showed a significant difference between exposure groups and CO. Across the duration of the experiment, no significant difference between CO and any exposure groups were established, suggesting a lack of concentration-related effect.

Mean hindlimb length (mm) increased progressively across the observation period in all treatment groups. On E4, mean hindlimb length showed notable difference. CO had a length of 1.450 ± 0.071, 1-NN-L had a length of 1.260 ± 0.088, 1-NN-M had the shortest length of 1.151 ± 0.074, and 1-NN-H had

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the longest length of 1.568 ± 0.133. E5 showed similar variability. CO had a length of 2.161 ± 0.143, 1-NN-L had the shortest length of 1.883 ± 0.057, 1-NN-M had the longest length of 2.394 ± 0.164, and 1-NN-H had a length of 2.067 ± 0.074. E6 showed decreased divergence. CO had a length of 3.191 ± 0.276, 1-NN-L had a comparable length of 3.221 ± 0.205, 1-NN-M had the shortest length of 2.852 ± 0.360, and 1-NN-H had the longest length of 3.384 ± 0.266. E7 showed similar difference. CO had a length of 5.682 ± 0.301, 1-NN-L had the shortest length of 5.257 ± 0.132, 1-NN-M had the longest length of 6.113 ± 0.368, and 1-NN-H had a length of 5.443 ± 0.757. E8 showed decreased difference. CO had the longest length of 8.413 ± 0.223, 1-NN-L had a comparable length of 8.196 ± 0.488, 1-NN-M had a length of 7.596 ± 0.256, and 1-NN-H had the shortest length of 7.519 ± 0.652. E9 showed increased variability. CO had the longest length of 11.866 ± 0.528, 1-NN-L had a length of 11.113 ± 0.862, 1-NN-M had the shortest length of 9.717 ± 0.430, and 1-NN-H had a length of 11.665 ± 2.137 (Figure 12).

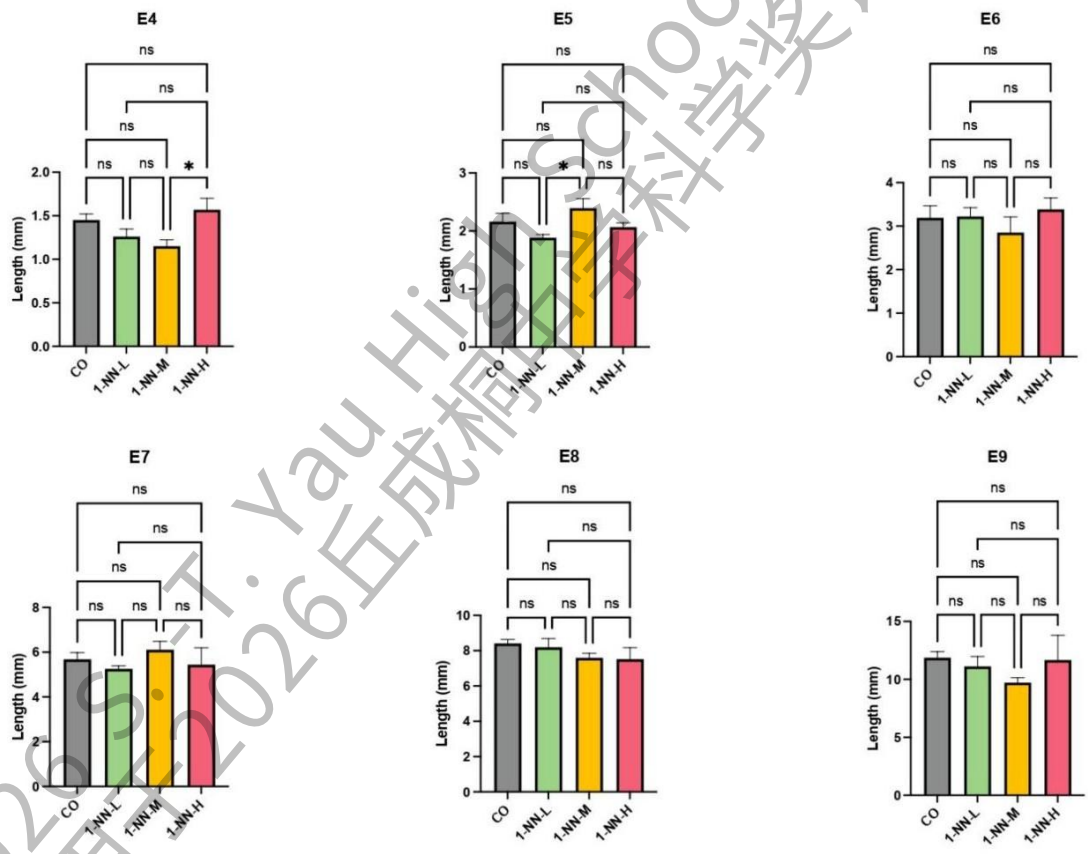


Figure 12. Effect of 1-NN on hindlimb length of chicken embryo, from E4-E9. CO: control; 1-NN-L: low concentration; 1-NN-M: medium concentration; 1-NN-H: high concentration. *p<0.05.

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3.3 Vascular parameters

No significant differences were seen in the heartbeat across all treatment groups, from E3 to E5. Additionally, no observable dose-dependent trend can be established.

Mean heartbeat (bpm) increased across the observation period in all treatment groups. At E3, mean heartbeat showed little difference. CO had the lowest value of 129 ± 3.9, 1-NN-L had the highest value of 133 ± 3.0, 1-NN-M had a comparable value of 132 ± 2.2, and 1-NN-H had a similar value of 131 ± 2.1. E4 showed similar variability. CO had the highest value of 171 ± 2.8, 1-NN-L had a similar value of 170 ± 8.5, 1-NN-M had a value of 166 ± 3.9, and 1-NN-H had the lowest value of 165 ± 4.3. At E5, divergence was notable. CO had a value of 172 ± 7.5, 1-NN-L had a value of 183 ± 4.3, 1-NN-M had the lowest value of 169 ± 12.1, and 1-NN-H had the highest value of 186 ± 0.6 (Figure 13).

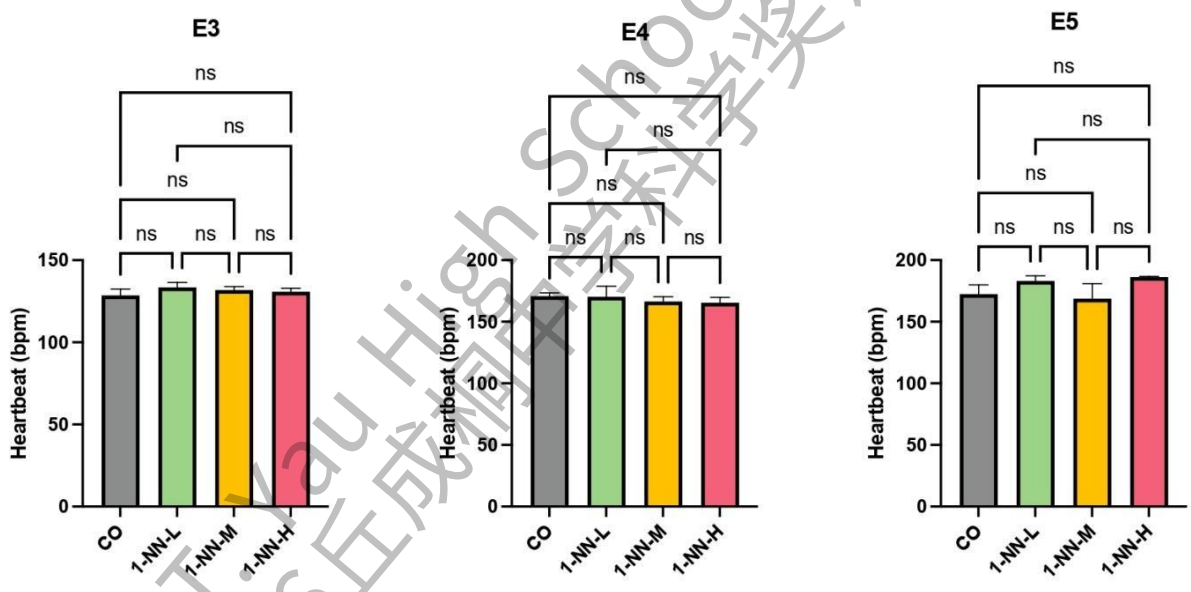


Figure 13. Effect of 1-NN on heartbeat of chicken embryo, from E3-E5. CO: control; 1-NN-L: low concentration; 1-NN-M: medium concentration; 1-NN-H: high concentration.

No significant differences were seen in vessel length on E3, though mean vessel length progressively decreased from CO to 1-NN-H. On E4, no dose-dependent pattern was observable, though 1-NN-H showed lower mean vessel length than CO.

Mean vessel length (mm) increased across the observation period in all treatment groups. At E3, mean vessel length showed moderate variability. CO had the longest length of 525.906 ± 76.532, 1-NN-L had a

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length of 456.033 ± 47.591, 1-NN-M had a comparable length of 483.432 ± 54.591, and 1-NN-H had the shortest length of 367.578 ± 36.980. E4 showed similar difference. CO had a length of 1511.599 ± 131.928, 1-NN-L had a length of 1118.820 ± 93.682, 1-NN-M had the longest length of 1269.719 ± 82.422, and 1-NN-H had the shortest length of 951.034 ± 72.846 (Figure 14).

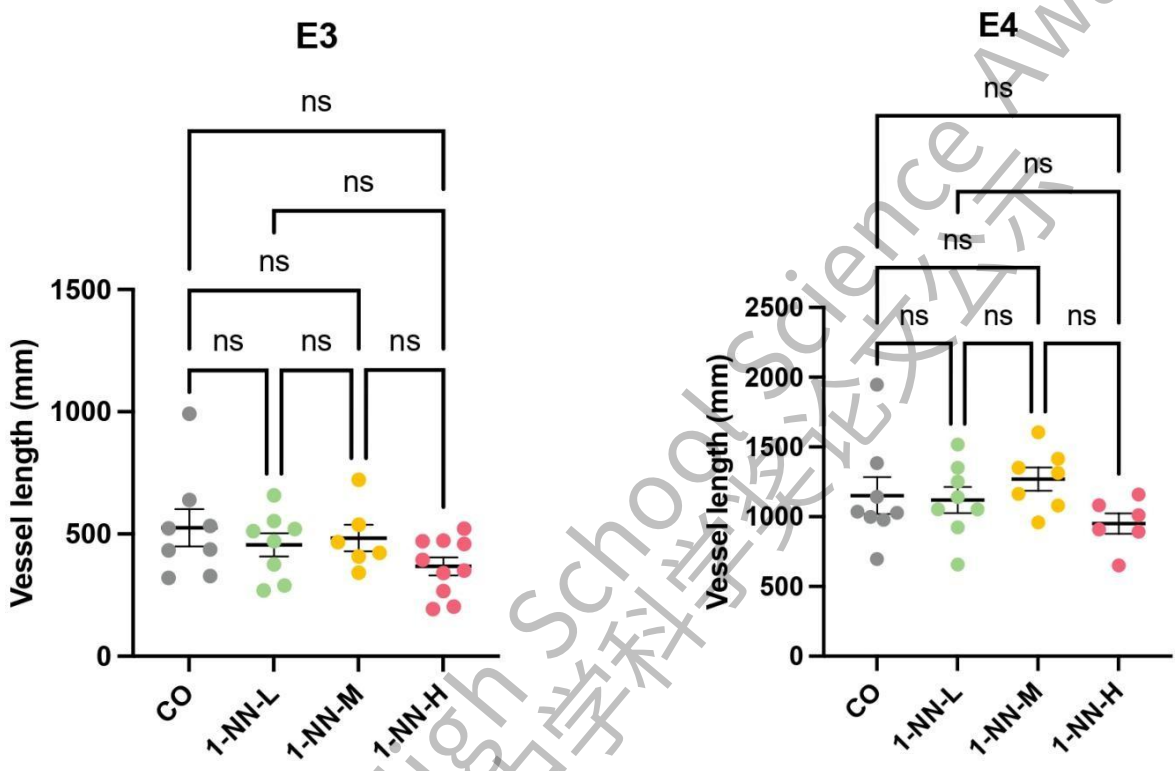


Figure 14. Effect of 1-NN on vessel length of chicken embryo, from E3-E4. CO: control; 1-NN-L: low concentration; 1-NN-M: medium concentration; 1-NN-H: high concentration.

Vessel junctions did not differ significantly between any treatment groups on E3 and E4. A decreasing trend was observable from CO to 1-NN-H on E3, but is not present on E4. On E4, 1-NN-H had lower mean junctions than CO, though overall no dose-dependent pattern is observable.

Mean vessel junctions increased across the observation period in all treatment groups. At E3, mean vessel junctions showed notable difference. CO had the highest number of 283 ± 78.0, 1-NN-L had a number of 209 ± 33.4, 1-NN-M had a comparable number of 232 ± 33.6, and 1-NN-H had the lowest number of 139 ± 29.1. E4 showed greater variability, CO had a number of 514 ± 150.4, 1-NN-L had a similar number of 481 ± 95.1, 1-NN-M had the greatest number of 708 ± 81.3, and 1-NN-H had the

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lowest number of 314 ± 52.4 (Figure 15).

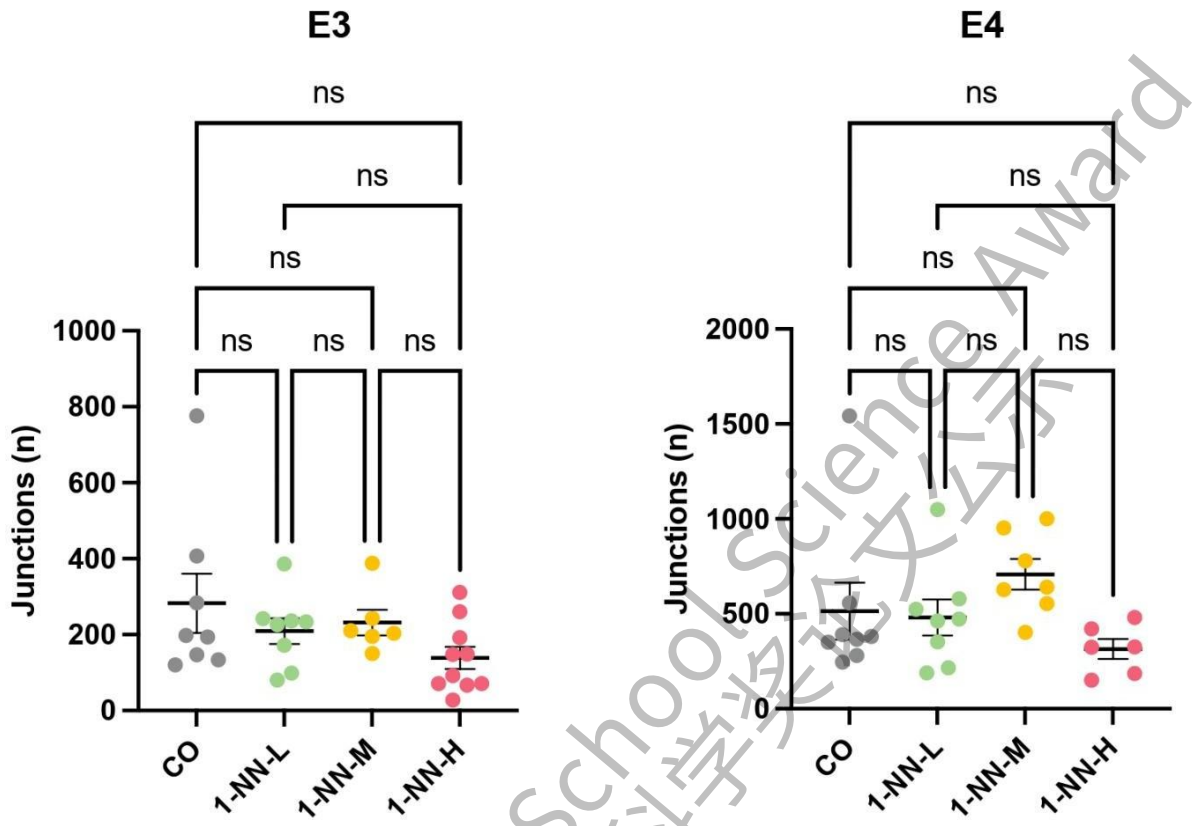


Figure 15. Effect of 1-NN on vessel junctions, from E3-E4. CO: control; 1-NN-L: low concentration; 1-NN-M: medium concentration; 1-NN-H: high concentration.

3.4 Organ morphology and weight

On dissection at E9, embryo weights across treatment groups showed no significant difference. Mean weight of embryo were relatively comparable. CO had the least weight of 1.53 ± 0.03 g, 1-NN-L had a weight of 1.57 ± 0.04 g, 1-NN-M had a similar weight of 1.55 ± 0.10 g, and 1-NN-H had the greatest weight of 1.61 ± 0.07 g (Figure 16).

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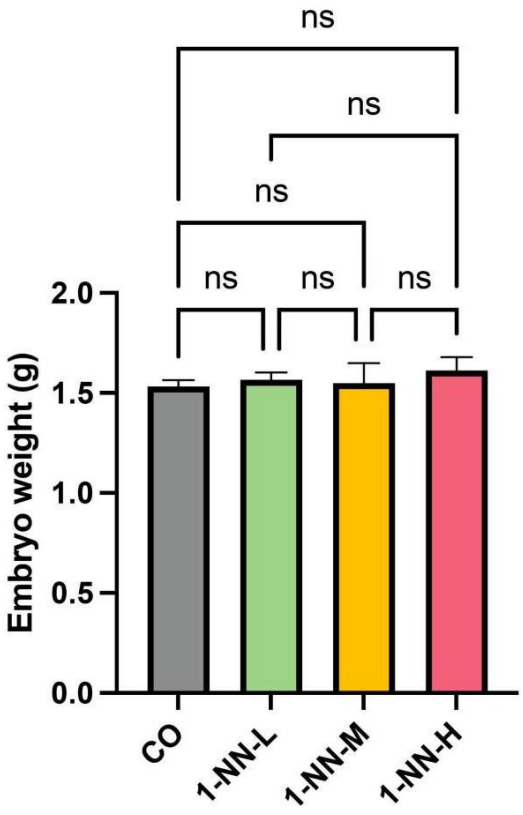


Figure 16. Effect of 1-NN on embryo weight of chicken embryo, at dissection on E9. CO: control; 1-NN-L: low concentration; 1-NN-M: medium concentration; 1-NN-H: high concentration.

On dissection on E9, the weight of the embryo's liver, brain, left, and right eye showed no significant difference across treatment groups. Similarly, the organ/weight ratio of the liver, brain, left, and right eye showed no significant difference across treatment groups. The liver weight showed little variability. CO had the lowest weight of 0.034 ± 0.005, 1-NN-L had a weight of 0.035 ± 0.006, 1-NN-M had a weight of 0.035 ± 0.005, and 1-NN-H had a weight of 0.035 ± 0.003. The liver / weight ratio showed similar difference. CO had a value of 0.022 ± 0.003, 1-NN-L had a value of 0.023 ± 0.004, 1-NN-M had a value of 0.023 ± 0.005, and 1-NN-H had a value of 0.022 ± 0.002. The left eye weight showed large variability. CO had a weight of 0.152 ± 0.010, 1-NN-L had a similar weight of 0.147 ± 0.010, 1-NN-M had the lowest weight of 0.115 ± 0.005, and 1-NN-H had the greatest weight of 0.158 ± 0.020. The left eye / weight ratio showed similar variability. CO had the greatest value of 0.099 ± 0.006, 1-NN-L had a value of 0.094 ± 0.007, 1-NN-M had the lowest value of 0.075 ± 0.002, and 1-NN-H had a value of 0.097 ± 0.010. The right eye weight showed notable divergence. CO had a weight of 0.162 ± 0.008, 1-NN-L had the greatest

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weight of 0.172 ± 0.007, 1-NN-M had the lowest weight of 0.130 ± 0.000, and 1-NN-H had a weight of 0.150 ± 0.020. The right eye / weight ratio showed comparable difference. CO had a value of 0.106 ± 0.006, 1-NN-L has the greatest value of 0.110 ± 0.005, 1-NN-M had the lowest value of 0.085 ± 0.006, and 1-NN-H had a value of 0.092 ± 0.010 (Figure 18). The brain weight showed great variability. CO had the lowest weight of 0.096 ± 0.007, 1-NN-L had the greatest weight of 0.123 ± 0.018, 1-NN-M had a comparable weight of 0.120 ± 0.020, and 1-NN-H had a weight of 0.108 ± 0.005. The brain / body ratio showed similar divergence. CO had the lowest value of 0.063 ± 0.004, 1-NN-L had the greatest value of 0.079 ± 0.012, 1-NN-M had a comparable value of 0.077 ± 0.008, and 1-NN-H had a value of 0.067 ± 0.003 (Figure 17).

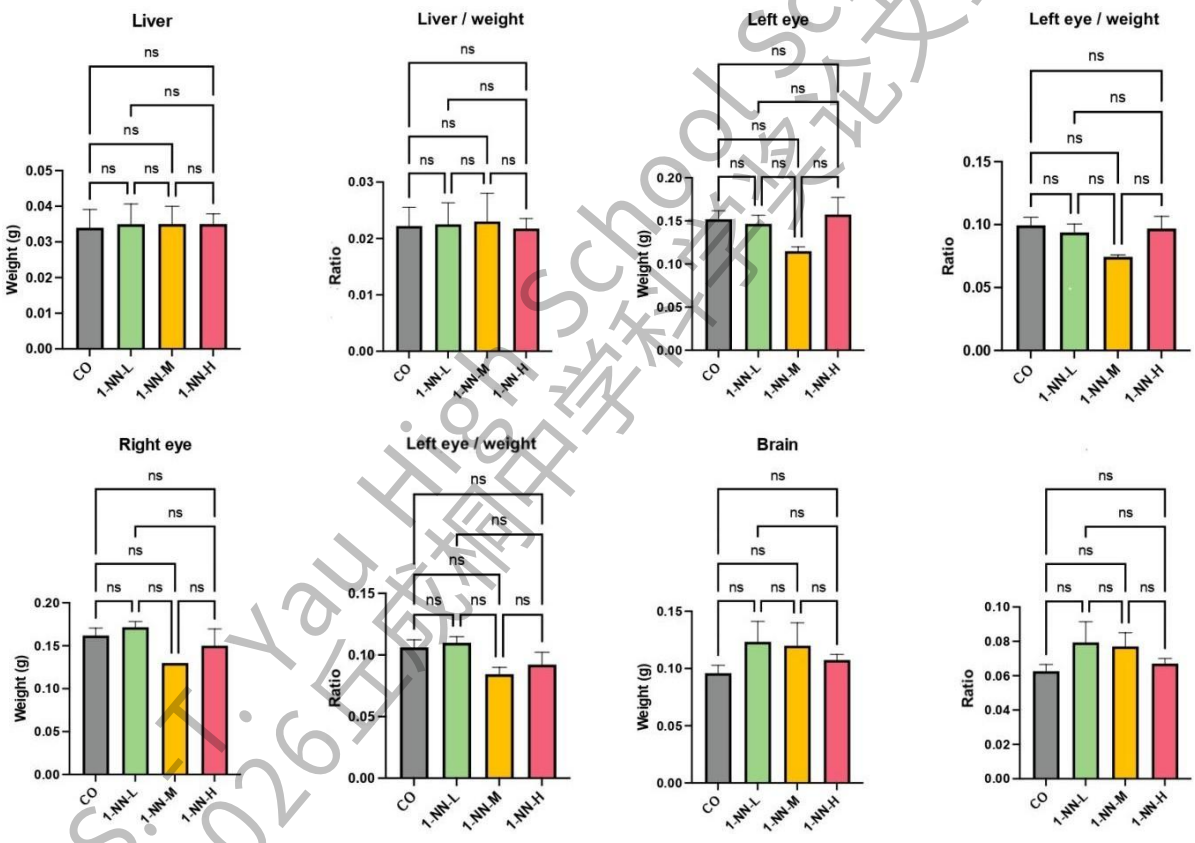


Figure 17. Effect of 1-NN on various organs (liver, brain, left eye, right eye) of chicken embryo and their respective organ/weight, at dissection on E9. CO: control; 1-NN-L: low concentration; 1-NN-M: medium concentration; 1-NN-H: high concentration.

Also at dissection at E9, both the weight of the embryo's heart ($p<0.05$) and the heart/weight ratio ($p<0.05$) showed a significant increase between CO and 1-NN-H. Additionally, the means of heart

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weight consistently increased from CO to 1-NN-H. A similar dose-dependent trend is seen in the heart/weight ratio, increased from CO to 1-NN-H .

At E9, the mean heart weight between CO and 1-NN-H showed significant difference, with the 1-NN-H group reaching the highest value. CO had the lowest weight of 0.024 ± 0.002 g, 1-NN-L had a weight of 0.027 ± 0.003, 1-NN-M had a weight of 0.030 ± 0.000, and 1-NN-H had the greatest weight of 0.040 ± 0.004. The heart / weight ratio also showed significant difference between CO and 1-NN-H. CO had the lowest value of 0.016 ± 0.001, 1-NN-L had a value of 0.017 ± 0.002, 1-NN-M had a value of 0.020 ± 0.002, and 1-NN-H had the greatest value of 0.025 ± 0.002 (Figure 18).

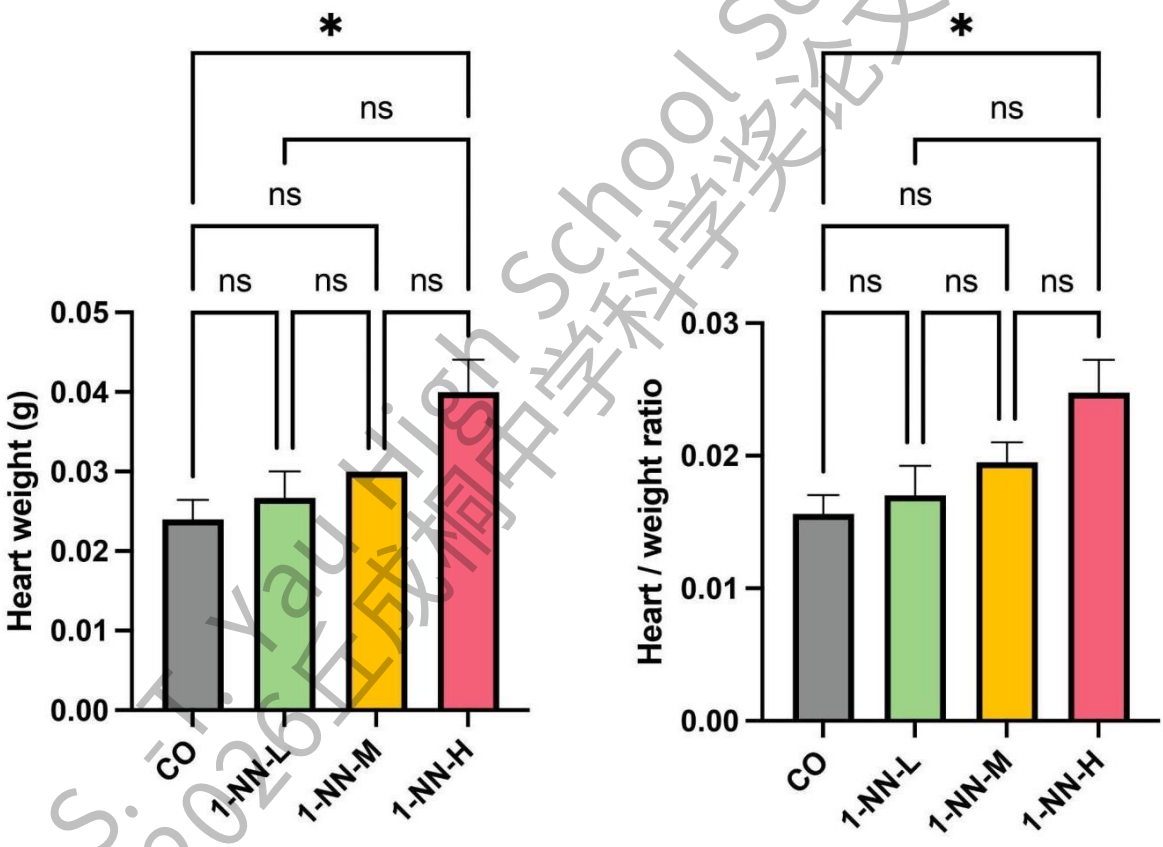


Figure 18. Effect of 1-NN on heart weight and heart/weight ratio of chicken embryo. CO: control; 1-NN-L: low concentration; 1-NN-M: medium concentration; 1-NN-H: high concentration. *p<0.05.

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3.5 Effect of 1-NN on gene expression

Across treatment groups, relative gene expression of *CYP1A5*, normalised to *18S*, showed no significant differences. While 1-NN-L and 1-NN-M had higher levels of CYP1A5 expression than CO, 1-NN-H had lower, suggesting no dose-dependent pattern.

Mean relative gene expression of *CYP1A5* (normalised to *18S*, fold change vs. control) showed great variability. CO had a value of 1.000 ± 0.000 , 1-NN-L had the highest value of 2.859 ± 2.129, 1-NN-M had a value of 1.798 ± 1.458 , and 1-NN-H had the lowest value of 0.379 ± 0.043 (Figure 19).

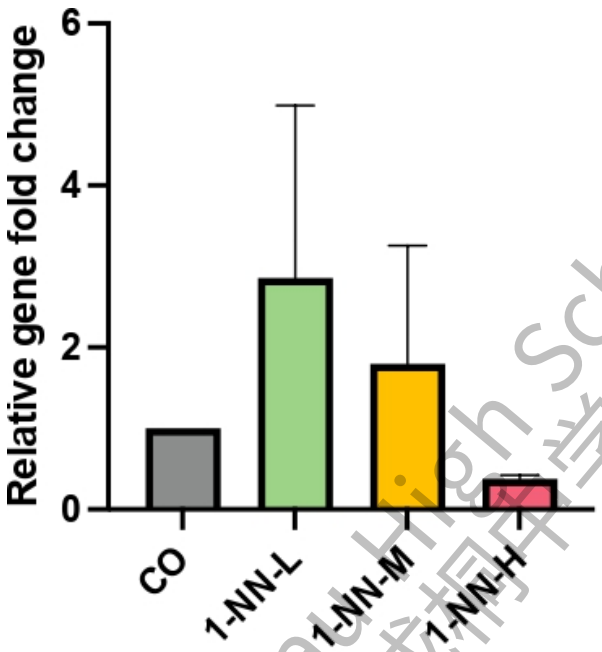


Figure 19. Result of RT-qPCR on relative fold change of gene *CYP1A5* following 1-NN exposure in chicken embryo, normalised to *18S*. CO: control; 1-NN-L: low concentration; 1-NN-M: medium concentration; 1-NN-H: high concentration.

3.6 Effect of 1-NN on protein expression

Across treatment groups, no significant differences are seen in the relative fold change of AhR, normalised to GAPDH. Additionally, no pattern suggesting a dose-dependent relationship is observable. The Western blot is shown in Figure 20.

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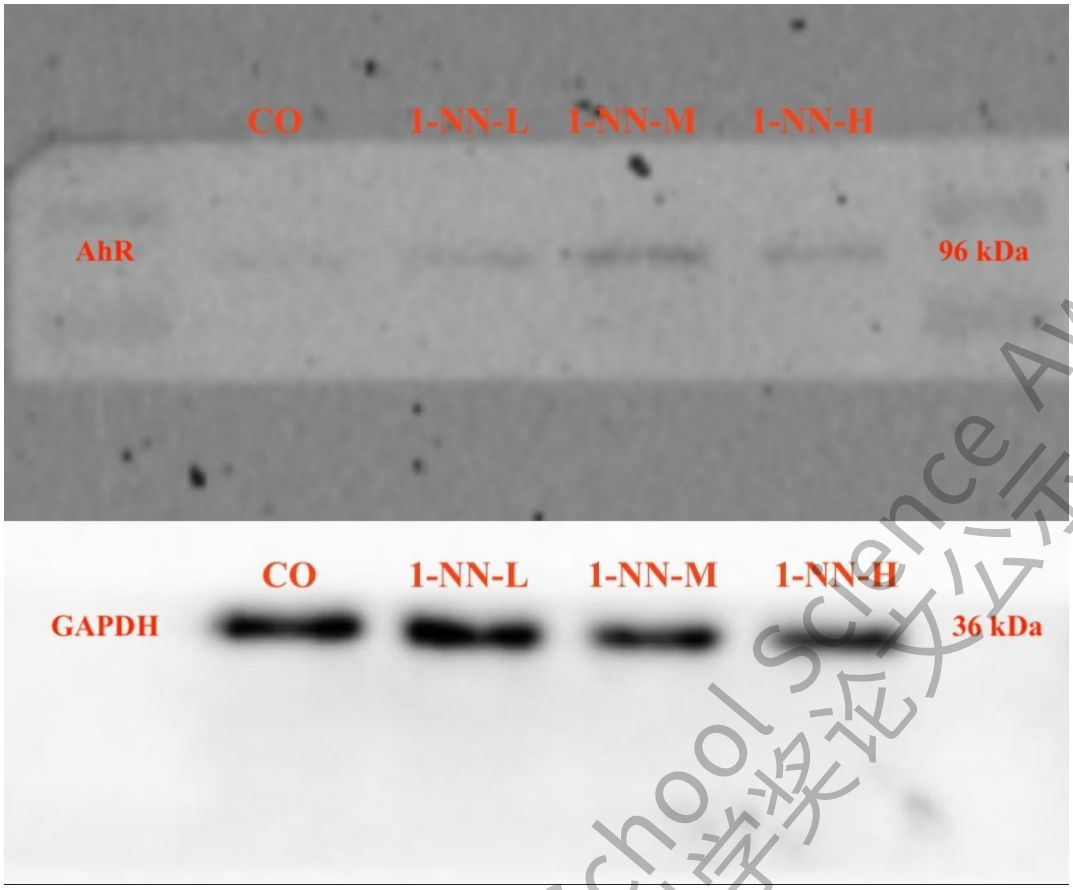


Figure 20. Representative images of Western blot results of chicken embryo protein. Top: AhR (96 kDa); bottom: GAPDH (36 kDa).

Mean relative AHR protein expression (normalized to GAPDH) showed notable difference. CO had the lowest value of 0.448 ± 0.139, 1-NN-L had the highest value of 0.535 ± 0.193, 1-NN-M had a comparable value of 0.503 ± 0.158, and 1-NN-H had a value of 0.470 ± 0.155 (Figure 21).

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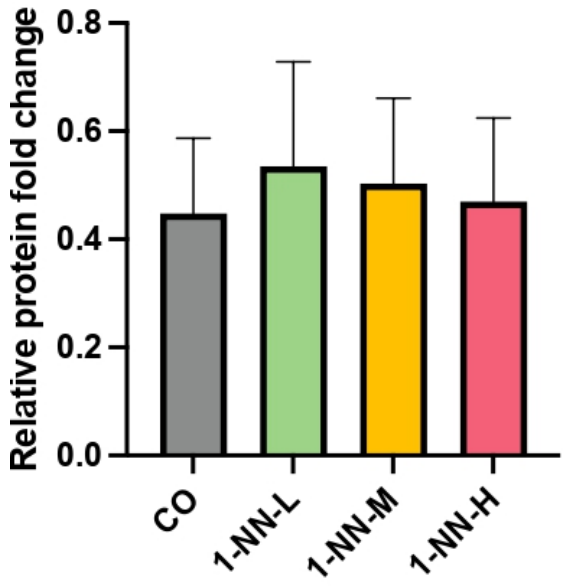


Figure 21. Results of Western blot on relative fold change of protein AhR following 1-NN exposure in chicken embryo, normalised to GAPDH. CO: control; 1-NN-L: low concentration; 1-NN-M: medium concentration; 1-NN-H: high concentration.

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4. Discussions

Our study characterised the effect of 1-NN on the development of *Ex ovo* chicken embryo. Developmental toxicity of 1-NN is limited, with only one prior study, conducted in mummichogs. The introduction of *Ex ovo* chicken embryo as a model therefore represents a novel approach not previously used in this context. Additionally, our study's data demonstrates a trend of impairment on vessel length and number of junctions, and significant cardiomegaly, both absolute mass and relative to total body mass. While 1-NN's reproductive toxicity, pneumotoxicity, genotoxicity, and carcinogenicity have been previously studied, its potential cardiotoxicity has not been previously identified. To our knowledge, this is the first study to report 1-NN induced cardiotoxicity, as suggested by a trend of abnormal vessel, and significant abnormal heart development.

A trend towards reduced vascular parameters was observed with increasing 1-NN concentration, though statistical significance was not reached. Although angiogenesis pathways were not directly assessed, PAHs decreasing vascularisation has been reported in mice embryos, with a 17% decrease in intraplacental arteries, and a sparser branching structure, resulting in lesser junctions [39]. Inhibition of angiogenesis has been linked to mediation via the AhR [40], NF-ƙB/TNF-α, and Nrf2/HO-1 (oxidative stress) pathways' activation [41]. If the trend reflects a genuine biological effect, it raises the possibility that 1-NN impairs angiogenesis through these mechanisms. However, this remains speculative without statistical significance, and would need both a larger sample size and direct molecular assays to confirm.

1-NN was shown, additionally, to affect cardiac development. Cardiomegaly, as observed in 1-NN-H, is similarly characterised by phenanthrene (PHE), which increases rat heart weight and cardiomyocyte size via DNA methylation [42]. PHE, a low-ringed PAH, is a similar molecule to 1-NN, and may show similar toxicological effects. Another study on benzo[a]pyrene (BaP) shows increased heart/weight ratios of zebrafish exposed during early life stages, accompanied with an elevated expression of CdC42, RhoA, phosphorylated ERK1/2 (p-ERK1, 2) and Rac1. BaP, another PAH, reflects cardiotoxicity also seen in 1-NN, while proposing alternate pathways for cardiomegaly [43]. Finally,

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tetrachlorodibenzo-p-dioxin (TCDD) caused hypertrophy in rat H9c2 cells (similar in behaviour to heart muscle cells), associated with significant induction of *CYP* genes. While not a PAH, TCDD is the most potent AhR ligand, hence inducing similar pathways [44]. However, cultured cell lines have very different environments than intact biological systems. The disparity, as demonstrated in a cardiomegaly study, can significantly impact gene expression, possibly explaining the inconsistency of *CYP* activity between the two studies [45]. Prior studies showed PAH induced increased heart rate in zebrafish embryos, which was paired with supexpression of sarcoplasmic/endoplasmic reticulum calcium ATPase 2a (atp2a2a) [46]. The heart rate of chicken embryos from E3-E4 showed no significant difference compared to CO in our study. However, effects were assessed in offspring of exposed parents rather than direct embryonic exposure, which may explain the discrepancy.

In our study, 1-NN showed no significant impact or trend on developmental outcomes. Survival rate, though not reflected in our study, was shown to decrease in mummichog embryos whose parents were implanted with 1-NN pellets [34]. The latter study utilised cholesterol as a carrier, compared to DMSO in our study, which would change the delivery of 1-NN and received internal doses. The exposure of 1-NN to parents, rather than embryos, may also affect the observed survival rate. Similarly, our study showed no significance in body length and malformation rates across treatment groups. A study conducted on zebrafish larvae showed that PHE reduced body length, and increased malformation rates, through alterations to the growth hormone/insulin-like growth factor 1 axis [47]. However, PHE administration was waterborne, which mimics environmental exposure with variable absorption, compared to our study's internal exposure, with near complete delivery. Additionally, the difference in molecules, despite similar properties and comparable molecular weight, could lead to vastly different biological effects.

RT-qPCR was conducted on *CYP1A5*, and WB was conducted for AhR, common proteins typically involved in PAH-induced toxicity. However, across treatment groups no significant difference was observed. Although 1-NN exhibited cardiotoxicity reminiscent of typical PAH exposure, RT-qPCR and WB have largely ruled out induction via AhR activation and *CYP1* family pathways that are commonly associated with PAH toxicity. Therefore, we suggest that 1-NN

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could have operated via a distinct, AhR/CYP independent pathway, such as those discussed above. Future work involving broader transcriptomic or proteomic testing could verify the mechanisms responsible for the observed phenotypes. Furthermore, this study utilised a relatively smaller sample size, which may limit statistical power to characterise more subtle dose-dependent effects. Larger-scale studies would help confirm the robustness of these findings, and detect treatment outcomes.

The findings presented in our study identified a previously unobserved cardiotoxicity of 1-NN on avian species, while contributing to a deeper understanding of its undercharacterised developmental toxicity. Potential cardiotoxicity is concerning, for both occupationally-exposed individuals and the general public, given 1-NN's role as a chemical intermediate and presence in diesel exhaust. Further verification of its toxicity profile is suggested, considering the sensitivity of cardiovascular development during early embryonic days. By suggesting that 1-NN's toxicity is independent of the AhR/*CYP1* pathway, this study could indicate that structurally-similar PAHs may involve a broader range of pathways and mechanisms than were previously appreciated, raising the need to reevaluate. This study also demonstrates the versatility of the *Ex ovo* chicken in observing cardiotoxicity and developmental toxicity, offering a scalable, cost-efficient model for future pollutant screening.

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5. Conclusion

Through examining the potential effect that 1-NN has on the development of *Ex ovo* chicken embryo, this study characterises the novel cardiotoxicity of 1-NN, cardiomegaly as observed at 10000 ng/g. Notably, these changes were induced without significant difference in AhR/*CYP* pathways, commonly observed in PAHs. This may suggest that 1-NN exerts toxicity via an independent pathway, though the limited sample size warrants further investigation. Trends towards reduced vascular parameters were also observed, though they did not reach statistical significance ($p>0.05$). This study highlights the necessity to further evaluate the toxicity of 1-NN, particularly for occupationally/environmentally-exposed groups, while highlighting the *Ex ovo* chicken as an excellent model to identify cardiotoxicity.

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7. Acknowledgements

For this study to conclude successfully, I would like to thank every teacher who has provided me with help along the way, and my family for always supporting me throughout.

The idea for this came to me seemingly suddenly, but looking back, it was obviously an accumulation of my passions and interests. For a very long time, I've always had a love for birds. I had a photo album dedicated to recording every unique species of bird I met whenever I was outside, and I prided myself in the sizeable collection. On walks, I would stand taking photos of sparrows for so long that my parents would call for me, thinking I was lost. Their agility fascinated me, and I thought they were the coolest animals. The idea of raising them was first implanted via videos online of people incubating chicks from day 1 until hatching. There was something magical about watching new life open its eyes on human hands. I often thought, when I was older, with enough time and knowledge, I would be content doing the exact same thing. With this project, I had an opportunity to work with chicks. From this, I wanted to answer an important question: what happens when the process of development goes wrong? Hatching felt magical to me, but it was also an incredibly delicate process. Embryonic development was sensitive to environmental stressors, a challenge wild birds face every day. So rather than raising chicks under perfect conditions, I chose to study what happened when they were exposed to environmental pollutants. It wasn't an easy choice. I loved these animals, and watching what happened was difficult. However, by studying the effect of pollutants, I could turn my fascination for birds into something useful, informing bird protection in the future.

My high school biology teacher, Mr. Dorsey, was super helpful in kick-starting me in my research process, helping me determine my direction, and offering me feedback in formatting and composing sections of my report, such as the background. Since the school did not have relevant equipment for this study, my research was conducted outside of school. Mr. Dorsey made sure to ensure my safety throughout the project and was involved throughout.

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Additionally, I would like to sincerely thank Professor Lei from the Shenzhen Institute of Advanced Technology Chinese Academy of Science, Research Center for Energy Metabolism and Reproduction, and my parents for helping me get in touch with a professor conducting relevant research after knowing my interest, to allow me to enter a professional lab. I am grateful for his support in granting me access to the research group's equipment, platform, and reagents for shell-less chicken incubation, embryo dissection, and molecular biology experiments (RNA extraction, PCR, Western blot). I was challenged by many things, including the difficult process of creating a sterile *Ex ovo* system and transferring eggs, both of which required hours of trial and error for me to learn the patterns for success. I would also like to acknowledge and thank my senior lab members for providing me with guidance on basic lab procedures. The decision for the topic of this study, the experimental design, data collection and analysis, as well as the writing process, was made independently by me, under the guidance of my high school biology teacher, Mr. Dorsey.

My parents have been the biggest supporters in my learning journey. Knowing my passion for science from a young age, they noted and fostered any interests I have, even sporadic ones. They encouraged me to pursue whatever I was interested in, with no restrictions or expectations for my exploration. My bookshelf was stacked to the brim with all kinds of books, from entomology to astrophysics, all at my request. As a young child with a strong curiosity and an even stronger urge to share it, I knew I would always find an open ear in the household. It is fully with their support that I've come to discover what I believe is my true calling. Without them, this project would have been impossible.

Since I knew I would be interested in pursuing the sciences, my foremost hope was to work in a proper lab. Walking into Professor Lei's lab was a whole new level of dream come true. Being surrounded by cutting-edge equipment, standing at the frontier of science, watching important innovations unfurl right beside me: it felt surreal. Researching my own topic as a high school student yet joining established scientists in their work felt like both a blessing and an honour.

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Commitments on Academic Honesty and Integrity

We hereby declare that we

1. are fully committed to the principle of honesty, integrity and fair play throughout the competition.
2. perform the research work ourselves and thus truly understand the content of the work.
3. observe the common standard of academic integrity adopted by most journals and degree theses.
4. have declared all the assistance and contribution we have received from any personnel, agency, institution, etc. for the research work.
5. have truthfully disclosed all AI tool usage in the research report and obtained prior approval from supervising teachers. All supporting documentation has been submitted as required. We hereby affirm that no improper use of AI has occurred at any stage of the research process.
6. undertake to avoid getting in touch with assessment panel members in a way that may lead to direct or indirect conflict of interest.
7. undertake to avoid any interaction with assessment panel members that would undermine the neutrality of the panel member and fairness of the assessment process.
8. observe the safety regulations of the venue(s) where we conduct the experiment(s), if applicable.
9. observe all rules and regulations of the competition.
10. agree that the decision of YHSA(Asia) is final in all matters related to the competition.

We understand and agree that failure to honor the above commitments may lead to disqualification from the competition and/or removal of award, if applicable; that any unethical deeds, if found, will be disclosed to the school principal of the team member(s) and relevant parties if deemed necessary; and that the decision of YHSA(Asia) is final and no appeal will be accepted.

X
Name of team member: **Connie Shien Jin**

X
Name of supervising teacher: **Adam Dorsey**

Michael Sheridan

Noted and endorsed by

Name of school principal: MICK (MICHAEL K.) SHERIDAN

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