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Ecotoxicological effects of microplastics on bird embryo development by hatching without eggshell

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TITLE:

Ecotoxicological effects of microplastics on bird embryo development by hatching without eggshell

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Keywords

Microplastics; Quail embryo; Embryonal toxicity; Hatching without eggshell; Particle size; Short-term exposure

Summary

This paper introduces a method of hatching without using an eggshell for toxicological studies of particle pollutants such as microplastics.

Abstract

Microplastics are an emerging global pollutant type that poses a great health threat to animals due to their uptake and translocation in animal tissues and organs. Ecotoxicological effects of microplastics on the development of bird embryos are not known. The bird egg is a complete development and nutrition system, and the entire embryo development occurs in the eggshell. Therefore, a direct record of bird embryo development under the stress of pollutants such as microplastics is highly limited by the opaque eggshell in traditional hatching. In this study, the effects of microplastics on quail embryo development were visually monitored by hatching without an eggshell. The main steps include the cleaning and disinfection of fertilized eggs, the incubation before exposure, the short-term incubation after exposure, and the sample extraction. The results show that compared with the control group, the wet weight and body

length of the microplastics-exposed group displayed a statistical difference and the liver proportion of the whole exposed group significantly increased. Additionally, we evaluated external factors that affect the incubation: temperature, humidity, egg rotation angle, and other conditions. This experimental method provides valuable information on the ecotoxicology of microplastics and a novel way to study the adverse effects of pollutants on the development of embryos.

Introduction

The production of plastic waste was about 6300 Mt in 2015, one-tenth of which was recycled, and the rest was burned or buried underground. It is estimated that about 12,000 Mt of plastic waste would be buried underground by 2050[1]. With the international community's attention to plastic waste, Thompson first proposed the concept of microplastics in 2004[2]. Microplastics (MPs) refer to small particle plastics with a particle diameter less than 5 mm. At present, researchers have detected the ubiquitous presence of MPs in the coastline of various continents, the Atlantic Islands, inland lakes, the Arctic, and deep-sea habitats[3-7]. Therefore, more researchers have begun to study the environmental hazards of MPs.

Organisms could ingest MPs in the environment. MPs were found in the digestive tract of 233 marine organisms worldwide (including 100% turtle species, 36% seal species, 59% whale species, 59% seabird species, 92 kinds of sea fish, and 6 kinds of invertebrates)[8]. Moreover, MPs may block the organisms' digestive system, accumulate, and migrate in their bobies[9]. It has been found that MPs can be transferred via the food chain, and their intake differs with the changes of habitat, growth stage, feeding habits, and food sources[10]. Some researchers reported the existence of MPs in the droppings of seabirds[11], which means that seabirds act as the carrier of MPs. In addition, ingestion of MPs can affect health of some organisms. For example, MPs can be entangled in the gastrointestinal tract, thus increasing the mortality of cetaceans[12].

MPs alone have toxic effects on organisms as well as joint toxic effects on organisms with other pollutants. Ingestion of environmental-related concentrations of plastic debris may disturb the endocrine system function of adult fish[13]. The size of microplastics is one of the important factors that affect their uptake and accumulation by organisms[14,15]. The small-size plastics, especially the nanosize plastics, are prone to interaction with cells and organisms with high toxicity[16-19]. Although the harmful effects of nano-particle size microplastics on organisms exceed the current research level, the detection and quantification of microplastics with sizes less than several micrometers, especially the submicron/nano-plastics in the environment, is still a great challenge. In addition, nano-plastics also have some effects on embryos. Polystyrene can damage the development of sea urchin embryos by regulating protein and gene profiles[20].

To explore the potential impact of MPs on organisms, we conducted this study. Due to the similarity between bird embryos and human embryos, they are usually used in developmental biology research[21] including angiogenesis and antiangiogenesis, tissue engineering, biomaterial

implant, and brain tumors[22-24]. Bird embryos have the advantages of low cost, a short culture cycle and easy operation[25,26]. Therefore, we chose quail embryos with a short growth cycle as the experimental animal in this study. Simultaneously, we can directly observe the morphological changes of quail embryos exposed to MPs during the embryonic development stage using an eggshell-free hatching technology. The experimental materials used were polypropylene (PP) and polystyrene (PS). Because PP and PS[27] account for the largest proportion of polymer types obtained in sediments and water bodies worldwide, the most common polymer types extracted from captured marine organisms are ethylene and propylene[28]. This experimental protocol describes the whole process for visual evaluation of toxicological effects of MPs on quail embryos exposed to MPs. We can easily extend this method to examine other pollutants' toxicity to embryo development of other oviparous animals.

Protocol

1. Preparation before exposure

1.1. Select fertilized quail eggs born on the same day for the exposure test.

1.2. Select quail eggs with similar weights. Each fertilized quail egg is about 10-12 g.

1.3. Fully clean all fertilized quail eggs from external feces and other debris.

1.4. Sterilize each pre-hatched fertilized quail egg and the eggs to be used with an antibiotic solution (penicillin and streptomycin, 1:1000, room temperature). Sterilize the incubator with 75% ethanol.

1.5. Open the eggs with the blunt end of a dental drill, leaving the eggshell at the tip for further use. The opening diameter of the egg was about 3 cm.

NOTE: To reduce the damage to the quail embryo, use a dental drill to open the blunt end of the egg and make the crack as smooth as possible.

1.6. After sterilization, place the fertilized quail eggs in a 38 °C incubator with 60% humidity for 24-36 h. Ensure that the blunt end of the quail egg faces up.

1.7. During the incubation of fertilized quail eggs, sterilize the tools needed in the subsequent experiments in a sterilization pot.

1.7.1. Use a film with a temperature tolerance high enough to avoid problems with the high-temperature sterilization.

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Commented [NN4]: Please specify the tools here.

2. Hatching the quail egg without a shell

2.1. Transfer the pre-hatched fertilized quail eggs from the incubator to a clean bench and lay them flat on the container to stabilize them for about 1-2 min.

2.2. Use scissors (12.5 cm surgical straight scissor) to cut a small opening (diameter 3 mm) in the central axis of the pre-hatched fertilized quail eggs and to cut 1-2 cm small opening. Carefully transfer the egg white and yolk of the fertilized quail eggs to the cut eggshell.

NOTE: When cutting a small opening with scissors, avoid touching of the yolk of quail eggs.

2.3. Add the control solution (without MPs) and the exposed solution of different masses (0.1, 0.2, and 0.3 mg) of microplastics with three particle sizes (100, 200, and 500 nm) to the egg contents by pipette. At the same time, add an appropriate amount of penicillin and streptomycin.

2.4. Cover the opening of the eggshell with the sterilized film (step 1.6).

2.5. Treat all the fertilized quail eggs.

2.6. Place the transferred quail embryos into the 38 °C incubator with 60% humidity for the necessary period. In this experiment, use an egg rotation angle of ±30°. Turn the eggs once an hour.

NOTE: The transfer should be as fast as possible, which requires more practice at the early stage.

3. Sample collection

3.1. After seven days of culture, remove well-developed embryos observed by the naked eye from the yolk and wash with phosphate buffered solution (PBS).

3.2. Dry the surplus solution outside the cleaned embryo with absorbent paper and weigh in a clean Petri dish.

3.3. Open the whole chest cavity, separate the liver and the heart from the viscera with needle-nose pliers, and place in 1.5 mL centrifuge tubes immediately after clearing.

3.4. Quickly record the weight on an electronic balance and calculate the hepatosomatic index (HIS = liver weight / body weight x 100). Measure the length of the sternum and body.

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Commented [NN6]: How much is added?

Commented [NN7]: How much is added?

Commented [NN8]: Treat how?

3.5. Based on the above indicators, evaluate the impact of MPs on embryonic development.

NOTE: Embryo quality here refers to the quality of yolk removal.

4. Data analysis

4.1. Report the experimental data in the form of mean ± standard error (SEM).

4.2. Use single-factor analysis of variance to compare the means of multiple groups of samples. The significant difference value was $\alpha = 0.05$.

Representative Results

For the analysis of experimental data, we compared wet weight, body length, sternum length and the change of hepatosomatic index between the control group and the 6 experimental groups, measuring and reflecting the quail embryos' growth and development from a macro perspective. We detected six normal quail embryos in each group. Each embryo reached the required Hamburger and Hamilton (HH) stage.

In **Figure 1**, we transferred the pre-hatched fertilized quail egg contents into the hemispheric eggshells and put them into the incubator. Then we recorded the development of embryos in the middle period of incubation for three days. As shown in **Figure 2**, A-A2 is the control group, and B-B2 is one treatment group. From the perspective of macroscopic embryo development, the embryos developed normally without the adverse effects of microplastics.

Table 1 and **Table 2** are the mean ± SEM of wet weight, body length, and sternum length of quail embryo after one-week exposure. The tables show that the wet weight and body length change significantly in different exposure groups. The weight and body length of the groups treated with 0.1 mg, 0.3 mg, 100 nm, and 500 nm MPs decreased slightly. The body weight and body length of 0.2 mg of 200 nm microplastic treated groups increased slightly ($P < 0.05$).

Hepatosomatic index (HIS) shows the proportion of liver in the quail embryo, which is an important sign to judge the degree of liver development. Moreover, HSI plays an important role in the pathogenesis of liver cell membrane injury and inflammatory infiltration. As shown in **Figure 3** and **Figure 4**, compared with the control group, the proportion of liver in the whole treatment group increased significantly after exposure to microplastics. However, there was no significant difference between the 0.2 mg and 0.3 mg of 100 nm MPs treatment groups and the control group.

Figure 1: Hatching quail eggs without shell.

Figure 2: The embryo development of quail on the 6th, 7th, and 8th day in the middle stage of

hatching without eggshell. The green arrow points to the eyes; the blue arrow points to the limbs.

Figure 3: Hepatosomatic index of quail embryos after exposure to MPs (nm) for 7 days. Significant differences between control and treatment groups are indicated by * $P < 0.05$.

Figure 4: Hepatosomatic index of quail embryos after exposure to MPs (μm) for 7 days. Significant differences between control and treatment groups are indicated by * $P < 0.05$.

Table 1: Wet weight, body length and sternum length of quail embryos after exposure to MPs (nm) for 7 days

Table 2: Wet weight, body length, and sternum length of quail embryos after exposure to MPs (μm) for 7 days. Compared with the control group, * indicates $P < 0.05$, ** indicates $P < 0.01$.

Discussion

This paper provides an effective experimental scheme to evaluate quail embryo development by detecting the basic development indexes. However, there are still some limitations to this experiment.

First, the mortality of quail embryos in the later stage of hatching is higher because of the shell-less hatching. There are artificially uncontrollable factors such as the destruction of normal protein ratio in the experimental process. We limited the exposure time of embryos to ensure the accuracy of the experiment. The research of embryotoxicity can only occur in the early and middle stages of embryo development. Second, the study of MPs on quail embryo development only occurs at the basic morphological analysis level. Thus, the conclusions are relatively simple and defects may exist. At the same time, the requirements for experimental conditions and operation are relatively high in the process of this experiment. Therefore, some noteworthy points are listed as follows:

It is very important to disinfect and sterilize fertilized quail eggs in the preparatory work due to the harmful pathogenic microorganisms on the surface of fertilized quail eggs. If disinfected, the microbes may intrude into the fertilized quail eggs during incubation, resulting in the quail embryos' death. Even if the transfer is successful, the death rate will be higher. Therefore, a good job should be done in disinfection and sterilization to reduce the experimental mortality.

When birds hatch eggs, they often change the position of the eggs and keep the air circulation to maintain a constant temperature for the eggs and the correct position for the fetus. This experiment used film to seal the eggshell. If the angle of egg rotation is too large, then the egg white will flow out. If it is too small, then adhesion between the embryo film and the eggshell film might occur, resulting in dead embryos. Therefore, set the rotation angle according to the

actual situation.

During transfer of quail embryos, the pre-fertilized quail eggs are placed horizontally and then cut in the middle of the eggshell. In this way, a small part of egg white easily flows out, which destroys the normal proportion and distribution of the thick and thin egg white. This makes the yolk, which should have been on the top, lean to one side, causing the embryo to die. Therefore, take care to make all the egg white flow into the new hemispheric eggshell to ensure the normal proportion and distribution during transfer.

After the successful transfer, the experimenter must be careful not to drop the liquid directly. The liquid should rely on the eggshell wall to make it flow slowly during addition of pollutants and antibiotics.

In addition to the four points mentioned above, strictly control the incubation conditions. Coordinate the balance of temperature, humidity, and ventilation. Keep the incubation laboratory quiet and dark to achieve the best incubation environment.

In conclusion, this experiment provides a basic protocol for studying the effects of environmental pollutants on the development of quail embryos. There are also other types of indicators in the study of embryonic growth and development, including vascular development, oxidative stress, and cell damage. The above experiment is only a simple macroscopic evaluation of embryonic development from the morphological aspect. Finally, the improved research idea and protocol in the future could provide a new method for the toxicological study of embryo growth and development.

Acknowledgements

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Disclosures

The authors have nothing to disclose. All authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work of this paper.

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Figure 1

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Figure 2

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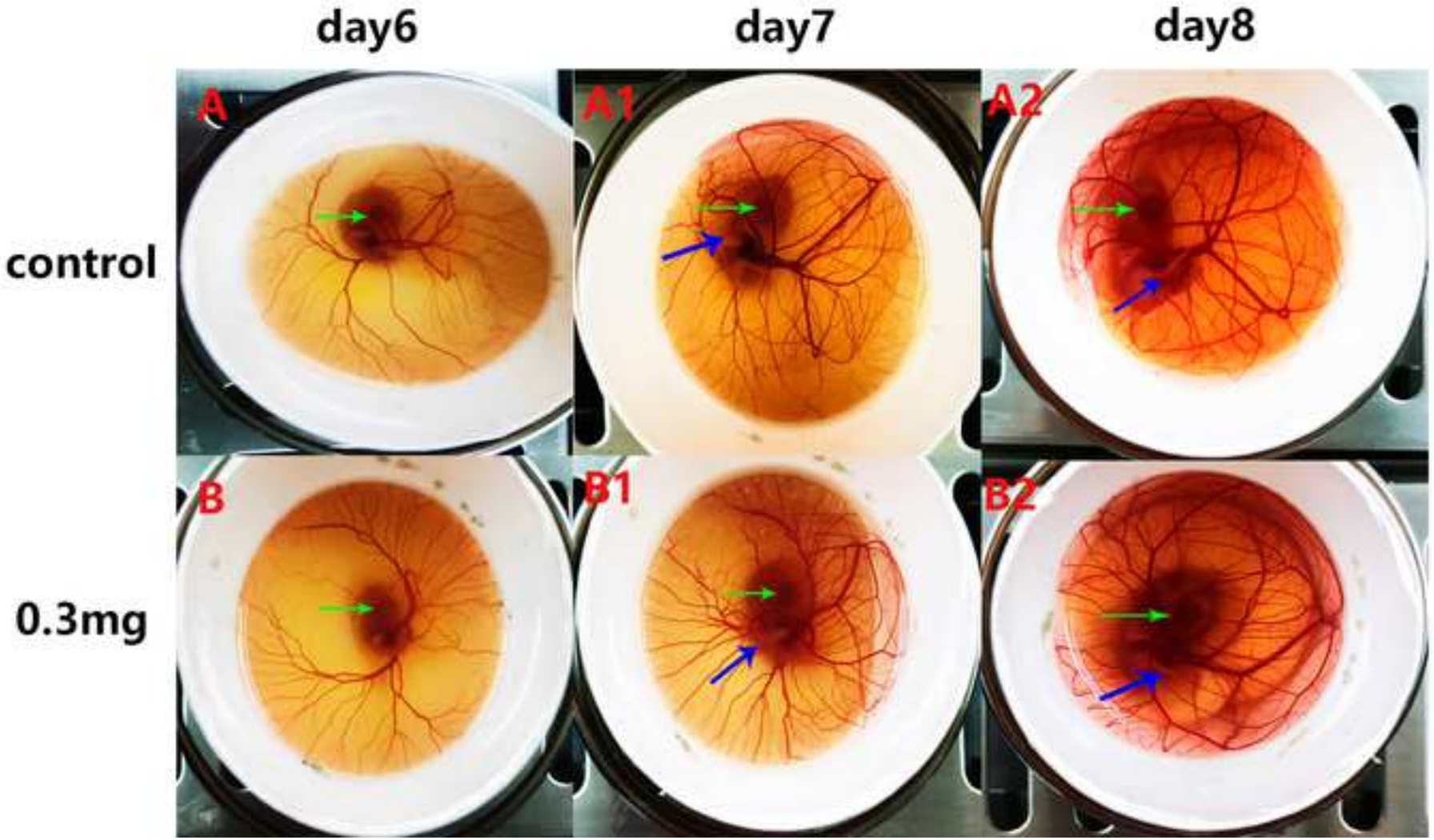

Figure 3

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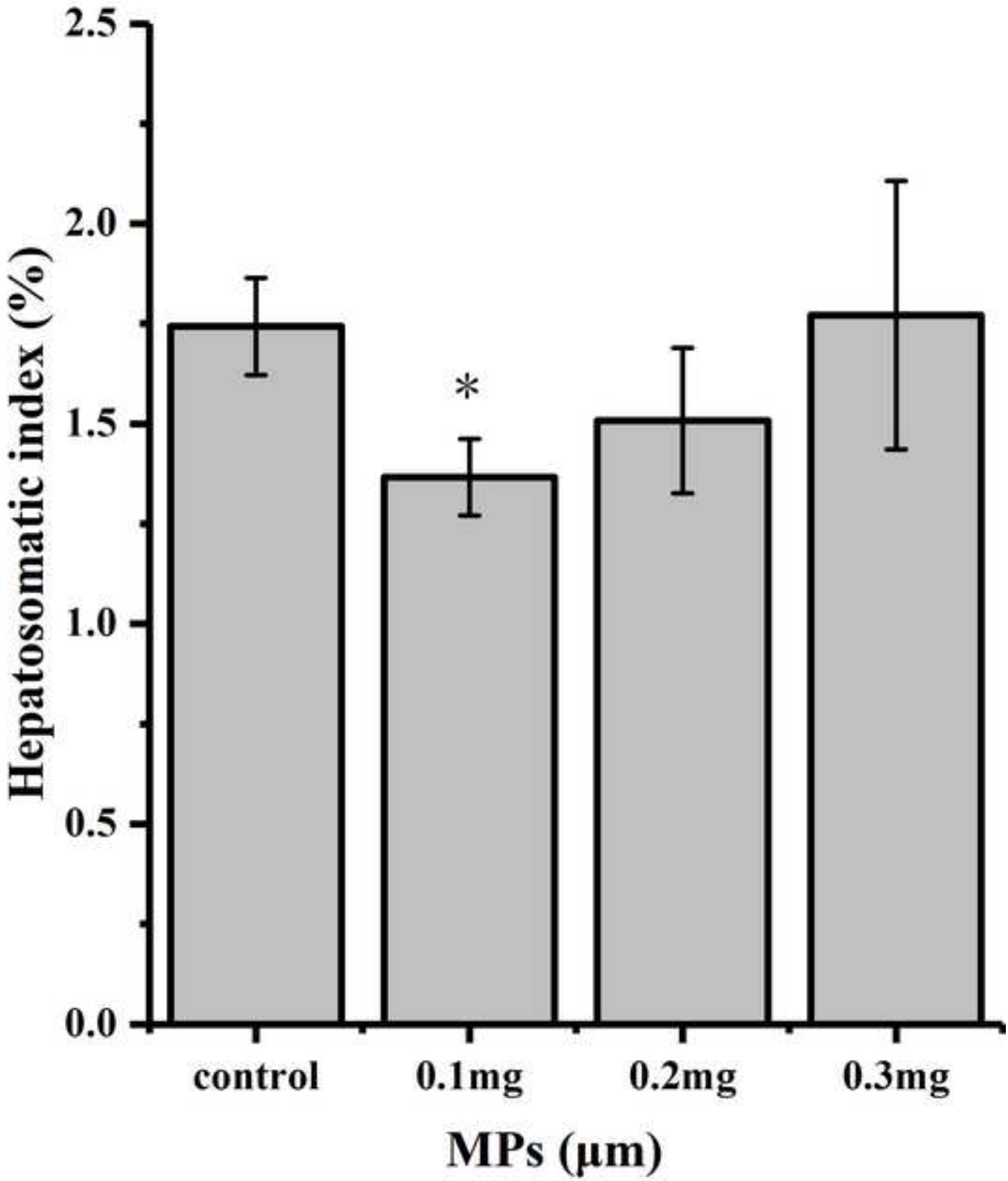

Figure 4

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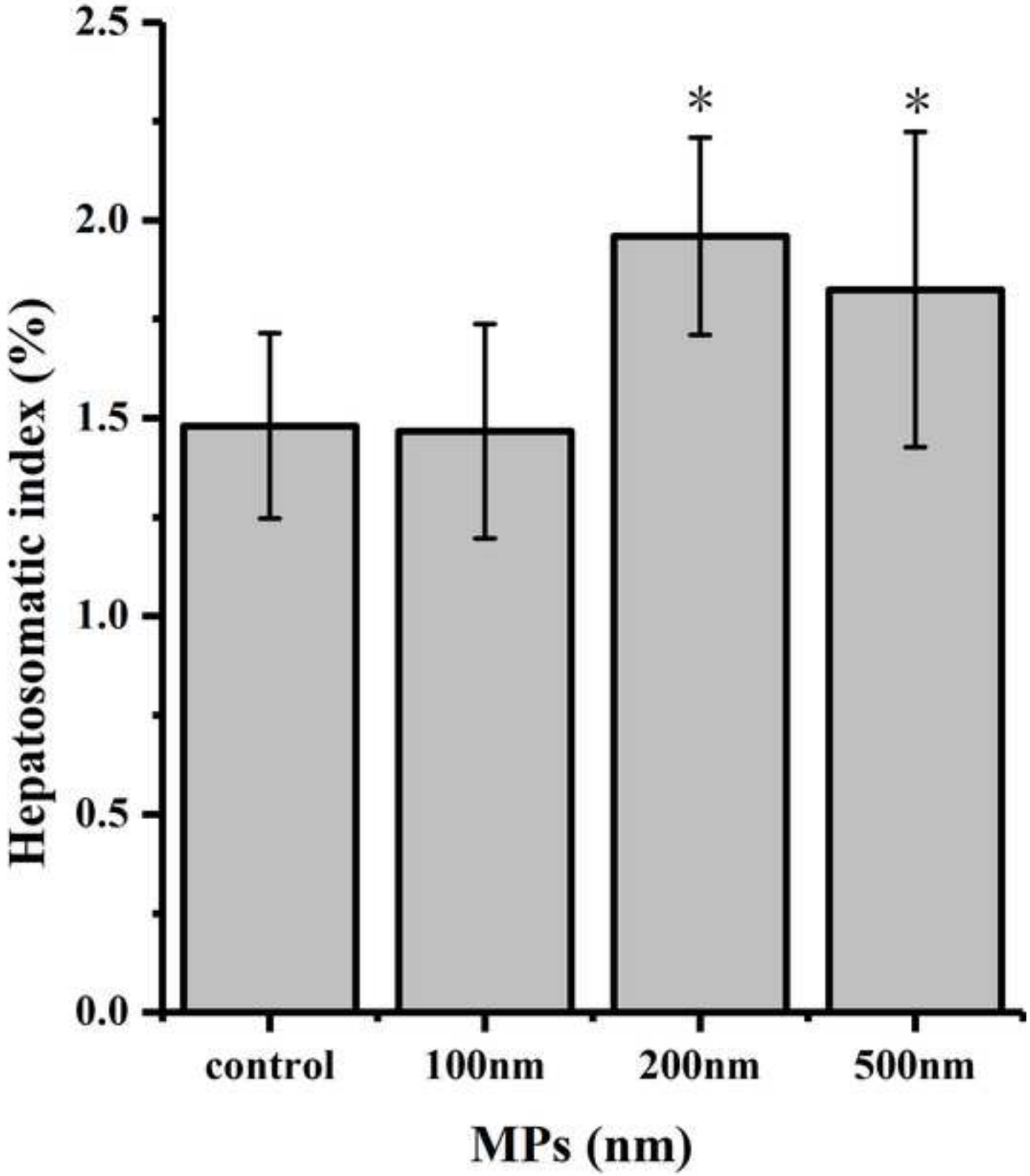

Table 1

MPs treatment	Weight (g)	Length (cm)	Sternum length
Control	2.509±0.324	5.425±0.477	1.025±0.094
100 nm	1.812±0.155*	4.632±0.315*	0.950±0.152
200 nm	2.272±0.368	5.297±0.268	1.025±0.076
500 nm	1.785±0.127*	4.892±0.154*	1.017±0.082

Table 2

Treatment	Weight (g)	Length (cm)	Sternum length
Control	2.161±0.166	5.23±0.26	1.10±0.04
0.1 mg	1.960±0.338*	4.82±0.75*	1.04±0.04
0.2 mg	2.410±0.366*	5.25±0.26	1.07±0.10
0.3 mg	1.901±0.759	4.95±0.15*	1.02±0.09

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Multi sample tissue grinder	Shanghai Jingxin Industrial Development Co., Ltd.	Tissuelyser-24	Grind large-sized plastics into small-sized ones at low temperature
Electronic balance	OHAUS corporation	PR Series Precision	Used for weighing
Fertilized quail eggs	Guangzhou Cangmu Agricultural Development Co., Ltd.		Quail eggs for hatching without shell
Fluorescent polypropylene particles	Foshan Juliang Optical Material Co., Ltd.		Types of plastics selected for the experiment
Incubator	Shandong, Bangda Incubation Equipment Co., Ltd.	264 pc	Provide a place for embryo growth and development
Nanometer-scale polystyrene microspheres	Xi'an Ruixi Biological Technology Co., Ltd.	100 nm, 200 nm, 500 nm	Types of plastics selected for the experiment
Steel ruler	Deli Group	20 cm	Used to measure length
Vertical heating pressure steam sterilizer	Shanghai Shenan Medical Instrument Factory	LDZM-80KCS-Ⅱ	Sterilize the experimental articles

Dear Editor Ph Nam Nguyen，

On behalf of my co-authors, we thank you very much for giving us an opportunity to revise our manuscript, we appreciate the editor and reviewers very much for their positive and constructive comments and suggestions on our manuscript entitled “Ecotoxicological effects of microplastic on bird embryo development by hatching without eggshell” (JoVE61696).

Thank you very much for your attention to our manuscript. We have studied reviewer’s comments carefully and have made revision which marked in red in the paper. Please kindly find the attachment of the revised version. Once again, we would like to express our great appreciation to you and the reviewers for the comments on our paper. Looking forward to your reply.

Thank you and best regards.

Yours sincerely,

Liyi Wang

The main corrections in the paper and the responses to the editorial, production, and reviewer’s comments are as flowing:

Responds to the editorial and production comments:

Changes to be made by the Author(s) regarding the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Reply: Thanks for your comments. We’ve got professional help with the technical and grammatical editing of our paper, and the corrections are marked in red.

2. Please submit each figure individually as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps.).

Reply: Thanks for your comments. All images were submitted as a vector image(.psd).

3. Please remove the brackets around the superscripted subtitles.

Reply: Thanks for your comments. All superscript brackets have been removed in the revised submission.

4. Is an ethics statement required. If so, please include it in the written manuscript and before the protocol section of the video as well.

Reply: Thanks for your comments. All the eggs we used were commercial eggs that can be sold. Ethics approval was not required for this research.

5. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?

Reply: Thanks for your comments. All the details were provided in the revised submission (Line 83-87, 90, 103, 113-115).

6. What batch of fertilized quail eggs are used? How are the eggs fertilized?

Reply: Thanks for your comments. Quail fertilized eggs were born the same day were used in this experiment. The quail fertilized eggs are obtained by natural mating, and the mating partners of male and female quails are fixed.

7. What are the sizes of the quail eggs used?

Reply: Thanks for your comments. Each quail egg is *about 10~ 12 g. Instead of size, we measure eggs by weight. The description in the manuscript has also been changed.*

8. 2.1.C: How is the sterilized with antibiotics done? What is the concentration used?

Reply: Thanks for your comments. After dissolving penicillin and streptomycin into ultrapure water, all eggs were soaked in water for 1-3 min, and then wiped with sterile paper to dry. The antibiotics solution dilution rate was 1:1000.

9. 2.1.D: What is the drill bit size? How large of an opening is made?

Reply: Thanks for your comments. The type of bit used in the experiment was cutting type, and the diameter of drill bit was 7.9 mm. The opening diameter of the egg was about 3 cm.

10. 2.2.B: How small of an opening? Please specify the size of the tool and the opening.

Reply: Thanks for your comments. The opening diameter of the fertilized egg is about 3 mm. The tool used was 12.5 cm Surgical straight scissor.

11. How is the HSI calculated?

*Reply: Thanks for your comments. HSI= Liver weight/ Body weight*100*

12. Please discuss limitations of the protocol in the discussion.

Reply：Thanks for your comments. In this experiment, because of the high mortality of late embryos, the exposure period was short. In addition, the experiment is still at the basic research level, and has not extended to the molecular level. The conclusion is not deep enough. Line 201-207: All the details were provided in the revised submission.

13. Please include an Acknowledgements section, containing any acknowledgments and all funding sources for this work.

Reply: Thanks for your comments. An Acknowledgement for this work has been added to the manuscript.

14. Please include a Disclosures section, providing information regarding the authors' competing financial interests or other conflicts of interest. If authors have no competing financial interests, then a statement indicating no competing financial interests must be included.

Reply: Thanks for your comments. The author's statement has been added to the manuscript.

Changes to be made by the Author(s) regarding the video:

1. Please increase the homogeneity between the video and the written manuscript. Please revise the narration to be more homogenous with the written manuscript. Ideally, the narration is a word for word reading of the written protocol.

Reply: Thanks for your comments. Video dubbing has been modified to increase consistency. According to the actual experimental situation, and experimenter hopes to explain the action as clearly as possible in the video. The narration of the video is slightly different from that of the manuscript.

2. Hold on the title cards for longer, all of them are only up for a second. Hold on the main title card for an additional 4-5 seconds, and the other cards for an additional

2-3.
Reply: Thanks for your comments. The problem of the main title card appears for a short time has been modified in the video.
3. Hold on the text for the tools at 2:53 for a few second longer so the viewer can catch everything.
Reply: Thanks for your comments. The time of the appearance of tool text in the video has been extended.
4. Some rough spots on the VO that should be edited out: at 0:21 I can hear papers being shuffled, and at 1:56 I can hear things being moved on a desk and a mouse being clicked.
Reply: Thanks for your comments. The noise in the video has been modified.
5. 6:00 = lease stabilize the egg videos on the different days.
Reply: Thanks for your comments. The video of the development period has been adjusted to the maximum extent.
6. 7:53: The hand waving at the end of the protocol can be removed.
Reply: Thanks for your comments. The wave at the end of the video has been removed.
7. Please include a short conclusion section with a conclusion title card.
Reply: Thanks for your comments. The conclusion has been added to the video.

Responds to the reviewer's comments:

Reviewer #1:
1. Except for hepatosomatic index, are there other morphology index to qualify the develop process? For example, early organs such as eyes…
Reply: Thanks for your comments. In addition to liver indicators, there are also indicators of the heart and brain. However, the experimental results cannot be released yet.
2. Format of x-, y-axis labeling, blank before unit in Fig. 3, 4
Reply: Thanks for your comments. The Fig. 3, 4 has been revised according to the comments.
3. Add size bar in Fig.2, and some arrows point out special organs or structures
Reply: Thanks for your comments. In Fig. 2, the organs that can be seen in the figure have been marked according to the comments. And *Figure 2 is only a picture to be displayed and is not quantified. So no size bar is added.*

Reviewer #2:
Manuscript Summary:
Reply: Thanks for your comments. Line:15-17: The summary has been added to the manuscript.
Abstract
Lines 17-22: The abstract does not contain a review of the results of the methods.
Reply: Thanks for your comments. Line 29-31: The results section has been added to the abstract.

Intro

Line 28: should provide the definition of microplastics here, for readers that might not know how they are defined

Reply: Thanks for your comments. Line 44: The definition of microplastics has been added to the manuscript.

Line 34: by migrate do you mean move through food webs?

Reply: Thanks for your comments. Yes, it can be moved not only in the food web but also in the body. Such as microplastics can pass through the intestinal wall into the circulatory system of the body and then into the organs (Farrell et al., 2013). And the top predator indirectly ingests microplastics through food webs (Alejo-Plata et al., 2019; Nelms et al., 2018).

Lines 36-40: these sentences seem disconnected, the first one discusses seabirds and the next cetaceans

Reply: Thanks for your comments. Two different kinds of organisms are selected as examples to explain that the organisms mentioned in the first sentence of this paragraph can ingest microplastics. At the same time, the two sentences are not linked smoothly, and I have revised them in the manuscript.

Lines 46-52: the transition from the third paragraph to the last paragraph does not make sense - there needs to be references to concerns over the impacts of MPs on organismal development in the embryonic stages, there are likely papers that voice this concern out there, or at least include this as an overarching question to put the research into context.

Reply: Thanks for your comments. Line 64-67: It has been modified in the manuscript.

Protocol

Line 64: when working with microplastics, it's really important to ensure cross contamination is not occurring in the lab. What steps were taken to ensure microplastics from outside sources (example - the air vents, researchers' clothes) were not cross contaminating the embryos in the experiment

Reply: Thanks for your comments. It has been modified in the manuscript. The microplastics were disinfected with absolute ethanol. And in the whole process of the experiment, we operated on the sterile table. It is added to the embryo in the form of sterile PBS.

Discussion

Line 176: if the scientists conducting this experiment did rotate the embryos during their observations, this should be included as a step in the methods section so that others can replicate it

Reply: Thanks for your comments. Line 114: The rotation angle of the egg has been added to the protocol.

Lines 177-182: this should also be addressed in the methods section to ensure others can replicate

Reply: Thanks for your comments. The answer to this question has been included in the protocol.

Lines 193-195: the incubation period used in this study is very short and might not be long enough to study certain pollutants' impacts on the embryos. Is any work

ongoing to try and extend the incubation time for experiments?

Reply: Thanks for your comments. There is indeed work in progress to try to develop it until hatching is complete. It's not easy to draw conclusions because of the small sample size. At the same time, we are improving the experimental conditions to achieve the expectations.