# Antibiotics Disrupt Embryonic Development in the Leech, *Helobdella stagnalis*

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Edited by Joshua Waters

## Abstract

Commercial livestock are often treated with antibiotics, some of which are known to cause adverse effects in humans, especially during human fetal development. Some antibiotics are retained in meat, but little is known about possible side effects of residual antibiotics in human development. Our project utilizes leech embryos as a model system, since embryos are large enough to visualize easily, and their developmental stages follow a definitive timeline. To identify effects of antibiotics in leech embryos, we tested tetracycline and tylosin, two common antibiotics used in livestock. The positive control, colchicine, has known developmental defects on leech embryos. Our findings show that embryos exposed to the antibiotics had an abnormal number and patterning of stem cells, as well as an increased death rate. These findings suggest that antibiotics have developmental defects on leech embryogenesis.

## Introduction

Physicians commonly prescribe antibiotics to treat infections and diseases caused by bacteria. Because antibiotics target specific bacteria, they are prescribed according to clinical practice guidelines. Different stages of infection are known to have an increased risk of transmission. Treatment with antibiotics reduces the spread of infection by minimizing the amount of bacteria present. While antibiotics have been one of the most revolutionary discoveries in medicine, their use may have drawbacks.

Although antibiotics are used with the intention of killing infectious bacteria, they are also known to kill the normal bacterial flora in the body. This can lead to additional infections, like thrush, due to an imbalance of bacteria (Dinsmoor et al., 2005). Some antibiotics are known to cause birth defects; for example, tetracycline is advised against after the fifth week of pregnancy because it disturbs the development of teeth and bones. There is also a link between the use of tetracycline and lethal liver damage, as well as tooth discoloration (Mylonas, 2011). Overuse of antibiotics has also created an abundance of antibiotic resistant bacteria. Many infections have become difficult to treat since the bacteria have evolved to resist treatment.

Several ethical guidelines are in place when considering humans for experimentation. Risk of harm and the general welfare of human participants must be taken into account. Additional undesirable factors are small sample size, lengthy gestation, and excessive costs. *Helobdella stagnalis*, a species of glossiphoniid leech, was selected as a model system for multiple reasons. With the experiment focusing on embryonic development, a single leech can provide access to many embryos. Leeches are not only cost effective and easily maintained, their embryos are also large enough to visualize, and their developmental stages are clear and well defined.

In this report, the effects of tylosin and tetracycline have been tested on *H. stagnalis* embryos. Tylosin is a feed additive that promotes growth, and is also used in veterinary medicine. This antibiotic is retained in meats like pork, chicken, and beef (Horie et al., 2003). Tetracycline is an antibiotic commonly used in human and animal veterinary medicine because of its effectiveness against a variety of bacteria (Tölgyesi et al., 2014). It has been proven that meats, such as pork, chicken, and beef, retain antibiotics that were given to them as livestock (Yamaguchi et al., 2015). Since many farmers treat their livestock with antibiotics, this may have effects on those who consume these products. Exposure to this information presented an opportunity to investigate possible developmental changes caused by these two antibiotics on the embryonic development of the leech *H. stagnalis.* We collected and exposed 12 gravid leeches to antibiotics to assess changes in embryonic development. The findings show that embryos exposed to the antibiotics expressed several abnormalities. These results suggest that antibiotics have developmental defects on leech embryogenesis.

## Materials & Methods

After the initial trials (data not shown) were contaminated with protozoa, the methods were adjusted to prevent contamination of follow up trials. Embryos contained in the gelatinous cocoon had a lesser chance of becoming infected due to the protective properties of the cocoon. Infection was introduced after unintentional removal of the embryos from the cocoon while the embryos were collected from the leech. The follow up trials were then modified to include the adult leeches with their respective embryo filled cocoons.

### *Leech culture conditions*

*H. stagnalis* specimens were initially screened to identify gravid leeches, which were placed in a separate container until eggs were laid. This separate container was left in room temperature conditions to promote growth. Fertilized eggs were secreted into a gelatinous cocoon positioned on the ventral side of the leech. After fertilization, each leech and their respective embryos were placed into separate 25 mL petri dishes. Before any treatment was administered, the stages of leech embryos were identified and documented accordingly (Huang et al., 2002). One dish served as the negative control, which contained 25 mL of leech water only. Leech water comprises 3 g of Instant Ocean (Product# SS15-10, Blacksburg, VA) and 15 L of distilled and charcoal filtered water. Any leech water used in this experiment was autoclaved to decrease the risk of contamination. The second dish was treated as the positive control, which contained 25 μg/ml of colchicine. The third dish was treated as one of the experimental treatments that contained 62.5 μg/ml of tetracycline. The last dish was treated as another experimental treatment of 62.5 μg/ml of tylosin, which was dissolved with a 1% ethanol solution to promote solubility.

### *Concentration of antibiotics*

The MRL is the maximum amount of residue legally permitted in food. The European Union has an MRL of 100 μg/kg, in Russia the MRL is 10 μg/kg and in the USA the MRL is 2,000 μg/kg (Tölgyesi et al., 2014). The tremendous difference in the maximum residue limits allowed in different countries is intriguing. The baseline concentration of the antibiotics used in this study is equal to the MRL for the USA, which converts to ~62.5 μg/ml.

### *Recording and estimating leech development*

Each treatment was applied to one leech with its respective embryos. After 2, 4, and 6 days of treatments, embryos were examined and photographed under a dissecting microscope. Stages were identified and documented at these points. All treatments were performed in triplicate. Only data for the sixth day after treatment is shown, since it was evident that the control reached a later stage while all other treatments arrested development prior to juvenile formation (Fig. 2A).

### *Morphological effects of antibiotics*

The morphology of the leech embryos was observed under the dissecting microscope.

### *Statistical Analysis*

A significance analysis was performed on Microsoft Excel using student's t-test. Data from the average stage progression for each treatment was used to determine any significant change from the control. Due to high numbers of embryo death, certain values were not included in data analysis. For example, in one trial, only one embryo survived in the colchicine treatment to stage 10/11. This value was not averaged since it is not representative of the data.

## Results

To test the effects of antibiotics on morphological development in embryos, we chose to use leech embryos because of their large size and well-defined developmental stages. We chose tetracycline and tylosin because both are often used in veterinary medicine. Furthermore, tylosin is a feed additive used to promote growth in livestock. We predicted that the development of the embryos would be altered after exposing them to antibiotics. As expected, there were visible differences in embryonic development among treated samples after 2, 4, and 6 days (Fig. 1). Once leech embryos were exposed to treatment, embryos were either evaluated as normal or abnormal, as shown in Table 1 (Materials and Methods). We found that both treatments (tetracycline and tylosin) and the positive control (colchicine) caused abnormalities in embryonic development (Table 1).

Second, we recorded the developmental stages of the leeches to determine if there was a negative effect of antibiotics on leech development (Materials and Methods). We found that there were negative developmental effects by antibiotics we tested (Fig. 2); tetracycline at day 2 ($p=0.079$), day 4 ($p=0.095$); tylosin treatment at day 2 ($p=0.079$, $\alpha=0.1$); tetracycline treatment after day 6 ($p=0.027$, $\alpha=0.05$). We also found that the samples treated with tetracycline had a significantly reduced growth rate (Fig. 2B; $p=0.079$, $\alpha=0.1$).

Third, we measured the survival rate of the embryos, and found both of the antibiotic treatments had a low survival rate, with tetracycline being lower at 12% after the 6-day incubation period (Fig. 3). According to the reported timeline of leech embryogenesis, stage 10 is the beginning of juvenile stage of the life cycle. We observed the juvenile leeches in the control after six days of treatment, whereas none of leeches in the treated samples grew to the juvenile stage (Fig. 1 and 3).

Antibiotic treated adult leeches varied in survivability in each trial (data not shown). The tetracycline treatment caused adult death in all three trials while the tylosin treatment resulted in death in two of the trials. The embryos also suffered a high mortality rate amongst the antibiotic treatments. The small number of embryos that survived (Fig. 3) showed abnormal development.

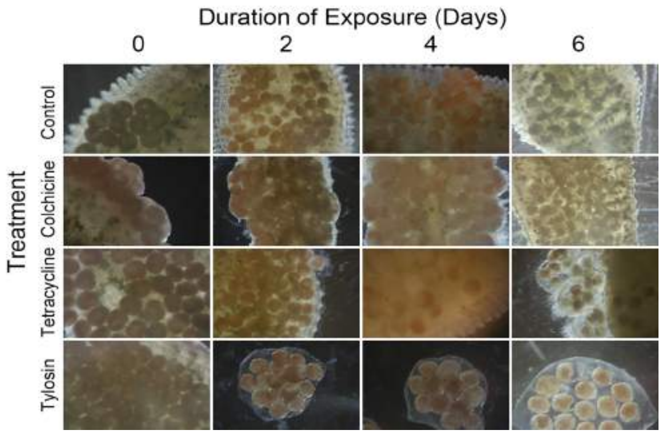


Figure 1. Images of leech embryos 0, 2, 4, and 6 days after treatments of control (normal leech water), colchicine 25 μg/ml, tetracycline 62.5 μg/ml, and tylosin 62.5 μg/ml.

Table 1: Effect of treatment on development of embryos at 0, 2, 4, and 6 days.

| Day | Control | Colchicine | Tetracycline | Tylosin |
|---|---|---|---|---|
| 0 | Normal | Normal | Normal | Normal |
| 2 | Normal | Abnormal | Abnormal | Abnormal |
| 4 | Normal | Abnormal | Abnormal | Abnormal |
| 6 | Normal | Abnormal | Abnormal | Abnormal |

## Discussion

In this study, we found that two common antibiotics, tetracycline and tylosin, had several negative effects on leech embryonic development: abnormal morphology, slow growth rate, and low survival rate. The exposure of antibiotics to leech embryos suggests that antibiotics have a negative effect on leech embryogenesis.

During the latest trial, all treatments except the control became infected with fungus between the fourth and the sixth day (data not shown). While the treatments of antibiotics and colchicine had an effect on the embryos up

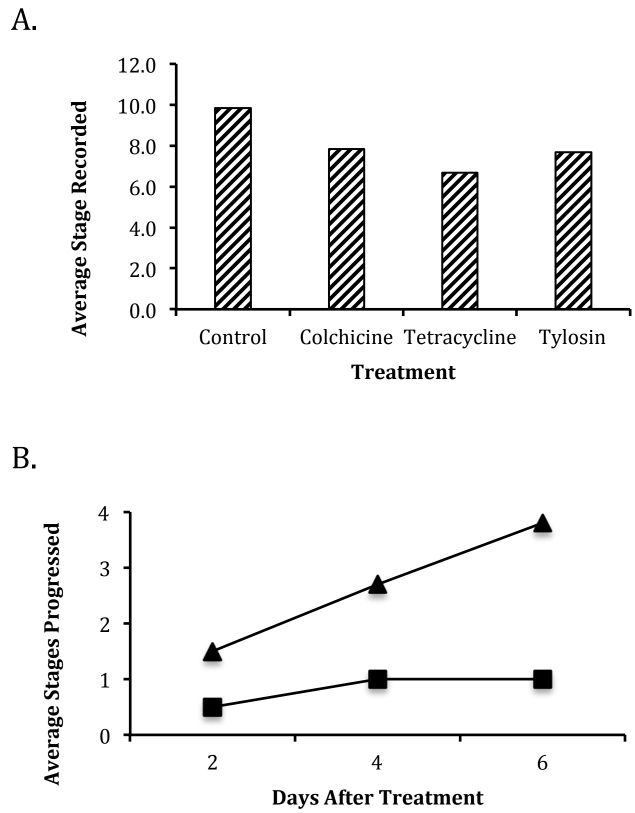


Figure 2: Negative effect of antibiotics on leech embryonic development. A. The average stage recorded of leech embryos at 6 days. Error bars represent s.e.m. B. Difference of growth rates between the control (triangle) and the tetracycline (square) treatment at 2, 4, and 6 days. The equation for the control is y=1.15x+0.3667 and the equation for the tetracycline treatment is y=0.25x+0.3333.

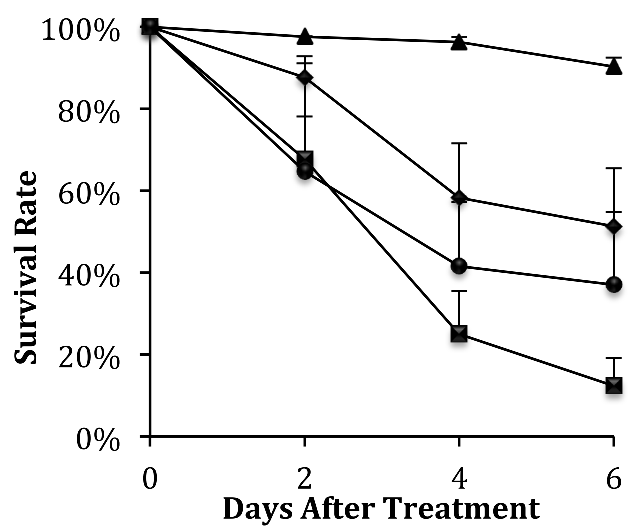


Figure 3: The average survival rate of each treatment after 0, 2, 4, and 6 days. Trangle=Negative Control, Square =Tetracycline, Circle=Tylosin, Diamond=Colchicine.

to the fourth day, the fungal infection may have skewed the results. Although infection did not contaminate the control, the embryos acquired an abnormal cell shape for unknown reasons. Because of time constraints, the three trials were performed during different days due to lack of gravid leeches. Leech embryos started at different stages when treatment begun for each trial, but clear abnormalities and a lack of progression were evident for all treatments in comparison with the control.

For ethical reasons, it is not feasible to study the role of antibiotics on human embryonic development. One of the concerns is the antibiotic carried over through the consumption of meats. A recent study recommended for women to intake 46 grams of protein per day (Bouvard et al., 2015). Proteins such as meats may contain residual antibiotics, which could be especially harmful to pregnant women, as they may be unknowingly harming the fetus due to these residual antibiotics. Considering the importance of the current research topic, more creative research is required to understand the implications of the usage of antibiotics.

## Acknowledgements:

We thank Dr. Kwangwon Lee and our TA, Joshua Waters, for making this project achievable. Special thanks to Dr. Daniel Shain, Dr. Saglam Naim, and Ralph Saunders for allowing us to use their laboratory, guiding, and assisting us in our research.

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