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Metabolic Inhibitor Effects on Eyespot Formation in Regenerating Planaria

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Metabolic Inhibitor Effects on Eyespot Formation in Regenerating Planaria

by

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Abstract

Planaria are flatworms known for their remarkable ability to regenerate. Glycolytic activity has been shown to increase during planarian regeneration (Osuma et al., 2017) and regeneration requires both an increase in mitotic activity and an increase in apoptotic activity to successfully regrow missing structures (Pellettieri et al., 2010; Wenemoser & Reddien, 2010). The objectives of this study are to determine if metabolic inhibitors affect the time needed to regenerate eyespots after head amputation as a measurement of regeneration and if there are any correlations between average numbers of mitotic and apoptotic cells in regenerating planaria in the presence or absence of metabolic inhibitors. Planaria (*D. dorotocephala* and *D. tigrina*) were exposed to non-lethal doses of metabolic inhibitors after amputation between the head and the pharynx to determine if metabolic inhibitors affect eyespot regeneration times. Mucus removal, fixation, and bleaching were used to prepare planaria for immunolabeling procedures. Mitotic cells were labeled to determine if metabolic inhibitors affect average numbers of mitotic cells in regenerating planaria. Immunolabelling procedures were used in an attempt to visualize apoptotic cells in planaria. Results from the exposure experiments suggest that the earlier planaria are exposed to metabolic inhibitors after amputation, the longer it takes to regrow eyespots. By comparing the average number of mitotic cells planaria in the presence or absence of metabolic inhibitors, it was determined that delays in eyespot formation are not associated with changes in the average number of mitotic cells. Further studies should be conducted to test for significant differences between apoptotic cells in inhibitor-treated and untreated planaria.

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Chapter 1: Introduction

Planaria Regeneration

Planaria are flatworms known for their remarkable ability to regenerate. The planarian regeneration response involves the formation of the blastema, an area of rapid growth of new cells and tissues produced from neoblasts (Reddien & Alvarado, 2004). Neoblasts are adult pluripotent stem cells found throughout the body of planaria and allow planaria to perform stem cell-mediated regeneration by both proliferating and differentiating at the blastema to grow and replace lost tissues (Reddien & Alvarado, 2004). These stem cells produce all cell types and organs in planaria and are directly responsible for the flatworms' regenerative capabilities (Rink, 2013). Two stem cell responses of increased mitotic activity occur in regenerating planaria. The early stem cell response occurs 4 to 12 hours post amputation, while the later stem cell response occurs 2 to 4 days post amputation (Wenemoser & Reddien, 2010). The early response appears to occur for any injury type, while the later response occurs due to tissue loss requiring regeneration (Wenemoser & Reddien, 2010). During regeneration after amputation, not only is there rapid proliferation and differentiation of stem cells, but also controlled cell death. In planaria, apoptosis occurs near the injury site 1 to 4 hours post amputation and throughout the amputated fragment 3 days after amputation (Pellettieri et al., 2010). Apoptosis is a method for remodeling preexisting tissue and has been found in planaria to occur in differentiated cells, not stem cells (Pellettieri et al., 2010). This suggests that regeneration requires both an increase in mitotic activity, as stem cells in the blastema generate new cells and tissues, along with apoptosis to remodel preexisting, differentiated tissue to successfully regrow missing structures in planaria (Pellettieri et al., 2010; Wenemoser & Reddien, 2010).

After an injury, planaria will form a blastema and begin the regeneration process (Reddien & Alvarado, 2004). The planarian regeneration response consists of three stages: the generic wound response (0-24 hours post injury), the regeneration response (approximately 30 to 70 hours post injury), and the differentiation phase (approximately 70 to 120 hours post injury) (Wurtzel et al., 2015). The generic wound response consists of the activation of about 224 genes that allow planaria to activate a regeneration response to any injury. Patterning factors and specialized neoblast genes are expressed in the regeneration response only if the injury results in tissue loss, allowing the regenerating tissues to eventually differentiate. The differentiation phase consists of the appearance of new, differentiated tissue (Wurtzel et al., 2015). Experimentally, it has been determined that glycolytic activity shows a significant increase by 24 hours post injury, reaching maximal levels by 72 – 96 hours post injury, coinciding with the onset of the differentiation phase (Osuma et al., 2017).

Rapidly proliferating stem cells have been shown to utilize glycolysis as the metabolic pathway of choice (Haroon et al., 2021). In planaria, a measured decrease in the ratio between oxygen consumption rate, a marker of mitochondrial activity, and extracellular acidification rate which measures lactic acid concentrations, a marker of glycolytic activity, suggests a reliance on glycolysis during regeneration (Osuma et al., 2017). Furthermore, the mitochondrial states in proliferating neoblasts and differentiated cells differ, suggesting that proliferating neoblasts with low mitochondrial mass have a greater affinity for pluripotency in comparison to cells with high mitochondrial mass (Haroon et al., 2021). Proliferating stem cells require more building blocks necessary for cell growth and division, which are supplied by glycolysis and the pentose phosphate pathway, reducing the need for mitochondria which is reflected by lower mitochondrial mass. Inhibiting oxidative phosphorylation-dependent metabolism has resulted in

increasing pluripotent stem cells in planaria (Haroon et al., 2021), suggesting that glycolysis-dependent metabolism is the preferred metabolic pathway utilized by neoblasts in regenerating planaria. Increases in glycolysis during regeneration may be favored due to regulation by phosphofructokinase of other metabolic shunts necessary for producing nucleotides, phospholipids, and amino acids required for cell division and growth (Osuma et al., 2017; Gibb et al., 2017).

Glycolysis begins with the conversion of glucose to glucose-6-phosphate (G6P) by the enzyme hexokinase. Through a series of reactions, G6P is converted into pyruvate, the product of glycolysis. Excess G6P can be converted into glycogen (glycogenesis), or it can be used in the pentose phosphate pathway. Byproducts of the pentose phosphate pathway include fatty acids from NAPDH production, nucleotides from the precursor ribose-5-phosphate, and aromatic amino acids from erythrose-4-phosphate (Voet et al., 2016). The pentose phosphate pathway also produces glyceraldehyde-3-phosphate and fructose-6-phosphate, intermediates of glycolysis. Another intermediate of glycolysis, dihydroxyacetone phosphate (DHAP), can become the backbone of triglycerides and glycerophospholipids (Voet et al., 2016). DHAP is produced in glycolysis from glucose and from pyruvate during gluconeogenesis (glucose synthesis); glyceraldehyde-3-phosphate from the pentose phosphate pathway can be reversibly converted into DHAP. Pyruvate can be converted back into glucose during gluconeogenesis, whereas in mitochondria, pyruvate can be converted to acetyl-CoA which is used in the TCA cycle. Acetyl-CoA is metabolized in a series of steps in the TCA cycle to produce NADH and $FADH_2$ for ATP production via the electron transport chain and ATP synthase complex, a part of oxidative phosphorylation. Excess acetyl-CoA is used for fatty acid, phospholipid, and cholesterol synthesis (Voet et al., 2016). The intermediates and reactants of the citric acid cycle, including

pyruvate, acetyl-CoA, alpha-ketoglutarate, succinyl CoA, fumarate, and oxaloacetate, can be used to synthesize amino acids. Monomers necessary for cell growth and division, such as amino acids, fatty acids, glycerol, and nucleotides, are produced from the byproducts of these interconnected metabolic pathways.

Metabolic Pathways and Inhibitors

Glycolysis consists of two phases: the investment phase where ATP is used to drive reactions and the payoff phase where ATP is generated. In the first step of the glycolysis investment phase, glucose is converted to G6P by hexokinase. Inhibition of hexokinase is expected to limit G6P production as well as production of byproducts for triglyceride synthesis, glycogenesis (glycogen synthesis), the pentose phosphate pathway, and the citric acid cycle. Lonidamine (LND) is a potent hexokinase inhibitor and inhibits hexokinase (HK II) found on the outer membrane of the mitochondria, which is not normally observed in differentiated cells but in cancerous and stem cells (Floridi & Lehninger, 1983; De Martino et al., 1984). Studies are lacking that utilize LND to inhibit glycolysis in planaria.

In the payoff phase of glycolysis, glyceraldehyde-3-phosphate is converted into 1,3-bisphosphoglycerate by glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Inhibition of GAPDH is expected to reduce pyruvate production and, consequently, reduce metabolic by-products formed during the TCA cycle, as well as potentially enhancing earlier reactions due to a buildup of metabolites. Heptelidic acid (HA, also known as koningic acid), dimethyl fumarate (DMF, also known as dimethyl formamide), and 4-octyl itaconate (4-OI), are known inhibitors of GAPDH. HA irreversibly inhibits glycolysis (Endo et al., 1985) by covalently binding to a cysteine residue at the active site involved in catalysis of glyceraldehyde-3-phosphate (Kato et al., 1992). HA contains an epoxy moiety which is thought to react readily with the sulfhydryl

group on the cysteine residue in GAPDH to induce inhibition (Sakai et al., 1988). Previous research has shown that the inhibition by HA is time-dependent and inhibits GAPDH within minutes. Sakai et al. (1988) demonstrated that GAPDH activity decreased by 50% within 20 seconds of exposure to 2 μM HA. HA has been shown to reduce extracellular acidity in planaria while minimally affecting the oxygen consumption rate (Osuma et al., 2017), suggesting that HA can be used to inhibit the payoff phase of glycolysis in planaria. The payoff phase of glycolysis can also be inhibited using DMF. Like HA, DMF inhibits GAPDH by covalently modifying a cysteine at the active site (Eming et al., 2021; Kornberg et al., 2018). GAPDH activity decreases in a time-dependent manner by 50% after about 50 minutes of exposure to concentrations of 25 μM DMF (Kornberg et al., 2018). Itaconate is a metabolite derived from cis-aconitate in the TCA cycle and is produced when respiration decreases and glycolysis increases, such as what is seen in Warburg-type metabolism (Eming et al., 2021). 4-OI is a cell-permeable derivative of itaconate and has been shown to also decrease enzymatic activity by alkylating a cysteine residue at a non-active site of GAPDH (Liao et al., 2019). Concentrations of 62.5 μM 4-OI have been shown to decrease GAPDH activity by 50% after about 3.5 hours of exposure (Liao et al., 2019). While each of the three inhibitors discussed above use covalent modifications to inhibit GAPDH, the rate at which they decrease enzyme activity differs.

The first step of the pentose phosphate pathway is the conversion of G6P to 6-phosphogluconolactone by the enzyme glucose-6-phosphate dehydrogenase and $NADP^+$. 6-aminonicotinamide (6-AN) is a known inhibitor of glucose-6-phosphate dehydrogenase (Arbe et al., 2020). Byproducts of the pentose phosphate pathway, including fatty acids, nucleotides, and aromatic amino acids are necessary components for cell growth and division. Inhibition of glucose-6-phosphate dehydrogenase has been shown to delay tail regeneration in *Xenopus*

tropicalis (Patel et al., 2022), suggesting the importance of the pentose phosphate pathway for species capable of regeneration, including planaria.

Chapter 2: Objectives

Research Questions

The objective of this study is to determine if potential metabolic inhibitors affect eyespot formation time post head amputation in planaria. This research aims to answer seven questions: (1) Do GAPDH inhibitors affect eyespot formation times in bisected planaria? (2) Are the effects of GAPDH inhibitors on eyespot formation times the same for the three stages of regeneration (generic wound response, regenerative response, and the differentiation phase)? (3) Does a second species of planaria display similar responses to GAPDH inhibitors? (4) Are there any observable differences in the number of cells undergoing cell division between control and inhibitor-treated regenerating planaria? (5) Does inhibitor treatment alter the number of apoptotic cells in regenerating planaria? (6) Does a potential inhibitor of the investment phase of glycolysis produce results similar to treatment with the GAPDH inhibitors? (7) Does a potential inhibitor of the pentose phosphate pathway produce similar results to treatment with GAPDH inhibitors?

Research Outline

To answer the seven questions outlined above, the objective will be achieved by: (1, 2) Applying non-lethal doses (a treatment concentration at which none of the exposed planaria die) of GAPDH inhibitor treatments to regenerating planaria during each of the three stages of regeneration (generic wound response, regeneration response, differentiation phase) for 24 hours each and measuring the length of time post-injury until eyespot formation is observed. (3) Measuring the length of time post-injury until eyespot formation is observed in a different species of planaria (*Dugesia tigrina*) and comparing these results to earlier data from *Dugesia dorotocephala*. (4) Utilizing an immunostaining assay to visualize the distribution and relative density of mitotic cells in regenerating planaria. (5) Using dyes or an immunostaining method to

compare apoptosis, or cell death, between inhibited and non-inhibited regenerating planaria. The 24-hour long exposure will be replicated with (6) an investment phase inhibitor of glycolysis and (7) an inhibitor of the pentose phosphate pathway.

Chapter 3: Materials and Methods

Planaria Care and Maintenance

Planaria [*D. dorotocephala* (Carolina Biological Supply) and *D. dugesia* (Flinn Scientific)] were maintained at room temperature in planaria water (0.5g/L Instant Ocean salts) and planaria water was replaced every other day.

Metabolic Inhibitor Exposure Pilot Experiment

Planaria (*D. dorotocephala*) were starved for one week prior to initiating the experiments. Planaria were bisected between the head and pharynx, and the posterior portion was transferred to a 24-well TC treated plate (USA Scientific). Six planaria were used per concentration of treatment solutions. Treatment solutions (100 µM, 50 µM, and 25 µM) of either DMF (Cayman Chemical), 4-OI (Cayman Chemical), HA (Focus Biomolecules), 6-AN (Cayman Chemical), or LND (Cayman Chemical) were added to each well with individual tail fragments at room temperature (RT). After 24 hours, the treatment solutions were removed and replaced with planaria water. The planaria were monitored over the next several days to determine the number of tail fragments that survived each treatment. If no planaria survived 25 µM of treatment solution, then the experiment was repeated with 20 µM, 15 µM, 10 µM, and 7.5 µM of treatment solution. If no planaria survived 7.5 µM of treatment solution, then the experiment was repeated with 5 µM, 2.5 µM, 1.0 µM, and 0.5 µM of treatment solution. After executing the experimental protocol, all planaria were euthanized by freezing.

Metabolic Inhibitor Exposure Experiment

Planaria (*D. dorotocephala*) were starved between 1 and 7 days before initiating the experiments. A sample size of 10 planaria per exposure timeframe treatment and control were bisected between the head and pharynx, and the posterior portion was transferred to a 24-well TC

treated plate. Immediately after amputation, the 0-24 hours post amputation (hpa) group were exposed to the treatment solution for 24 hours. The 24-48 hpa, 48-72 hpa, and control groups were treated with planaria water for 24 hours. After 24 hours post amputation, the 0-24 hpa, 48-72 hpa, and control groups' solutions were removed and replaced with planaria water and the 24-48 hpa group solution was removed and replaced with treatment solution. After 48 hours post amputation, the 0-24 hpa, 24-48 hpa, and control groups' solutions were replaced with planaria water and the 48-72 hpa group solution was replaced with treatment solution. After 72 hpa, all solutions across all treatment groups were removed and replaced daily with planaria water until eyespot regrowth was observed. The exposure protocol was repeated for a total of six replicates for each inhibitor tested. Treatment solutions used include: 10 µM HA [0.014% (v/v) DMSO], 15 µM DMF [0.0083% (v/v) DMSO], 20 µM 4-OI [0.0071% (v/v) DMSO], 2.5 µM LND [0.0033% (v/v) DMSO], and 100 µM 6-AN [0.1% (v/v) DMSO]. The protocol was repeated with an interspecies comparison, *D. tigrina* with the following adjustments: the control groups were treated with control solution (planaria water matched to DMSO concentration of the treatment solution), the sample size of each exposure time frame treatment and control groups was 20 planaria, and the experiment was repeated for a total of three replicates per inhibitor.

Separately, bisected planaria (*D. dorotocephala*) were exposed to concentrations of 0.1%, 0.014%, 0.0083%, and 0.00707% DMSO in planaria water 0-24 hpa. Each group treated with DMSO solutions contained 40 planaria. After 24 hpa, the DMSO solution was removed and replaced daily with planaria water until eyespot regrowth was observed.

Mucus Removal, Fixation, and Bleaching

Mucus removal, formaldehyde fixation, and bleaching protocols were adapted from Forsthoefel et al. (2014). Planaria were treated with HA, DMF, and 4-OI according to the

metabolic inhibition exposure experiment protocol. Mucus removal, fixation, and bleaching was performed immediately after 24 hours of exposure to the treatment solutions. Treatment solutions were removed and planaria were rinsed with planaria water. Planaria were chilled on ice for 1 minute. Planaria water was replaced with ice-cold 2% (v/v) HCl (J.T. Baker). The 24-well plate was shaken moderately by hand for 1 minute. Planaria were chilled on ice for 1 minute. The 24-well plate was shaken again moderately by hand for 1 minute. HCl was removed and planaria were rinsed with 1X PBS (ATCC).

Planaria were covered in 4% (v/v) formaldehyde (Sigma-Aldrich) in PBSTx [1X PBS plus 0.3% (v/v) Triton X-100 (Sigma)] and gently rocked for 20 minutes at RT. The fixative solution was removed, then planaria were rinsed twice with PBSTx and once with 1X PBS. The PBS was removed and replaced with bleaching solution [6% (v/v) H_2O_2 (Alfa Aesar) in methanol (BDH)]. The 24-well plates containing planaria were placed in a foil-lined container 45 cm below a fluorescent light at RT for 24 hours. After 24 hours, the bleaching solution was removed and planaria were rinsed three times in methanol (BDH), then stored at -20 °C. Planaria were rehydrated in 1:1 methanol:PBSTx, then washed three times in PBSTx for 5 minutes each before use in immunolabeling experiments.

Mitosis Immunolabeling

Planaria were exposed 0-24 hpa, 24-48 hpa, and 48-72 hpa with metabolic inhibitors (HA, DMF, and 4-OI) and mucus removal, fixation, and bleaching occurred. Each exposure time-frame group contained 5 planaria. Planaria were washed three times with PBSTx, then blocked in Blocking Solution [1X PBS, 1% (w/v) IgG-free BSA (Sigma Life Science), 0.3% (v/v) Triton X-100] overnight at 4 °C with gentle rocking. Blocking Solution was removed and the planaria were incubated overnight at 4 °C with gentle rocking with rabbit anti-

phosphohistone H3 (S10) primary antibody (Cell Signaling Technology) diluted 1:2000 in Blocking Solution. The primary antibody solution was removed and the planaria were washed six times for 30 minutes each with PBSTx at RT with gentle rocking, then washed three times for 30 minutes each with Blocking Solution at RT with gentle rocking. Planaria were incubated with goat anti-rabbit poly-HRP-conjugated secondary antibody (ThermoFisher Scientific) diluted 3:5 in Blocking Solution at 4 °C overnight with gentle rocking. The secondary antibody solution was removed and the planaria were washed eight times for 30 minutes each wash with PBSTx at RT with gentle rocking. The final PBSTx wash was removed and planaria were incubated with Tyramide Working Solution (1:1:100, tyramide stock solution:H_2O_2:reaction buffer) (ThermoFisher Scientific) for 10 minutes at RT with gentle rocking. After 10 minutes of incubation with Tyramide Working Solution, Reaction Stop Reagent (ThermoFisher Scientific) was added to the planaria, then the planaria were washed three times for 20 minutes each with PBS at RT with gentle rocking. The wash solution was removed and planaria were mounted in Vectashield (Vector Laboratories). Mitosis immunolabeled planaria were stored at 4 °C in foil-wrapped 24-well plates. The number of mitotic cells per area were counted 7 mm from the amputation site in photographic images and were standardized per area as measured in pixels using ImageJ software.

Apoptosis Immunolabeling

Planaria were exposed 0-24 hpa, 24-48 hpa, and 48-72 hpa to metabolic inhibitors (HA, DMF, and 4-OI) and mucus removal, fixation, and bleaching occurred. Each exposure time-frame group contained 5 planaria. Planaria were washed three times in 1X PBS, then quenched in 3.0% (v/v) H_2O_2 for 5 minutes each at RT. After, the planaria were washed twice with PBS for 5 minutes each at RT. Excess solution was removed and Equilibration Buffer (Millipore Sigma)

was applied directly to each well with planaria for 10 seconds at RT. Excess solution was removed and planaria were incubated in Working Strength TdT enzyme (Millipore Sigma) at 37 °C overnight.

The Working Strength TdT enzyme was removed and planaria were agitated for 15 seconds before incubating for 10 minutes at RT with Working Strength Stop/Wash Buffer (Millipore Sigma). Planaria were washed eight times for 30 minutes each with Blocking Solution (1X PBS, 1% IgG-free BSA, 0.3% Triton X-100) at RT with gentle rocking. Afterwards, planaria were washed three times in PBS for 1 minute each before incubating in Anti-Digoxigenin Peroxidase Conjugate (Millipore Sigma) overnight at 4 °C with gentle rocking.

Excess solution was removed and planaria were washed eight times for 30 minutes each with Blocking Solution at RT with gentle rocking. The planaria were incubated in Tyramide Working Solution for 10 minutes at RT with gentle rocking. Reaction Stop Reagent was applied, then the planaria were rinsed three times for 20 minutes each with PBS at RT with gentle rocking. Apoptosis immunolabeled planaria were stored at 4 °C in foil-wrapped 24-well plates.

Immunolabeling Imaging

Planaria were observed using a Leica WILD M2Z dissecting microscope and immunofluorescence was observed using NightSea Add-on Light and Filter Set with Royal Blue Light Head. Mitosis images were obtained using a phone camera attachment and a Leica inverted microscope with a CoolLED *p*E-100 excitation light source set at 100% intensity and 470 nm.

Chapter 4: Results

Statistical analysis was performed to determine unequal variances using SPSS statistical software with help from the Statistical Consulting and Research Center at St. Cloud State University. Analyses to determine statistically significant differences in the means between each metabolic inhibitor exposure group and the control groups and statistical analyses to determine differences between the average number of immunofluorescent cells present were performed using JMP statistical software with help from Dr. Richard Sundheim.

Metabolic Inhibitor Exposure Pilot Experiment

Planaria (both *D. dorotocephala* and *D. tigrina*) treated from 0-24 hpa with 10 µM HA, 15 µM DMF, or 20 µM 4-OI, survived and regrew eyespots. Planaria treated with 100 µM, 50 µM or 25 µM 6-AN from 0-24 hpa survived and regrew eyespots. *D. dorotocephala* treated with 2.5 µM LND from 0-24 hpa survived and regrew eyespots.

From the ranges of concentrations used, the lethal doses at which 50% of the planaria died (LD50) were determined for the metabolic inhibitors HA, DMF, 4-OI and LND. The concentration of the GAPDH inhibitors used in the exposure experiments (10 µM HA, 15 µM DMF, and 20 µM 4-OI) were calculated to be 48%, 40%, and 17.25% below LD50, respectively (see Fig. 1 in Appendix). The concentration of the hexokinase inhibitor used in the exposure experiment (2.5 µM LND) was calculated to be 41.3% below LD50. The LD50 value was not determined for 6-AN because planaria exposed to the highest concentration tested (100 µM 6-AN, 0.1% DMSO) did not die. Higher concentrations of 6-AN were not tested to prevent exposing planaria to concentrations of DMSO greater than 0.1% (see Table 3).

Metabolic Inhibitor Exposure Experiment

Data collected from the *D. dorotocephala* metabolic inhibitor exposures were analyzed with Dunnett's multiple comparison test at the level of significance $p = 0.05$ (see Table 1). The average number of days until eyespot formation for each exposure group are shown in Fig. 2 and Fig. 3 in the Appendix. The general trend suggests that the average number of days until eyespot formation increases with earlier exposure to the metabolic inhibitors HA, DMF, 4-OI, and LND, with no trend observed for exposure to 6-AN. For DMF exposures, statistically significant differences between the means of the exposure groups and the control group were found for exposures between 0-24 hpa and 24-48 hpa, with levels of significance $p < 0.0001$ for both. Statistically significant differences between the means of the 0-24 hpa exposure and the control groups for 4-OI occurred with the level of significance $p < 0.0001$. For LND exposures, statistically significant differences were found between the 0-24 hpa exposure and control groups, with levels of significance $p = 0.0131$. Surprisingly, no statistically significant differences were found between the exposure groups and the control groups when *D. dorotocephala* were exposed to either HA or 6-AN.

Table 1

D. dorotocephala *metabolic inhibitor exposure multiple comparison test.*

	p - Value		
	0-24 hpa	***24-48 hpa***	***48-72 hpa***
HA	0.4376	0.5960	0.9872
DMF	< 0.0001*	< 0.0001*	0.2225
4-OI	< 0.0001*	0.2079	0.5350
6-AN	0.9020	0.2969	1.0000
LND	0.0131*	0.1866	0.5109

Note. Means were compared to the control group using Dunnett's test at p = 0.05. Asterisks mark statistically significant differences in the mean in comparison to the control group. Heptelidic acid (HA), dimethyl fumarate (DMF), 4-octyl itaconate (4-OI), 6-aminonicotinamide (6-AN), lonidamine (LND).

Data collected from the *D. tigrina* metabolic inhibitor exposures were analyzed with Dunnett's multiple comparison test at p = 0.05 (see Table 2). The average number of days until eyespot formation for each exposure group are shown in Fig. 4 and Fig. 5 in the Appendix. The general trend suggests that the average number of days until eyespot formation increases with earlier exposure of the metabolic inhibitors HA, DMF, and 4-OI. Similar to the results from the *D. dorotocephala* exposure experiments, exposure to DMF during 0-24 hpa or 24-48 hpa resulted in statistically significant differences between the means of the exposed groups and the control group, with levels of significance p = 0.0005 for the 0-24 hpa exposure group and p = 0.0001 for the 24-48 hpa exposure group. For the 4-OI exposure groups, statistically significant differences were found with levels of significance p = 0.0002 and p = 0.0011 for the 0-24 hpa and 24-48 hpa exposure groups, respectively. Similar to the results obtained from the *D. dorotocephala* exposures, statistically significant differences were not found between the exposure groups and the control groups when *D. tigrina* were exposed to either HA or 6-AN.

Table 2

D. tigrina *metabolic inhibitor exposure multiple comparison test.*

	p - Value		
	0-24 hpa	***24-48 hpa***	***48-72 hpa***
HA	0.1822	0.7621	0.8947

DMF	0.0005*	0.0001*	0.2901
4-OI	0.0002*	0.0011*	0.5887
6-AN	1.0000	0.1137	0.6185

Note. Means were compared to the control group using Dunnett's test at p = 0.05. Asterisks mark statistically significant differences in the mean in comparison to the control group. Heptelidic acid (HA), dimethyl fumarate (DMF), 4-octyl itaconate (4-OI), 6-aminonicotinamide (6-AN).

Planaria (*D. dorotocephala*) were exposed to planaria water or to a DMSO solution (either 0.1%, 0.014%, 0.0083%, or 0.00707% DMSO in planaria water) and the average number of days until eyespot regrowth was compared using Dunnett's multiple comparison test at p = 0.05 (see Table 3). Planaria treated with 0.1% DMSO solution took significantly longer to regrow their eyespots in comparison to untreated planaria. Significant differences were not found between planaria treated with concentrations less than or equal to 0.014% DMSO and untreated planaria.

Table 3

D. dorotocephala *DMSO solutions vs. planaria water multiple comparison test.*

	p-Value
0.1% DMSO	0.0182*
0.014% DMSO	0.3055
0.0083% DMSO	0.8420
0.00707% DMSO	0.5463

Note. Means of the average number of days until eyespot regrowth during treatment with DMSO solutions were compared to the control group exposed to planaria water using

Dunnett's test at p = 0.05. Asterisks mark statistically significant differences in the mean in comparison to the control group.

Mitosis Immunolabeling

The average number of mitotic cells for each exposure group were compared between the other timeframes within each exposure group using Tukey-Kramer test at p = 0.05 (see Table 4). The only statistically significant differences observed were between the 0-24 hpa DMF exposure group compared to the 24-48 hpa DMF exposure group and 48-72 hpa DMF exposure group. Statistically significant differences were not observed between the exposure timeframes within each inhibitor group among the other metabolic inhibitors used.

Table 4

D. dorotocephala *all-pairs Tukey-Kramer analysis.*

	p - Value				
	HA	**DMF**	**4-OI**	**Control**	**Planaria Water**
0-24 hpa vs 24-48 hpa	0.1069	0.0050*	0.1906	0.4170	0.8379
0-24 hpa vs 48-72 hpa	0.1789	0.0038*	0.0828	0.0512	0.9002
24-48 hpa vs 72-48 hpa	0.9789	0.9871	0.8465	0.3475	0.9618

Note. Means of mitotic cell count (cells/area) were compared between all exposure time groups using Tukey-Kramer test at the level of significance p = 0.05. Asterisks mark statistically significant differences in the mean in comparison to groups exposed to the same inhibitor. The average number of mitotic cells in the 0-24 hpa DMF exposure group was found to be significantly different than the average number of mitotic cells in the 24-48 hpa and 48-

72 hpa DMF exposure groups. Heptelidic acid (HA), dimethyl fumarate (DMF), 4-octyl itaconate (4-OI), control (planaria water with 0.014% DMSO), planaria water (0.5 g/L Instant Ocean salts in dH_2O).

The average number of mitotic cells were compared to the control group (planaria water with 0.014% DMSO) using Dunnett's multiple comparison test at p = 0.05 (see Table 5). Statistically significant differences were not observed in average number of mitotic cells between the metabolic inhibitor treated planaria (exposed to either HA, DMF, or 4-OI) and untreated planaria (exposed to planaria water or planaria water with 0.014% DMSO) in any exposure time group.

Table 5

D. dorotocephala *mitotic activity multiple comparison test.*

	p - Value		
	0-24 hpa	***24-48 hpa***	***48-72 hpa***
HA	1.0000	0.9251	0.6683
DMF	0.9179	0.1592	0.9988
4-OI	0.8739	1.0000	0.8083
Planaria Water	0.6748	0.9091	0.7282

Note. Means of mitotic cell count (cells/area) were compared to the control (planaria water with 0.014% DMSO) using Dunnett's test at the level of significance p = 0.05. No statistically significant differences were found. Heptelidic acid (HA), dimethyl fumarate (DMF), 4-octyl itaconate (4-OI), planaria water (0.5 g/L Instant Ocean salts in dH_2O).

Apoptosis Immunolabeling

No clear labeling of apoptotic cells was observed.

Chapter 5: Discussion

Metabolic Inhibitor Exposure Experiment

Planaria eyespot formation time post-head amputation was compared to determine if statistically significant differences were observed between the average number of days until eyespot formation among metabolic inhibitor treated and untreated planaria. Treated exposure groups found to be statistically different from the control group suggest that the metabolic inhibitors used delay eyespot formation time.

In both *D. dorotocephala* and *D. tigrina*, statistically significant differences were not found in average number of days until eyespot formation when exposed to HA. This is unexpected, as previous literature has shown that HA decreases glycolysis activity for at least 25 minutes after exposure to the inhibitor (Osuma et al., 2017) and because the other GAPDH inhibitors tested (DMF and 4-OI) were shown to significantly delay eyespot formation during the 0-24 hpa exposure groups. The percentage below the lethal dose at which half of the planaria tested died when exposed to HA or DMF were relatively similar, 48% and 40% below LD50, respectively, suggesting that the differences in significant results observed between exposure to HA or DMF are not due to differences in the concentrations of HA and DMF used. However, comparing non-lethal concentrations of HA, DMF, and 4-OI with the same percentage below LD50 would reduce confounding variables from influencing the results of the study.

For *D. dorotocephala* and *D. tigrina* exposed to DMF, statistically significant differences between the average number of days needed for eyespot formation between the 0-24 hpa and 24-48 hpa exposure groups and the control group were found, suggesting that exposure to DMF delays eyespot formation time. Metabolic inhibitor exposure during 0-24 hpa overlaps with the generic response, while exposure during 24-48 hpa covers most of the regeneration response

outlined by Wurtzel et al. (2015). Because exposure to DMF during the generic response and most of the regeneration response resulted in significant delays in eyespot regeneration time, this may suggest a connection between glycolytic activity and the onset of gene expression in response to injury in planaria. Further studies should be conducted to test how DMF affects glycolytic activity, such as by measuring ECAR to provide evidence that glycolytic activity does decrease in planaria exposed to DMF. As an inexpensive GAPDH inhibitor, DMF is an accessible option for labs studying metabolism in regenerating planaria.

Statistically significant differences in the average number of days needed until eyespot formation when *D. dorotocephala* was exposed to 4-OI were found to occur between the 0-24 hpa exposure group and the control group. For *D. tigrina* exposed to 4-OI, statistically significant differences were found to occur in the 0-24 hpa and 24-48 hpa exposure groups in comparison to the control group. These results suggest that potential GAPDH inhibition by 4-OI during the first 0-24 hpa in planaria produces delays in eyespot formation time. However, there appear to be interspecies differences in timings of exposure to 4-OI during 24-48 hpa, since significant delays in eyespot formation occurred during 4-OI exposure 0-24 hpa in both *D. dorotocephala* and *D. tigrina*, but only *D. tigrina* had significant delays in eyespot formation during 4-OI exposure 24-48 hpa. On average, untreated *D. dorotocephala* regrew eyespots in 3.13 days, while *D. tigrina* regrew eyespots in 4 days. This suggests that perhaps the timing of gene expression and the pace of regeneration outlined in the three stages of planarian regeneration (Wurtzel et al., 2015) may be different in different species of planaria.

Both *D. dorotocephala* and *D. tigrina* showed no significant differences in average number of days until eyespot formation when exposed to 6-AN. This suggests that inhibition of the pentose phosphate pathway does not result in a delay in eyespot formation time and,

furthermore, that pentose phosphate pathway activity is not essential for planarian regeneration. This is unexpected, since inhibition of the pentose phosphate pathway has been shown to delay tail regeneration in other regenerative animals (Patel et al., 2022) and because many building blocks necessary for cell growth and division, an essential process to regenerate missing tissue, such as fatty acids, nucleotides, and aromatic amino acids, are produced in this pathway. However, production of fructose-6-phosphate and glyceraldehyde-3-phosphate during glycolysis can be converted in the pentose phosphate pathway to the precursors of the building blocks necessary for cell growth and division. It is possible that potential early inhibition of the pentose phosphate pathway by 6-AN did not result in a delay in eyespot formation in planaria because products from glycolysis were converted to the precursors of the building blocks necessary for cell growth and division. However, a toxic dose of 6-AN was not achieved in the exposure pilot experiments, suggesting that 6-AN was not taken up by the planarian cells. This may explain why no significant delays in eyespot regeneration were observed. Further studies should be conducted testing whether higher concentrations of 6-AN affect eyespot formation time in planaria.

The hexokinase inhibitor LND was shown to significantly delay eyespot formation during exposure 0-24 hpa in *D. dorotocephala*, suggesting that, along with significant delays in eyespot regrowth time when exposed to DMF or 4-OI, potential inhibition of glycolysis does impact regeneration in planaria and that inhibition of other enzymes in glycolysis may also delay eyespot formation time in regenerating planaria. Testing the effects of inhibitors of other enzymes in glycolysis on eyespot formation times in planaria should be studied to further confirm that potential inhibition of glycolysis does impact regeneration in planaria.

The rate at which the GAPDH inhibitors decrease enzyme activity differs: HA decreases enzyme activity by half in less than a minute (Sakai et al., 1988), DMF decreases enzyme activity by half in a little under an hour (Kornberg et al., 2018), and 4-OI decreases enzyme activity by half after about 3.5 hours of exposure (Liao et al., 2019). Perhaps delays in eyespot regeneration were not observed in the planaria exposed to HA because GAPDH activity decreases significantly within a minute after exposure to HA, but delays in eyespot regeneration do occur in planaria exposed to DMF or 4-OI because GAPDH activity decreases slower when exposed to these two inhibitors. It is possible that the relatively fast, and nearly complete, inhibition of GAPDH by HA triggers GAPDH synthesis to replace any GAPDH that was irreversibly inhibited by HA and prevent a long-term decrease in glycolytic activity. Further studies should be conducted testing the effects on enzyme activity of planarian GAPDH when exposed to HA, DMF, or 4-OI to determine differences between the reaction rates of these metabolic inhibitors.

HA, DMF, and 4-OI are covalent inhibitors of GAPDH. However, there are differences in the mechanisms of inhibition for each inhibitor. DMF and HA would be expected to produce similar exposure results, since the mechanism of inhibition is similar, as both bind to a cysteine residue at the active site of GAPDH, yet HA exposure did not result in significant delays in planarian eyespot regeneration while DMF exposure did. The discrepancies between the results of HA and the other two inhibitors may also suggest off-target effects of the inhibitors. Unlike HA, both DMF and 4-OI are not specific inhibitors of GAPDH and affect cysteine residues on kelch like ECH associated protein 1 (KEAP1), a sensor of oxidative and electrophilic stress, resulting in the release of nuclear factor – erythroid factor 2 – related factor 2 (Nrf2), a transcription factor that regulates the antioxidant response element, an enhancer associated with

the activation of genes in cells exposed to oxidative stress (Kornberg et al., 2018; Liao et al., 2019; Pålsson-McDermott & O'Neill, 2020). Downstream effects of Nrf2 release from KEAP1 include promoting expression of NAD(P)H quinone dehydrogenase 1, which reduces and detoxifies reactive quinones and protects cellular membranes, and heme-oxygenase 1, which is an antioxidant and reduces levels of IL-12, IL-16, p40, and TNFα (Pålsson-McDermott & O'Neill, 2020). IL-12 and IL-16 are interleukins, which are cytokines expressed by white blood cells, and p40 is a protein subunit on IL-16 and may be important in CD4+ T cell responses (Pålsson-McDermott & O'Neill, 2020). TNFα is also a cytokine that is involved with regulating inflammatory responses (Pålsson-McDermott & O'Neill, 2020). These off-target effects may have important implications in the wound and regeneration responses in planaria because there was a significant delay in eyespot formation in planaria exposed to DMF or 4-OI. However, many of these off-target effects are associated with organisms containing conventional respiratory systems with red and white blood cells. Planaria do not have a conventional respiratory system and may not have the same response associated with Nrf2 release from KEAP1 when exposed to either DMF or 4-OI. Further studies should be conducted to determine what the downstream effects of DMF or 4-OI exposure in planaria are.

Mitosis Immunolabeling

A significant difference in average number of mitotic cells near the amputation site was found in the 0-24 hpa DMF exposure group in comparison to DMF exposure between the 24-48 hpa and 48-72 hpa groups (see Table 3). This suggests that the average number of mitotic cells near the amputation site was significantly higher during the first 0-24 hpa of exposure to DMF in comparison to exposure of DMF during either 24-48 hpa or 48-72 hpa. Previous literature suggests that stem cell proliferation occurs in two bursts in regenerating planaria: between 4 to

12 hours post amputation and between 2 to 4 days post amputation (Wenemoser & Reddien, 2010). The increase in mitotic cell activity between 4 to 12 hours noted by Wenemoser and Reddien (2010) may have been captured by the significant increase in average number of mitotic cells in the DMF exposure 0-24 hpa. However, the within-group mitosis comparison results observed in the DMF group are not replicable to other groups where similar results would be expected, such as for 4-OI exposure, which also had significant effects on delaying eyespot formation. If the within-group DMF mitosis comparison results were associated with delays in eyespot formation, then similar within-group mitosis comparison results should be observed with the 4-OI treatments but were not. This suggests that the within-group mitosis differences are not associated with the results of the metabolic inhibitor exposure experiments. Furthermore, the between-group mitosis results comparing each of the metabolic inhibitor exposures to the control group did not find any significant differences, suggesting that delays in eyespot formation are not associated with the number of mitotic cells when exposed to metabolic inhibitors.

Apoptosis Immunolabeling

No conclusions can be determined due to lack of results gathered from testing apoptosis in metabolically inhibited planaria. Further studies should be conducted to measure apoptosis in metabolically inhibited planaria using other methods of apoptosis labelling.

Summary

Results from the exposure experiments suggest that the earlier planaria are exposed to GAPDH inhibitors after amputation, the longer it takes to regrow eyespots. By comparing the average number of mitotic cells in inhibitor treated planaria to untreated planaria, it has been found that delays in eyespot formation are not associated with changes in the number of mitotic cells when exposed to metabolic inhibitors. Further studies should be conducted to examine the

effects of metabolic inhibitors on enzyme activity of GAPDH purified from planaria and to measure apoptosis in planaria exposed to metabolic inhibitors.

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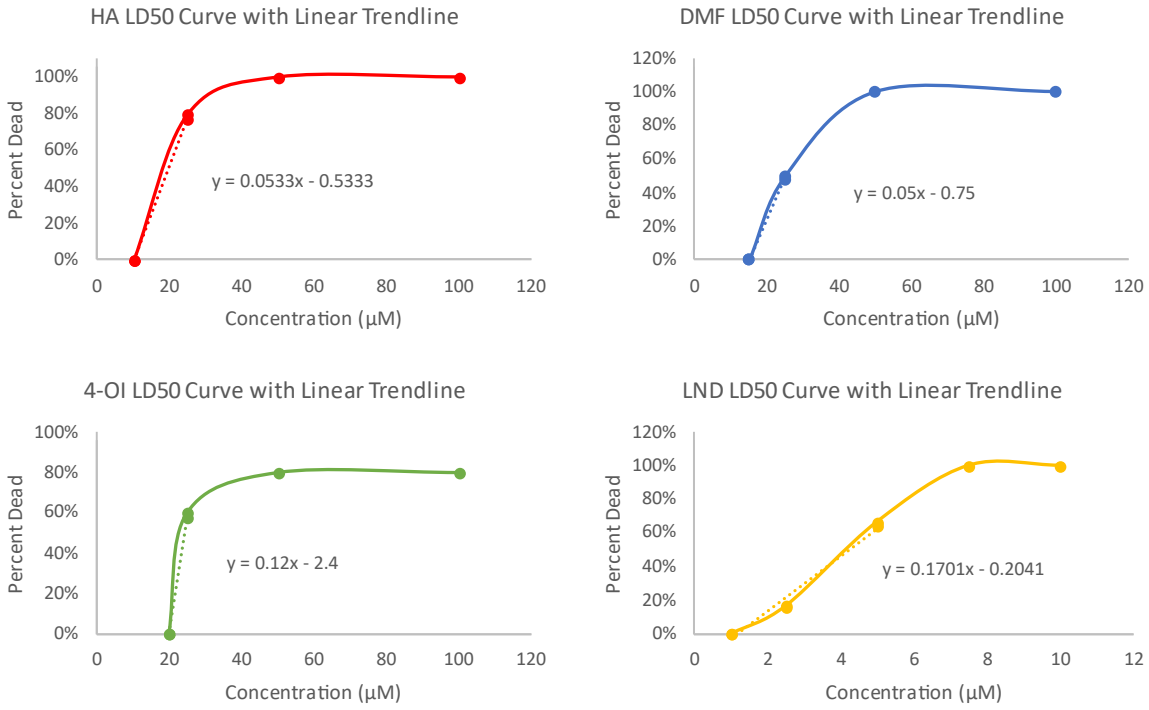
Appendix A: Figures

Figure 1.

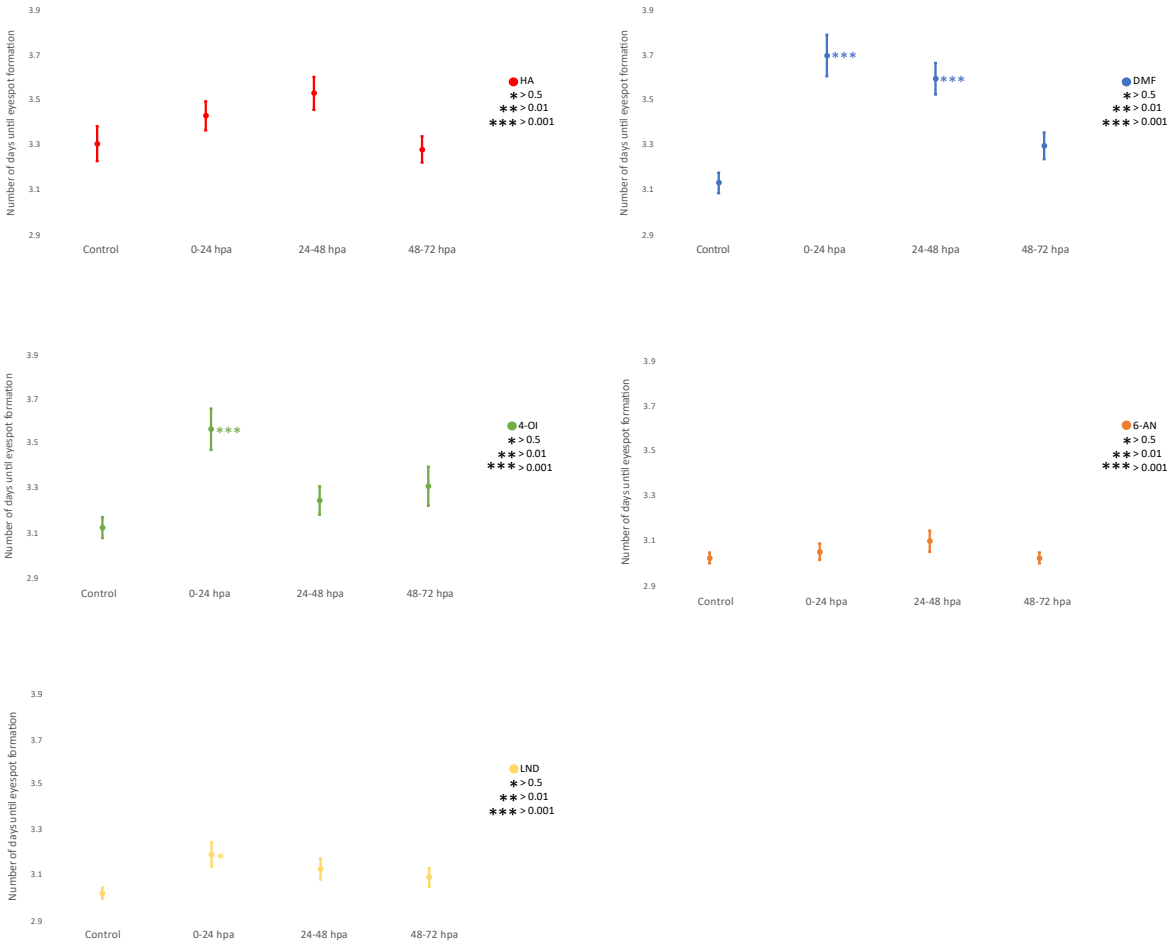
LD50 curves and trendlines from pilot experiment exposures of metabolic inhibitors in D. dorotocephala.



Note. Planaria were exposed to metabolic inhibitors for 0-24 hpa at varying concentrations. Linear trendlines were used to calculate the percentage below LD50 for each metabolic inhibitor concentration used. Heptelidic acid (HA), dimethyl fumarate (DMF), 4-octyl itaconate (4-OI), lonidamine (LND).

Figure 2.

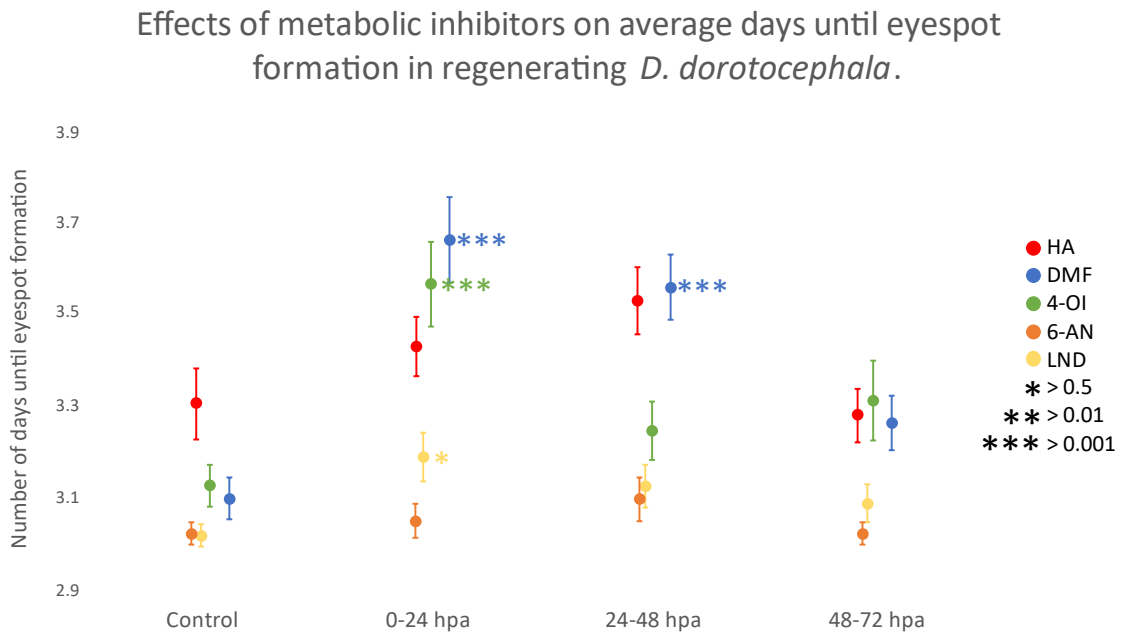
Effects of metabolic inhibitors on average days until eyespot formation in regenerating D. dorotocephala.



Note. Planaria were exposed to metabolic inhibitors for 0-24 hpa, 24-48 hpa, and 48-72 hpa. Means were compared to the control group using Dunnett's test at $p = 0.05$. Average number of days until eyespot formation are represented for each exposure group as a circle and error bars were calculated using standard error of the mean ($n = 60$). Heptelidic acid (HA), dimethyl fumarate (DMF), 4-octyl itaconate (4-OI), 6-aminonicotinamide (6-AN), lonidamine (LND).

Figure 3.

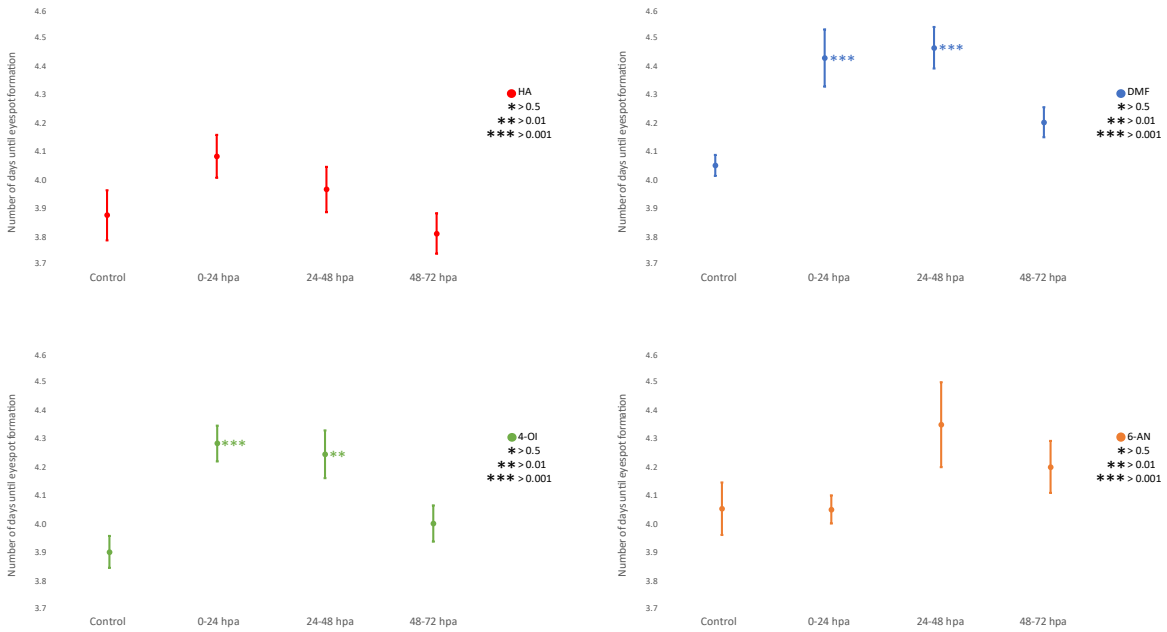
Combined image of the effects of metabolic inhibitors on eyespot formation in D. dorotocephala.



Note. Planaria were exposed to metabolic inhibitors for 0-24 hpa, 24-48 hpa, and 48-72 hpa. Significant delays in eyespot formation occurred in planaria exposed to DMF, 4-OI, or LND from 0-24 hpa and in planaria exposed to DMF from 24-48 hpa. Average number of days until eyespot formation are represented for each exposure group as a circle and error bars were calculated using standard error of the mean (n = 60). Heptelidic acid (HA), dimethyl fumarate (DMF), 4-octyl itaconate (4-OI), 6-aminonicotinamide (6-AN), lonidamine (LND).

Figure 4.

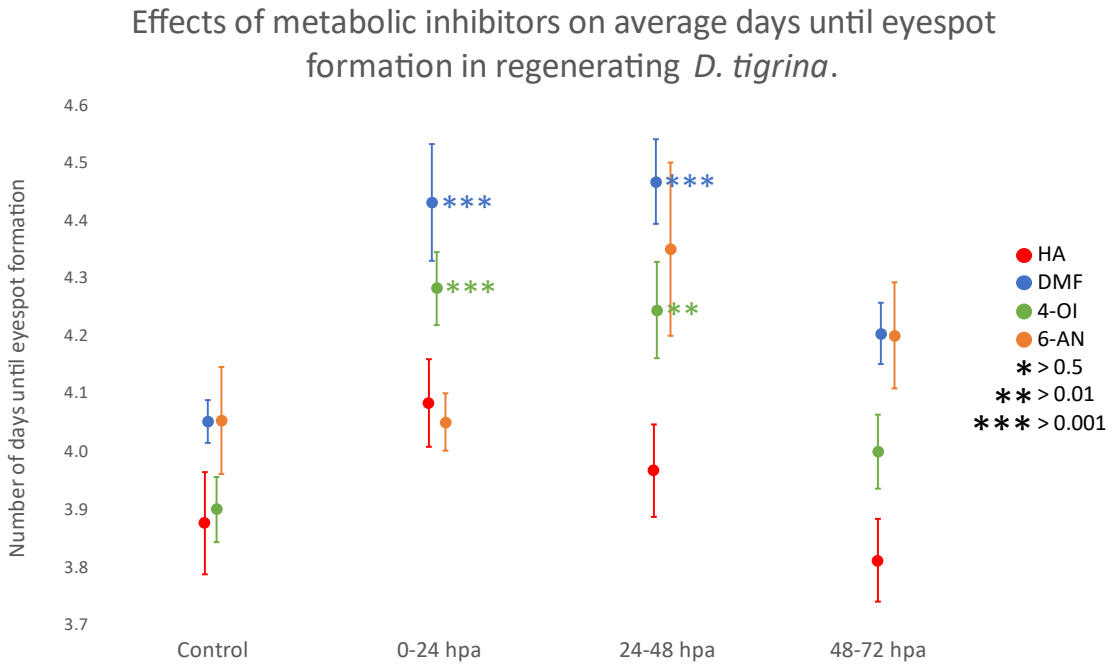
Effects of metabolic inhibitors on average days until eyespot formation in regenerating D. tigrina.



Note. Planaria were exposed to metabolic inhibitors for 0-24 hpa, 24-48 hpa, and 48-72 hpa. Means were compared to the control group using Dunnett's test at $p = 0.05$. Average number of days until eyespot formation are represented for each exposure group as a circle and error bars were calculated using standard error of the mean ($n = 60$). Heptelidic acid (HA), dimethyl fumarate (DMF), 4-octyl itaconate (4-OI), 6-aminonicotinamide (6-AN).

Figure 5.

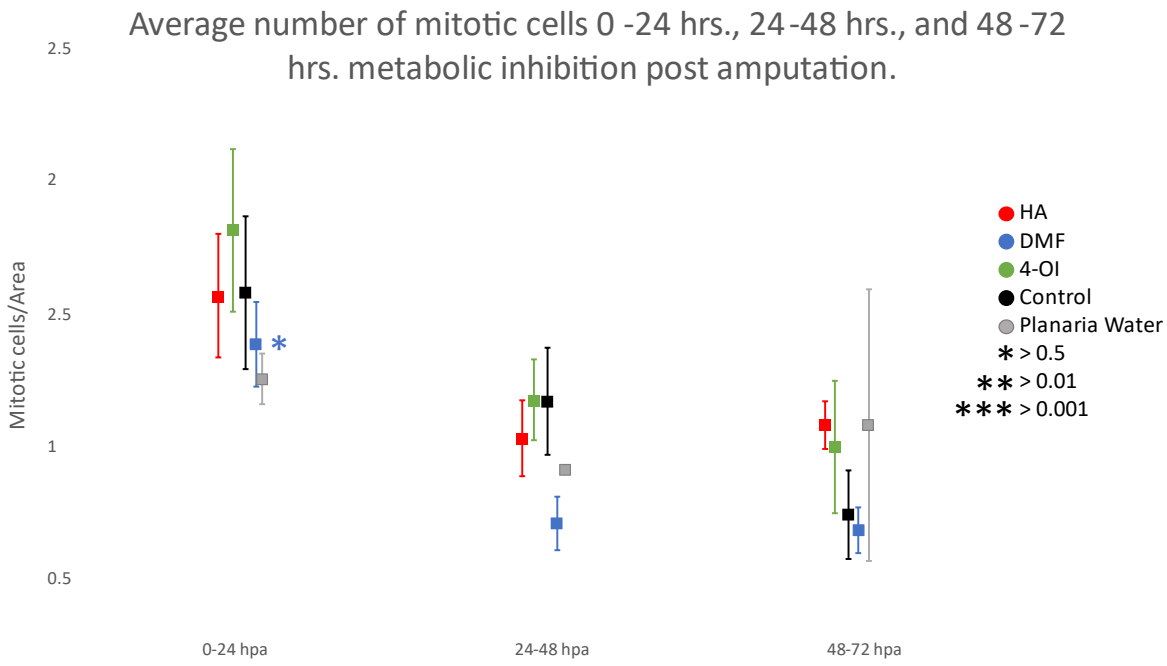
Combined image of the effects of metabolic inhibitors on eyespot formation in D. tigrina.



Note. Planaria were exposed to metabolic inhibitors for 0-24 hpa, 24-48 hpa, and 48-72 hpa. Significant delays in eyespot formation occurred in planaria exposed to DMF or 4-OI from 0-24 hpa and from 24-48 hpa. Means were compared to the control group using Dunnett's test at p = 0.05. Average number of days until eyespot formation are represented for each exposure group as a circle and error bars were calculated using standard error of the mean (n = 60). Heptelidic acid (HA), dimethyl fumarate (DMF), 4-octyl itaconate (4-OI), 6-aminonicotinamide (6-AN).

Figure 6.

Combined image of the effects of metabolic inhibitors on mitotic cells in D. dorotocephala.

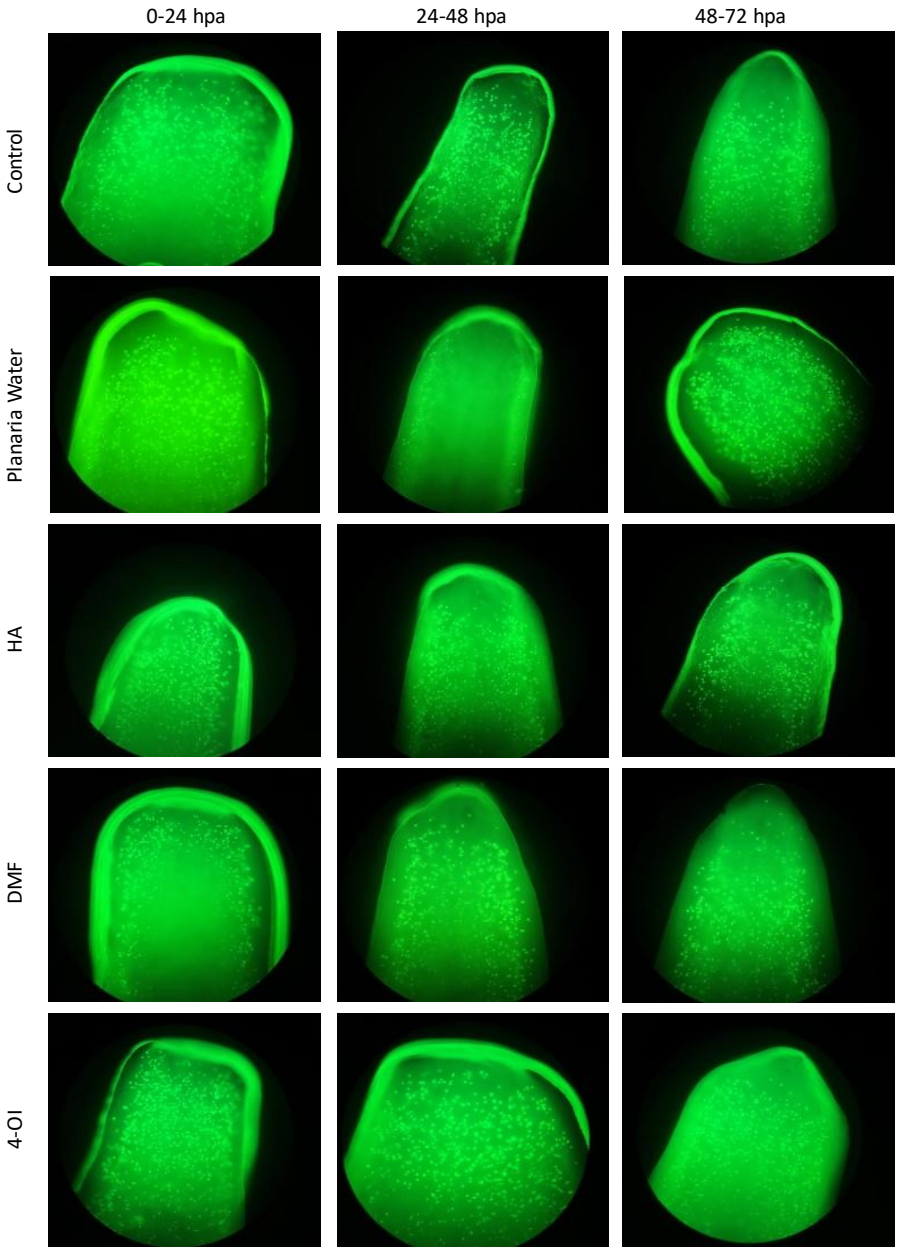


Note. Planaria were exposed to metabolic inhibitors for 0-24 hpa, 24-48 hpa, and 48-72 hpa. before immunolabeling for mitotic cells. Planaria exposed to DMF from 0-24 hpa had significantly more cells, as indicated by the blue asterisk, in comparison to planaria exposed to DMF from 24-48 hpa and 48-72 hpa. Statistically significant differences were not found between planaria exposed to metabolic inhibitors and the control group. Means of the exposure groups were compared to the control group using Dunnett's test at $p = 0.05$. Means were compared within each exposure group using Tukey-Kramer's test at $p = 0.05$. The average number of mitotic cells are represented for each exposure group as a square and error bars were calculated using standard error of the mean ($n = 5$). Heptelidic acid (HA), dimethyl fumarate (DMF), 4-

octyl itaconate (4-OI), control (planaria water with 0.014% DMSO), planaria water (0.5 g/L Instant Ocean salts in dH_2O).

Figure 7.

Sample images of immunolabeled mitotic cells in D. dorotocephala.



Note. Planaria were exposed to metabolic inhibitors for 0-24 hpa, 24-48 hpa, and 48-72 hpa. before immunolabeling for mitotic cells. The number of mitotic cells per area were counted 7 mm from the amputation site and were standardized per area as measured in pixels using ImageJ

software. Heptelidic acid (HA), dimethyl fumarate (DMF), 4-octyl itaconate (4-OI), control (planaria water with 0.014% DMSO), planaria water (0.5 g/L Instant Ocean salts in dH_2O).