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Butafenacil: A positive control for identifying anemia- and variegate porphyria-inducing chemicals

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ABSTRACT

Butafenacil is an herbicide that inhibits protoporphyrinogen oxidase (PPOX), an enzyme that catalyzes oxidation of protoporphyrinogen IX to protoporphyrin IX during chlorophyll and heme biosynthesis. Based on a high-content screen, we previously identified butafenacil as a potent inducer of anemia in zebrafish embryos. Therefore, the objective of this study was to begin investigating the utility of butafenacil as a positive control for identifying anemia- and variegate porphyria-inducing chemicals. Static exposure to butafenacil from 5 to 72 h post-fertilization (hpf) in glass beakers resulted in a concentration-dependent decrease in arterial circulation at low micromolar concentrations. At 72 hpf, the magnitude of butafenacil-induced anemia was similar when embryos were exposed in the presence or absence of light, whereas protoporphyrin accumulation and acute toxicity were significantly lower or absent when embryos were exposed under dark conditions. To identify sensitive developmental windows, we treated embryos to butafenacil from 5, 10, 24, or 48 hpf to 72 hpf in the presence of light, and found that anemia and protoporphyrin accumulation were present at 72 hpf following initiation of exposure at 5 and 10 hpf. On the contrary, protoporphyrin accumulation – but not anemia – was present following initiation of exposure at 24 hpf. Lastly, protoporphyrin accumulation at 72 hpf after exposure from 24 to 48 hpf suggests that protoporphyrin was not eliminated over a 24-h recovery period. Collectively, our data suggests that butafenacil may be a reliable positive control for identifying anemia- and variegate porphyria-inducing chemicals.

1. Introduction

Butafenacil is a broad-spectrum imide herbicide that inhibits protoporphyrinogen oxidase (PPOX), an enzyme that catalyzes oxidation of protoporphyrinogen IX to protoporphyrin IX – the last common step of chlorophyll and heme biosynthesis in plants and animals, respectively. Similar to other post-emergent herbicides, butafenacil is phytotoxic on contact and provides rapid knockdown of broadleaf and grass weeds [14]. Butafenacil has been registered for agricultural use within Australia, Argentina, Brazil, Japan, Switzerland, and Thailand since the early 2000s, and, within these countries, is sold as a pre-mixed formulation containing other post-emergent herbicides such as triasulfuron and glyphosate [14].

As butafenacil targets PPOX in both plants and animals, exposure of rodents (rats and mice) and aquatic plants (algae, diatoms, and duckweed) to butafenacil results in hematologic abnormalities and inhibition of growth, respectively, at relatively low concentrations [14]. However, due to low application rates (one application of <50 g ai/ha per year), rapid environmental degradation ($t_{1/2}$ < 5 days), and little to no potential for bioaccumulation ($t_{1/2}$ < 3 days), butafenacil is not expected to pose a significant human health nor ecological risk following agricultural use according to the label [14]. Based on a high-content screen using zebrafish embryos, we previously revealed that exposure to butafenacil from 5 to 72 h post-fertilization (hpf) abolished blood cell and hemoglobin production in the absence of effects on survival, body length, cardiac function, and blood vessel development [13]. Therefore, based on this study, we identified butafenacil as a potential positive control for identifying chemicals that induce blood disorders such as anemia and variegate porphyria.

Anemia is an inherited or acquired disorder that is characterized by (1) abnormally low levels of circulating red blood cells due to decreased red blood cell production, blood loss, or hemolysis or (2) normal levels of circulating red blood cells that are micro-

Abbreviations: ai, active ingredient; DMSO, dimethyl sulfoxide; EM, embryo media; hpf, hours post-fertilization; MS-222, tricaine methanesulfonate; PPOX, protoporphyrinogen oxidase; RO, reverse osmosis.

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cytic and/or hypochromic. As a result of decreased oxygen delivery throughout the body, patients with anemia experience a variety of symptoms (the most common being fatigue) and, in severe and chronic cases, irreversible damage to vital organs [7]. Within the United States, anemia is the most prevalent blood disorder across all age, racial, and ethnic groups. Iron-deficiency anemia is the most common form of anemia within American children, where prevalence is as high as 14% within children between 1 and 2 years of age (http://www.cdc.gov/nchs/fastats/anemia.htm). Anemia can also be caused by chronic exposure to certain drugs used to treat cancer and rheumatoid arthritis [11].

Porphyrias are a rare class of disorders caused by abnormal heme production, leading to accumulation of porphyrins or porphyrin precursors within the liver and other organs [17]. Within the United States, less than 200,000 people are diagnosed with acute or cutaneous porphyria, with the most prevalent form (porphyria cutanea tarda) resulting from chemically-induced deficiencies in uroporphyrinogen decarboxylase [1]. Variegate porphyria is a hepatic porphyria caused by a human PPOX mutation that decreases oxidation and conversion of protoporphyrinogen IX to protoporphyrin IX [2]. During acute attacks, patients with variegate porphyria can experience abnormal skin reactions to sunlight (or photodermatitis) due to reactive singlet oxygen formation and oxidative stress following photooxidation of protoporphyrinogen to protoporphyrin [2]. Moreover, certain drugs (e.g., barbiturates) and antibiotics (e.g., sulfonamides) can trigger acute symptoms of porhyrias such as photodermatitis, underscoring the importance of screening for potential effects on heme production during drug development.

As the identification of potential adverse hematologic effects is an important consideration during drug development, the objective of this study was to begin investigating the utility of butafenacil as a positive control for identifying anemia- and variegate porphyria-inducing chemicals. Our working hypothesis was that, similar to variegate porphyria, butafenacil-mediated inhibition of PPOX in transparent zebrafish embryos results in accumulation of protoporphyrin and increased acute toxicity when reared under light conditions. To accomplish the overall objective of this study, we conducted experiments to [1] determine whether butafenacil-induced anemia and variegate porphyria-like conditions occurs in the presence or absence of light; [2] identify developmental stages susceptible to butafenacil exposure within the first 72 h of embryogenesis; and [3] determine whether butafenacil-induced anemia and variegate porphyria-like conditions are reversible following recovery within clean water until 72 hpf. In addition to quantifying potential effects on survival, body area, arterial circulation, and pericardial area, for all three experiments we quantified the magnitude of protoporphyrin accumulation using fluorescence microscopy, as protoporphyrin autofluoresces between 600 and 660 nm [1,15] and can be detected in situ within zebrafish PPOX mutants [5].

2. Materials and methods

2.1. Animals

For all assays described below, we relied on a robust line of transgenic zebrafish (*fli1:egfp*) that stably express enhanced green fluorescent protein within vascular endothelial cells [12]. Although we did not assess the potential impacts of butafenacil exposure on angiogenesis within this study, we relied on *fli1:egfp* zebrafish to analyze body length, pericardial area, and arterial circulation using previously optimized protocols [18], as well as protoporphyrin accumulation using procedures described below. Adult *fli1:egfp* zebrafish were maintained on a 14-h:10-h light:dark cycle within a five-shelf stand-alone system (Aquatic Habitats, Inc., Apopka, FL, USA) containing photoperiod light-cycle enclosures and recirculating conditioned reverse osmosis (RO) water. Dissolved oxygen, pH, conductivity, salinity, alkalinity, and temperature within recirculating water were maintained at 4-6 mg/L, 6.5–7.5, 425–475 μS, <1 ppt, 50–100 mg/L, and 27–28 °C, respectively; in addition, levels of ammonia, nitrite, and nitrate within recirculating water were consistently below 0.1 mg/L, 0.05 mg/L, and 2 mg/L, respectively. Adult females and males were bred directly on-system using in-tank breeding traps suspended within 3-L tanks. For all experiments described below, newly fertilized eggs were staged according to previously described methods [10]. All fish were handled and treated in accordance with approved Institutional Animal Care and Use Committee protocols at the University of South Carolina – Columbia.

2.2. Chemicals

Butafenacil (99.3% purity) was purchased from Sigma–Aldrich (St. Louis, MO). Stock solutions of each chemical were prepared by dissolving chemicals in high performance liquid chromatography-grade dimethyl sulfoxide (DMSO) (50 mM), and then performing two-fold serial dilutions into DMSO to create stock solutions for each working solution. All stock solutions were stored at room temperature within 2-mL amber glass vials containing polytetrafluoroethylene-lined caps. For each exposure, working solutions of all treatments were freshly prepared by spiking stock solutions into embryo media (EM) (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$), resulting in 0.1% DMSO within all vehicle control and treatment groups.

2.3. Embryonic exposures

Newly fertilized eggs were collected immediately after spawning and placed in groups of approximately 100 per petri dish within a light- and temperature-controlled incubator until 5 hpf. Prior to each experiment, 50-mL glass beakers were thoroughly rinsed with DMSO and RO water. For exposures in the presence of light, viable *fli1:egfp* embryos were exposed to vehicle (0.1% DMSO) or butafenacil (0.025–0.78 μM) in triplicate glass beakers (20 per replicate beaker) under a 14-h:10-h light:dark cycle and static conditions at 28 °C from 5 to 72 hpf. For exposures in the absence of light, embryos were exposed using identical treatment groups and experimental conditions as above, except embryos were reared in the dark from 5 to 72 hpf.

To identify developmental windows susceptible to butafenacil exposure within the first 72 h of zebrafish embryogenesis, *fli1:egfp* embryos were exposed to vehicle (0.1% DMSO) or 0.09 μM butafenacil in triplicate glass beakers (20 per replicate beaker) using the following static exposure scenarios: [1] 5–72 hpf; [2] 10–72 hpf; [3] 24–72 hpf; and [4] 48–72 hpf. All exposures were conducted under a 14-h:10-h light:dark cycle and static conditions at 28 °C.

To determine whether butafenacil-induced effects were reversible, *fli1:egfp* embryos were first placed in triplicate glass beakers (20 per replicate beaker) and exposed to vehicle (0.1% DMSO) or 0.09 μM butafenacil from [1] 5–72 hpf, [2] 24–48 hpf, or [3] 48–72 hpf. For the 24–48-hpf exposure scenario, embryos were rinsed three times with clean EM, and maintained in clean glass beakers containing fresh EM until 72 hpf. All exposures were conducted under a 14-h:10-h light:dark cycle and static conditions at 28 °C.

2.4. Imaging procedures

At 72 hpf, hatched embryos were anesthetized with 100 mg/L MS-222 and up to 16 surviving embryos were individually loaded

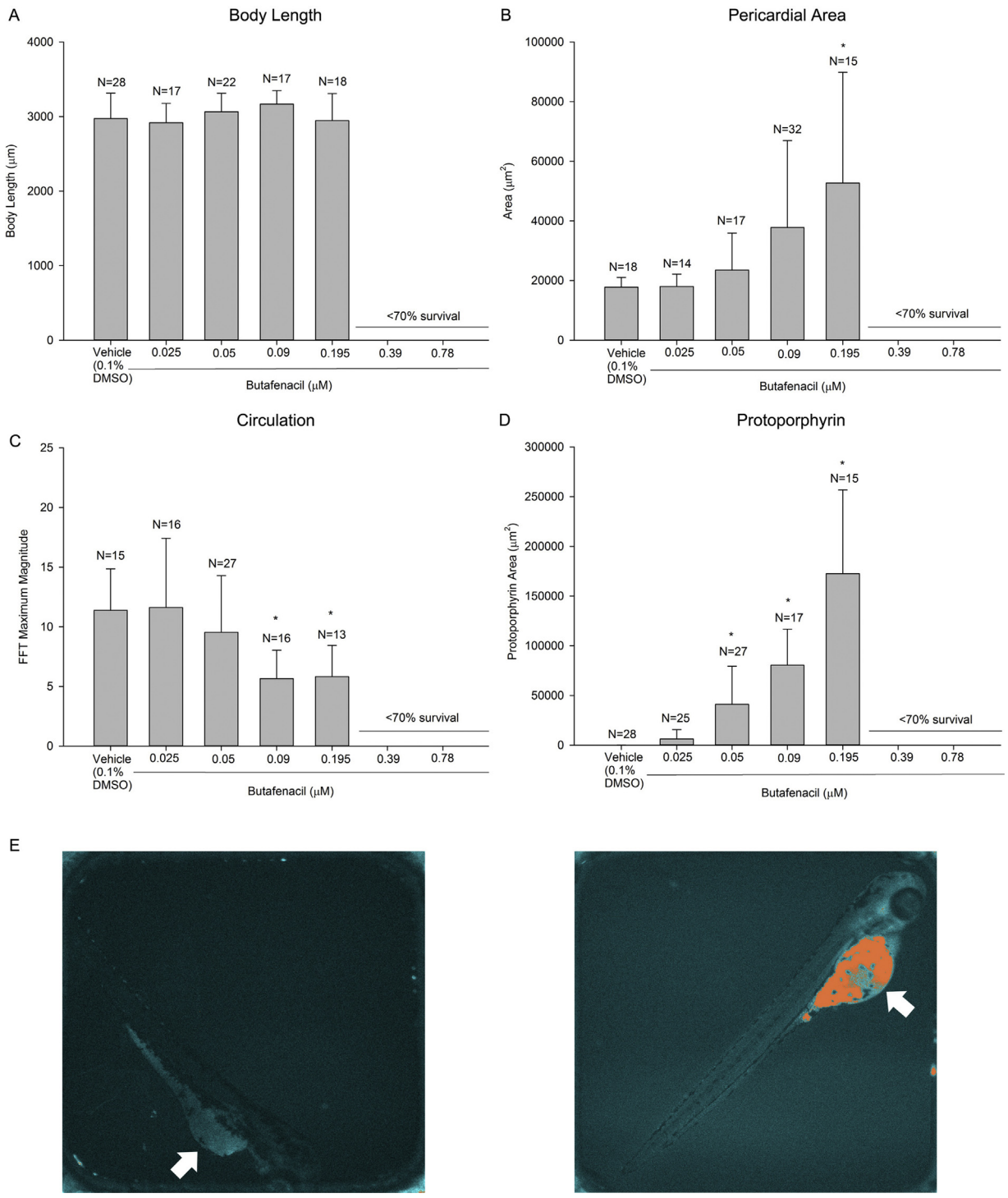

Fig. 1. Butafenacil exposure from 5 to 72 hpf under normal light conditions results in decreased circulation, increased pericardial area, and increased protoporphyrin accumulation. (A) Mean body length (±standard deviation), (B) mean pericardial area (±standard deviation), (C) mean circulation (±standard deviation), and (D) mean protoporphyrin accumulation (±standard deviation) following exposure to vehicle (0.1% DMSO) or 0.025–0.78 μM butafenacil. Numbers above each bar denote the number of embryos analyzed across three replicate beakers per treatment and 20 initial embryos per replicate beaker. Asterisk (*) denotes significant difference from vehicle controls ($p < 0.05$). (E) Representative images of protoporphyrin accumulation within a 72-hpf embryo exposed to vehicle (left) or 0.09 μM butafenacil (right) under light conditions; orange color represents autofluorescence above a threshold of 115 relative fluorescent units within the yolk sac (white arrow). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

into wells of a black 384-well glass-bottom microplate (Matrical Bioscience, Spokane, WA). The plate was then centrifuged at 300 rpm for 8 min to help orient hatched embryos into right or left lateral recumbency on the bottom of the well. Using automated image acquisition procedures within our ImageXpress Micro Widefield High-Content Screening System (Molecular Devices, Sunnyvale, CA), each embryo was imaged to analyze the following endpoints: body length, arterial circulation, pericardial area, and protoporphyrin accumulation. Previously optimized protocols and parameters were used to quantify body length, arterial

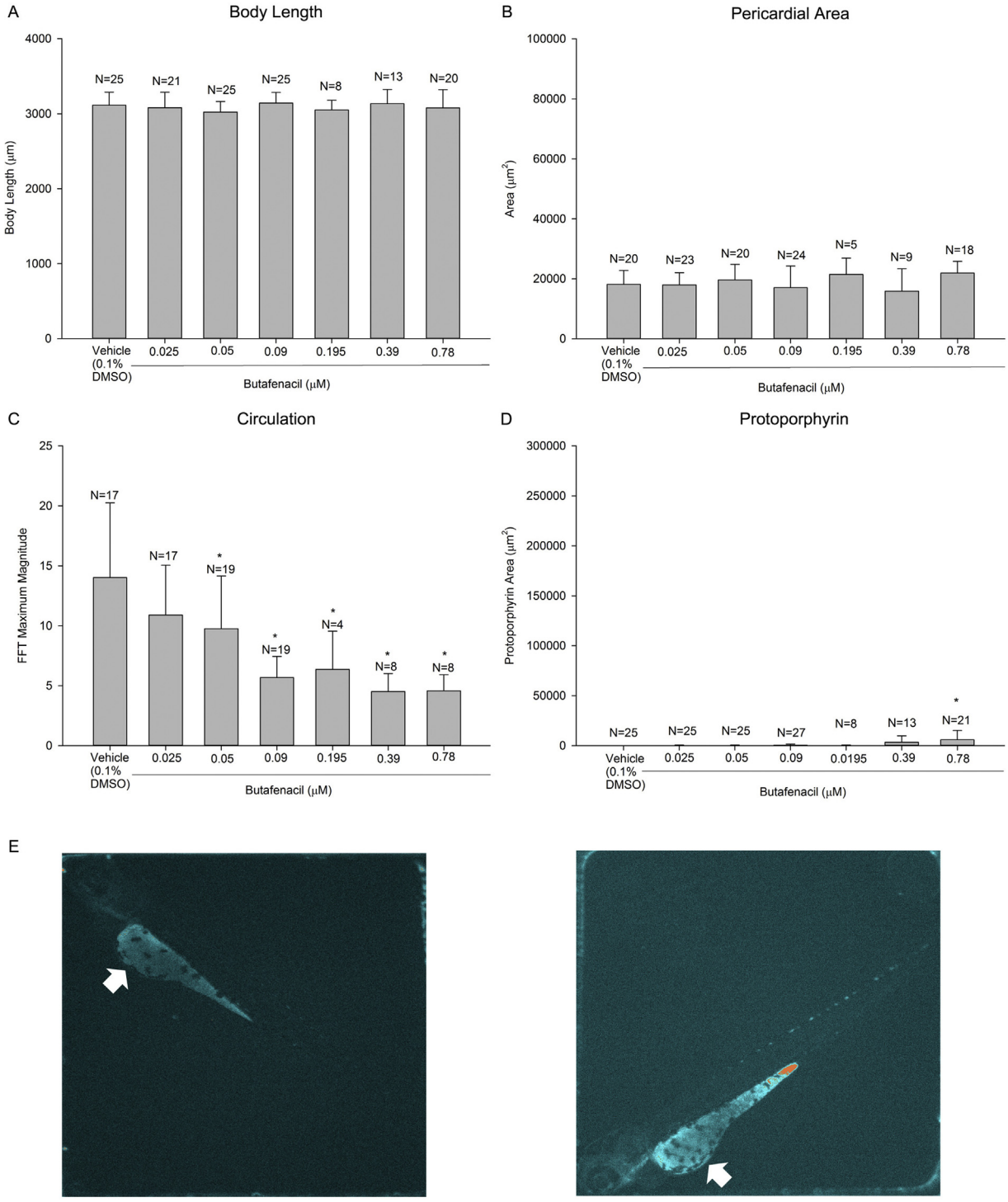

Fig. 2. Butafenacil exposure from 5 to 72 hpf in the dark resulted in decreased circulation in the absence of effects on survival, pericardial area, and protoporphyrin accumulation. (A) Mean body length (±standard deviation), (B) mean pericardial area (±standard deviation), (C) mean circulation (±standard deviation), and (D) mean protoporphyrin accumulation (±standard deviation) following exposure to vehicle (0.1% DMSO) or 0.025–0.78 μM butafenacil. Numbers above each bar denote the number of embryos analyzed across three replicate beakers per treatment and 20 initial embryos per replicate beaker. Asterisk (*) denotes significant difference from vehicle controls ($p < 0.05$). (E) Representative images of protoporphyrin accumulation within a 72-hpf embryo exposed to vehicle (left) or 0.09 μM butafenacil (right) under dark conditions; orange color represents autofluorescence above a threshold of 115 relative fluorescent units within the yolk sac (white arrow). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

circulation, and pericardial area [18]. Using a tetramethylrhodamine (TRITC) filter and 2X objective, one image per well was acquired to detect autofluorescence of accumulated protoporphyrin; these data were then analyzed by quantifying the threshold area within a range of 115–4000 relative fluorescent units.

2.5. Statistical analyses

All statistical procedures were performed using SPSS Statistics 22.0 (Chicago, IL). A general linear model (GLM) analysis of variance (ANOVA) ($\alpha = 0.05$) was used for all data, as these data did not meet the equal variance assumption for non-GLM ANOVAs.

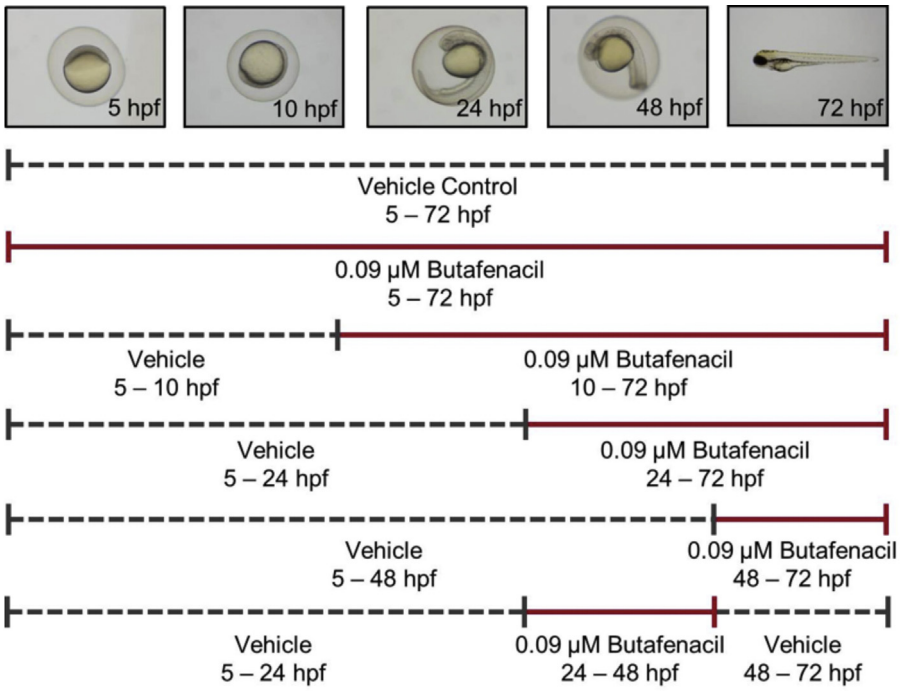

Fig. 3. Experimental design for identifying sensitive developmental windows of butafenacil exposure as well as the potential for recovery following transfer to clean water.

Pair-wise Tukey-based multiple comparisons of least-squares means were performed to identify significant differences among treatment groups.

3. Results

3.1. Butafenacil-induced anemia occurs in the presence or absence of light

In the absence of effects on survival (97 ± 6%), body length (Fig. 1A), or pericardial area (Fig. 1B), static exposure of 5-hpf zebrafish embryos to 0.09 μM butafenacil in glass beakers resulted in a significant decrease in arterial circulation at 72 hpf. This nominal concentration was approximately ~4-fold lower than the concentration required to significantly decrease circulation (in the absence of teratogenic effects) within 384-well glass-bottom plates [13], a difference that was likely due to higher sorption of butafenacil to plastic well walls relative to glass beakers. In addition, we detected a significant increase in protoporphyrin accumulation following exposure to 0.05 μM butafenacil (Fig. 1D and E), suggesting that protoporphyrin accumulation was a more sensitive endpoint relative to circulation.

3.2. Butafenacil-induced protoporphyrin accumulation only occurs in the presence of light

Similar to exposures under a 14-h:10-h light:dark cycle, exposure to butafenacil in the dark resulted in a concentration-dependent decrease in circulation in the absence of effects on survival (>80%), body length (Fig. 2A), and pericardial area (Fig. 2B), with a significant decrease in circulation starting at 0.05 μM butafenacil (Fig. 2C). However, unlike embryos exposed under light conditions, protoporphyrin accumulation was absent in embryos exposed to 0.025–0.39 μM (Fig. 2D and E) and, although significant protoporphyrin accumulation was present following exposure to 0.78 μM butafenacil, this effect was orders of magnitude lower than embryos exposed to butafenacil under light conditions.

3.3. Embryonic zebrafish are not susceptible to butafenacil exposure following completion of pharyngula

To identify developmental stages susceptible to butafenacil-induced anemia within the first 72 h of zebrafish embryogenesis, we initiated exposure of embryos to vehicle or 0.09 μM butafenacil at 5, 10, 24, and 48 hpf (Fig. 3), and then analyzed body length, pericardial area, circulation, and protoporphyrin accumulation at 72 hpf. No significant effects on body length were detected across all treatment groups (Fig. 4A), and a significant increase in pericardial area was detected within exposures starting at 10 and 24 hpf (Fig. 4B). While a significant decrease in circulation was only observed when exposures were initiated at 5 and 10 hpf (Fig. 4C), a significant increase in protoporphyrin accumulation was observed when exposures were initiated at 5, 10, and 24 hpf (Fig. 4D).

3.4. Zebrafish embryos require >24 h to eliminate and recover from protoporphyrin accumulation following exposure to butafenacil during pharyngula

To determine whether butafenacil-induced effects on circulation and protoporphyrin accumulation were reversible, embryos were then exposed to butafenacil from 24 to 48 hpf or 48–72 hpf; as a positive control, embryos were also exposed to butafenacil from 5 to 72 hpf (Fig. 3). No significant effects on body length were detected within any of the treatment groups (Fig. 5A), and pericardial area was only increased following a 24–48-hpf exposure (Fig. 5B). As expected, exposure from 5 to 72 hpf resulted in a significant decrease in circulation and a significant increase in protoporphyrin accumulation (Fig. 5C and D). While no other exposure scenario resulted in a significant decrease in circulation, exposure from 24 to 48 hpf resulted in a significant increase in protoporphyrin accumulation relative to vehicle controls (Fig. 5D).

4. Discussion

Using zebrafish as a model, this study demonstrated that, while butafenacil-induced anemia occurred in the presence or absence

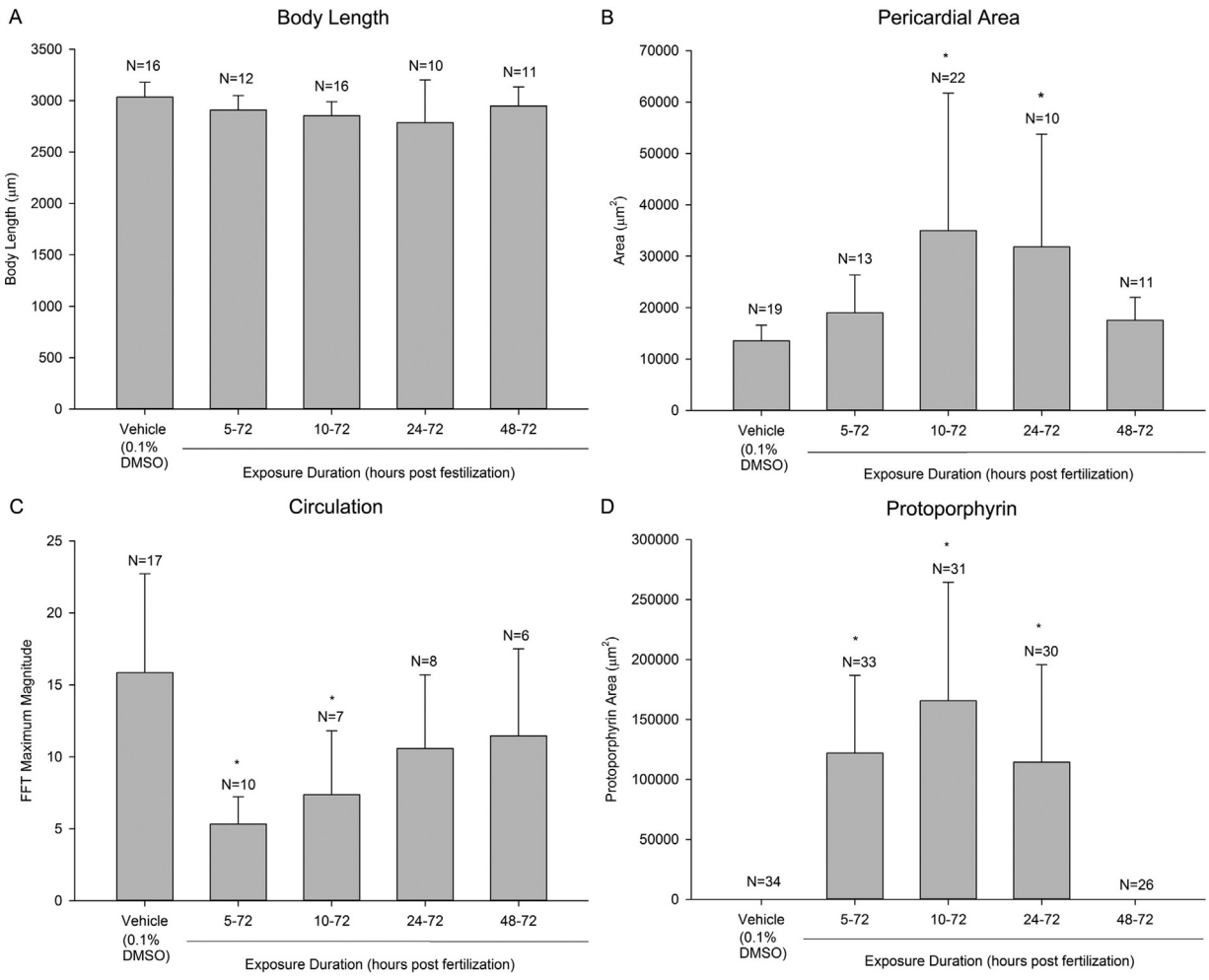

Fig. 4. Zebrafish embryos are not susceptible to butafenacil exposure following completion of pharyngula (24–48 hpf). (A) Mean body length (±standard deviation), (B) mean pericardial area (±standard deviation), (C) mean circulation (±standard deviation), and (D) mean protoporphyrin accumulation (±standard deviation) following exposure to vehicle (0.1% DMSO) or 0.09 μM butafenacil from 5 to 72, 10 to 72, 24 to 72, or 48 to 72 hpf. Numbers above each bar denote the number of embryos analyzed across three replicate beakers per treatment and 20 initial embryos per replicate beaker. Asterisk (*) denotes significant difference from vehicle controls ($p < 0.05$).

of light, butafenacil-induced protoporphyrin accumulation only occurred in the presence of light. Based on the known mode of action of PPOX-inhibiting herbicides in various organisms [9], butafenacil exposure of zebrafish embryos in the presence of light may have resulted in cellular accumulation and photooxidation of protoporphyrinogen to protoporphyrin. Moreover, direct interaction of light with protoporphyrin precursors may have resulted in increased acute toxicity under light conditions. Protoporphyrin can lead to reactive singlet oxygen formation in the presence of light, and photodermatitis within variegate porphyria patients is thought to be caused by photooxidation of protoporphyrinogen and increased production of reactive oxygen species within skin fibroblasts [2]. Similarly, as embryonic zebrafish are transparent within the first few days of development, proliferating and differentiating cells throughout the embryo are exposed to light and may be highly susceptible to oxidative stress caused by accumulated protoporphyrin.

During zebrafish embryogenesis, red blood cell development (or erythropoiesis) commences at early-segmentation (~11 hpf) with the migration of erythroid precursors toward the trunk midline [4]. Following migration, erythroid precursors undergo extensive proliferation until early-pharyngula (~25–26 hpf) to form approximately 300 proerythroblasts [4]. Following intravasation into developing blood vessels, proerythroblasts begin readily expressing genes required for red blood cell development (such as heme biosynthesis genes) and, by ~30–36 hpf, rapidly mature into erythrocytes that remain in circulation until a second definitive wave of erythropoiesis begins at 120 hpf [6,8]. While we did not confirm butafenacil uptake following initiation of exposures at different developmental stages, our data suggest that 72-hpf zebrafish embryos are not susceptible to butafenacil exposure following completion of pharyngula (24–48 hpf), as [1] initiation of exposure at 10 and 24 hpf resulted in a similar magnitude of effect on pericardial area and protoporphryin accumulation and [2] initiation of exposure at 48 hpf resulted in no effect on circulation, protoporphryin accumulation, or pericardial area. Although the exposure duration was shorter relative to initiation of exposure at 5 hpf, increased pericardial area and protoporphryin accumulation following exposure from 10 to 72 hpf or 24–72 hpf may be due to higher initial doses of butafenacil during the migration, proliferation, and/or maturation of erythroid precursors from early-segmentation (~11 hpf) to mid-pharyngula (~30–36 hpf).

Interestingly, protoporphyrin accumulation was primarily localized to the yolk sac of 72-hpf embryos, and embryos exposed to butafenacil during pharyngula (24–48 hpf) failed to eliminate protoporphyrin after a 24-h recovery period in clean EM. These findings suggest that either [1] butafenacil persisted within the embryo following transfer to clean water and/or [2] protoporphyrin was highly lipophilic and moderately persistent within zebrafish embryos. Nevertheless, as the yolk sac is transparent and large

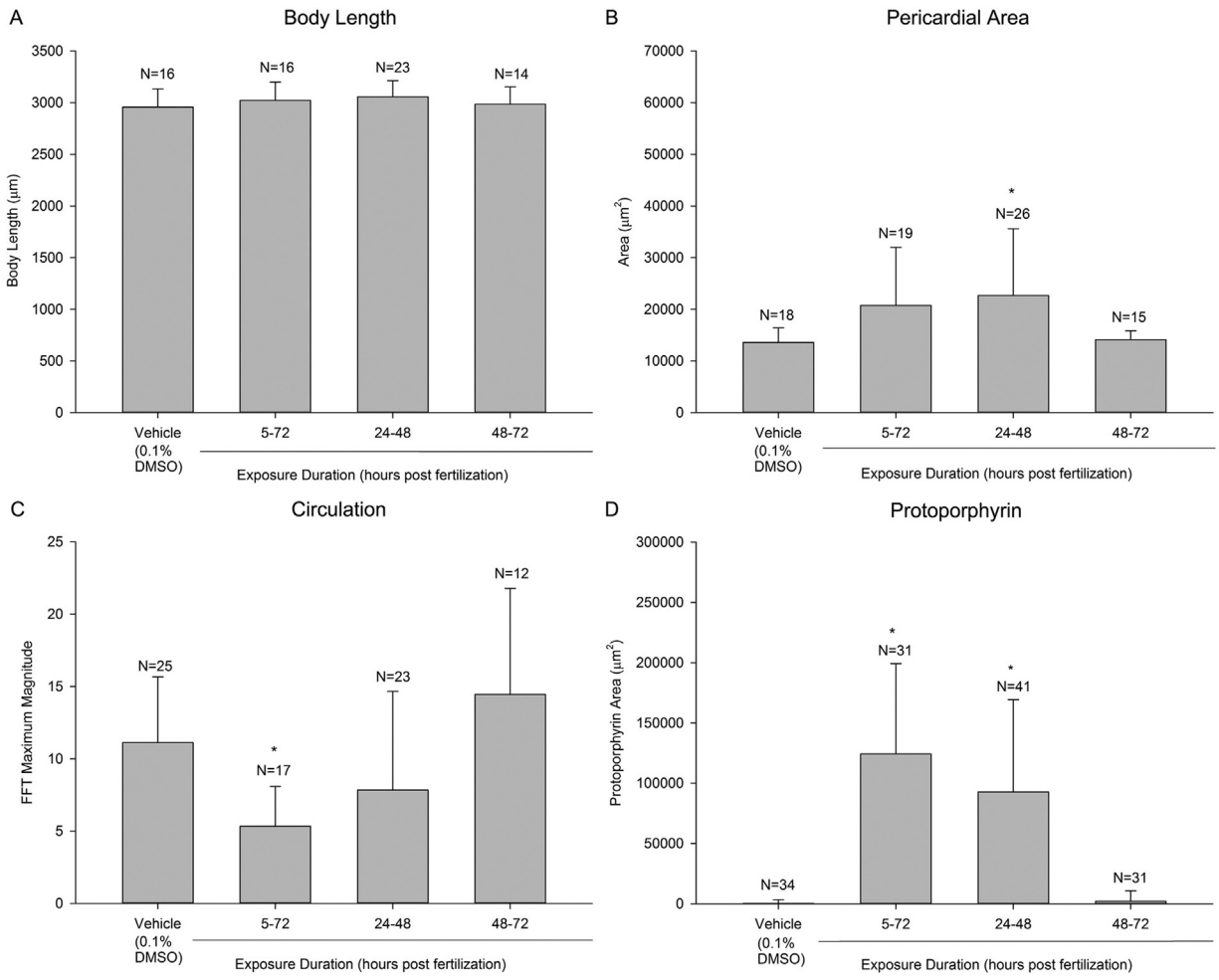

Fig. 5. Embryos require >24 h to eliminate and recover from protoporphyrin accumulation following exposure to butafenacil during pharyngula (24–48 hpf). (A) Mean body length (±standard deviation), (B) mean pericardial area (±standard deviation), (C) mean circulation (±standard deviation), (D) mean protoporphyrin accumulation (±standard deviation) following exposure to vehicle (0.1% DMSO) or 0.09 μM butafenacil from 5 to 72, 24 to 48, or 48 to 72 hpf. Numbers above each bar denote the number of embryos analyzed across three replicate beakers per treatment and 20 initial embryos per replicate beaker. Asterisk (*) denotes significant difference from vehicle controls ($p < 0.05$).

(relative to the total body area) within 72-hpf embryos, the yolk sac is also susceptible to light exposure and, as such, a probable source of oxidative stress in the presence of protopophyrin accumulation. Therefore, future research is needed to determine whether reactive oxygen species are generated within the protoporphyrin-containing yolk sac of butafenacil- and light-exposed embryos.

In summary, this study resulted in four major findings: (1) butafenacil-induced anemia occurs in the presence or absence of light; (2) butafenacil-induced protoporphyrin accumulation only occurs in the presence of light; (3) embryonic zebrafish are not susceptible to butafenacil exposure following completion of pharyngula; and (4) zebrafish embryos require >24 h to eliminate and recover from protoporphyrin accumulation following exposure to butafenacil during pharyngula. While our findings suggest that butafenacil inhibits PPOX during erythropoiesis, future research is needed to confirm that butafenacil is a potent inhibitor of recombinant zebrafish PPOX and butafenacil impacts red blood cells alone (and not other blood cells such as macrophages, neutrophils, etc.). In addition to identifying potential adverse hematologic effects during drug development, it is also important to ensure that drugs do not exacerbate conditions of diseases such as anemia and variegate porphyria. Therefore, the ability to identify anemia- or variegate porphyria-inducing side effects would be a valuable addition to screening used for drug discovery or development, as well as other chemical screening applications. While certain zebrafish mutant strains with disrupted blood cell development or differentiation are available [5,16], these strains have mainly been used to investigate the molecular mechanisms of hematopoiesis and may not be a viable option for pre-clinical screening applications. In addition to low fecundity, these mutant zebrafish strains usually do not live beyond 2–3 weeks, requiring maintenance as a heterozygous strain [5,16]. Therefore, the use of wildtype or homozygous transgenic reporter zebrafish lines (such as *fli1:egfp*) and butafenacil as a positive control has potential utility in drug discovery and development, as both anemia and variegate porphyria can be easily induced by butafenacil and rapidly identified within an intact organism.

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