Supplementary Material 1

Details for methods - FACETS

Fathead minnow exposed to environmentally relevant concentrations of metformin for one life cycle show no adverse effects.

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Methods for metformin delivery to exposure aquaria

Flow-through exposures of fathead minnows to metformin were accomplished with three peristaltic pumps. Water flowed through 20 needle valves into the mixing flask that received metformin stock solutions from 3 peristaltic pumps attached to 3 separate glass Boston bottles containing the metformin solutions. The metformin was prepared from metformin-HCl (metformin hydrochloride $C_4H_{11}N_5$–HCl, MW165.62, Catalog # PHR1084-500 mg, Lot# LRAB3694, CAS# 1115-70-4, Pharmaceutical Secondary Standard Certified Reference Material) purchased from Sigma-Aldrich (https://www.sigmaaldrich.com/catalog). Target nominal concentrations in aquaria were 3.0, 30, and 300 µg/L of metformin. A super-stock solution of 374 mg/L metformin was prepared by adding the entire 500-mg vial (approx. 500 mg of metformin-HCl) to 1042 mL of deionized MilliQ water. Three stock solutions of metformin (0.374, 3.7, and 37.4 mg/L) were prepared daily in fish lab water. No solvent controls were required for the exposures as the metformin super-stock and stock solutions were prepared in water.

For the highest metformin exposure concentration (300 µg/L), 100 mL of the 374 mg/L-

super-stock was mixed with 900 mL of fish lab water to prepare 1 L of stock solution in dechlorinated water (in an 1-L amber glass Boston bottle). Nominal concentrations in the stock were 37.4 mg/L. During each day for each exposure aquaria, a total of 200 mL of this stock flowed via peristaltic pump to 4 separate mixing chambers (250 mL Erlenmeyer flask), which also each received 24,000 mL lab water. The flow rates per hour were 8.33 mL stock mixed with 1000 mL lab water. The aerated Erlenmeyer mixing flasks delivered metformin solution (through an overflow tube via gravity) to each aquaria at a rate of 24,200 mL per day (or 1.008 L/hour). For the middle metformin treatment (30 µg/L nominal), 10 mL of the super-stock solution was diluted with 990 mL fish lab water in a separate 1 L Boston bottle, while for the lowest metformin treatment (3.0 µg/L nominal), 1 mL of the super-stock solution was diluted with 999 mL of fish lab water into a 1 L Boston bottle. As described above for the highest treatment, a separate peristaltic pump for each treatment was used to deliver 200 mL per day of stock to the mixing chambers. The mixing chambers supplied each replicate aquarium with metformin solutions mixed with lab water, or control aquaria with lab water, at a rate of 24.2 L per day into each 12 L aquarium. The solution replacement time in each replicate aquarium was about 2 tank volumes (24 L) per day. Flows of metformin stock solution and flows of lab water to each aquarium were checked daily and adjusted if necessary, and metformin stock solutions were replaced daily. New metformin super-stock solutions were prepared every 9 days.

Methods for fathead minnow exposures to metformin

Fathead minnow eggs from Aquatox (Aberfoyle, ON) and Ontario Ministry of the Environment Lab (Etobicoke, ON) were rolled from tiles, assessed for fertility and counted into mesh-bottom 500-mL egg cups. Three cups (each containing 10 eggs) were placed in each aquarium. Each aquarium contained 10 eggs sourced from OMOE (sourced from 4 breeding tiles),

and 20 eggs sourced from Aquatox (sourced from 14 breeding tiles). Exposures started on May 22 and 23 of 2018. Aquaria were housed in a 25°C water bath, aerated with air stones, and covered with plexiglass lids. Effective tank volumes were approximately 12 L. Exposure to the metformin began with eggs that were <12 hours post-fertilization. The photoperiod was set at 16 h light:8 h dark (lights on at 4 a.m., lights off at 8 p.m.) with gradual lighting and dimming mimicking dawn and dusk. Dilution water for the metformin was fish lab water, which was municipal water for the City of Burlington, ON, Canada that was dechlorinated by charcoal filtration, followed by filtration for particulates, and UV sterilization. Water temperature, dissolved oxygen, pH, conductivity, and ammonia were measured once a week in all fish exposure aquaria.

Eggs in egg cups were checked daily for mortality, and dead eggs were removed to discourage fungal growth. At 16 d post-hatch, larvae were counted and released from egg cups to the 12 L aquaria. Larval fish were fed 2 x per day newly-hatched brine shrimp, then 2 day-old hatched brine shrimp, with additional frozen and thawed brine shrimp added as fish grew to juveniles, then switched completely to frozen brine shrimp as adults, as described in Supplementary Material 2 Table S3. Random culling of fish over time, using a random-number generator, gave all fish in the tank an equal chance of being culled, and maintained appropriate densities for remaining fish to grow. At 30 days post-hatch (i.e. 30 dph), aquaria were randomly culled to 20 fish; at 64 dph, the fish were randomly culled to 15; and at 78 dph fish were randomly culled to 12 per tank. All culled fish were euthanized by an overdose of buffered tricane methane sulfonate (TMS) (500 mg/L), weighed, and measured.

At 78 dph, three breeding tiles constructed from PVC plastic pipes cut in half were added to each tank to promote maturation of males, and breeding/spawning behaviours. At 92 dph fish were culled to 10 fish per tank, with culling purposefully aimed at leaving at least 3 males and 5

females in each tank. A final cull down to 8 fish (3 males and 5 females) occurred at 141 dph. Breeding started in tanks with sexually mature individuals at 89 dph to 120 dph. Fish were left undisturbed every day until 10 AM to promote breeding. Any eggs deposited on tiles were removed at 10 AM, rolled from tiles, counted and assessed for egg stage, deformities and fertilization. Breeding was monitored until 168 dph. Up to 100 fertilized eggs from each batch were placed in screen-bottomed egg cups in separated flow-through aquaria containing clean lab water (aerated) and observed until hatch. For the entire experiment we observed and counted over 192,700 eggs. We observed a total of 46,495 eggs from the 3.0 µg/L metformin treatments, 38,834 eggs from the 31 µg/L metformin treatments, and 31,780 eggs from the 322 µg/L metformin treatments, which were distributed among the 4 replicate aquaria for each metformin exposure concentration. For the control treatment, we observed 75,666 eggs, as there were 8 replicate control breeding aquaria. Flow-through egg-hatching aquaria were temperature controlled at 25 °C and housed in a standing rack (for easy observation of eggs) that was separate from the metformin exposure. Egg production and hatch were monitored daily, and data were collected on the day to first breeding event with a clutch size ≥ 20 eggs, daily egg production, % fertilization, % dead eggs, % hatch, % deformed fry, and % dead fry. Percentages of deformed fry were also assessed as the total number of all deformed fry in a tank compared to the total number of eggs that hatched in that tank. Similarly, percentages of unfertilized eggs, dead, and mutant eggs were assessed as from the totals per tank. These values gave a second overall assessment of the deformities (or unfertilized eggs, dead, mutant eggs) in each tank and treatment that was not biased by the sizes of egg batches that were laid. Dead eggs were defined as eggs that were fertilized and had begun cell division, but then subsequently died. Mutant eggs were fertilized eggs that had failed to develop properly and had coagulated or were misshaped. Normal eggs were healthy eggs that were assumed viable.

Deformed fry had hatched but had severe (or mild-moderate) deformities of the spine, or edema of the heart or yolk sac.

Growth of F1 fish until 9 and 16 dph was assessed by transferring four fertilized batches of 100 eggs from each tank to clean water flow-through aquaria (at 25 °C) in a water bath. Eggs were held in egg cups and assessed at hatch, then 20 fry from each batch were randomly selected to grow out to 9 and 16 dph. Larval fish were fed 2 x daily as per feeding chart (Supplementary Material 2 Table S3). At 9 dph up to 10 fish were randomly culled from each egg cup and weight, length, and condition factor (CF) were measured. The remaining 10 larval fish in each egg cup were raised to 16 dph and then sampled to measure weight, length, and CF.

Methods for fish sampling and dissection

At 169-172 dph, all of the adult fish from the controls and metformin-exposure aquaria were sampled. Fish were anesthetized in TMS solution. Fish were weighed, measured, blood samples were taken from the dorsal aorta using a heparinized capillary tube, and then fish were euthanized by spinal severance. Fish were dissected as described previously by (Parrott and Metcalfe 2018), with separate dissection stations for male and female fish. Secondary sex characteristics were assessed and the phenotypic sex of the fish was determined from these characteristics. The measure of female secondary sex characteristics included ovipositor length and width measured under a dissecting microscope and the area of the approximately triangular ovipositor was calculated in mm^2. Ovipositor area ranged from 0.68 to 2.39 mm^2 for individual females. Male secondary sex characteristics that were assessed included the presence of a dorsal fin dot (graded as present/absent), and the appearance of the dorsal head pad, which was rated on a scale of 0 (no pad) to 4 (very large, well developed pad). Nuptial tubercles were graded as normal

or large and total and large tubercles were counted under a dissecting microscope. Male banding pattern was scored from 0 (no banding) to 3 (dark banding with black head). Tubercle index was calculated as (the total number of tubercles + 2 x the number of large tubercles)/10, and for individual males in this experiment it ranged from 0 to 4.7. The male index was calculated as the sum of: 1 point for fin dot + band points (out of a possible 3) + pad points (out of a possible 4) + tubercle index. The male index of individual males in this experiment ranged from 2.0 to 11.0.

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Methods for measuring vitellogenin in plasma

Concentrations of vitellogenin (VTG) in individual fish plasma samples was determined using a TECO Cyprinid Vitellogenin ELISA kit (TECO Medical Group, Sissach, Switzerland). The kit provided all of the reagents, controls, standards and a Cyprinid VTG antibody coated plate to run the assay. Six-point standard curves were run with each assay, and r^2 values were ≥ 0.98 (mean $r^2 = 0.9968$ s.d. 0.0054). Assay steps are described briefly below. Frozen plasma samples were thawed slowly on crushed ice to preserve sample integrity. Samples were diluted with buffer in borosilicate glass tubes (12 x 75 mm, Thermo Fisher Scientific) at room temperature. The optimal dilution for female plasma was 1:10000 or 1:250000. For males, the optimal dilution was most often 1:50-1:100. All samples were loaded into the antibody coated plate in duplicate in less than 10 minutes. Further binding, rinsing and developing steps were as described in the provided manual, with the exception of incubation time. We determined that 30 minutes was too long, as the reaction had gone past the peak and was giving a weaker signal. So after 15 minutes 100 µL of the stop solution was added to each well and the plate was read immediately at 450 and 405 nm on a microplate reader (ELx808, BioTek® Instruments Inc.).

Methods for assessing gonads histologically

Gonad sampling and processing methods were based on published methods (Ankley 2006; Tetreault et al. 2011). Methods for scoring and counting of gonads were based on (Ankley 2006). Gonad tissue was dissected and pairs of ovaries or testes were collected in histology cassettes. Cassettes with samples were fixed in Davidson's solution (200 mL formaldehyde, 300 mL ethanol,

200 mL glycerol, 100 mL glacial acetic acid, 300mL DI Water) for 24 h before transferring to 70 % ethanol for storage. Samples were subsequently split, with one sample kept as backup, and one dehydrated and cleared through a tissue processor (E150, Somagen Diagnostic) then embedded in paraffin. Female tissues were trimmed on a microtome (RM2155, Leica) to the approximate centre of the tissue and placed on ice for approximately 4 h to rehydrate. Two sets of three to six 5-µm thick serial sections were then taken and adhered onto a glass microscope slide. Male tissues were sectioned such that serial slides were created and the entire gonad was sliced in sections smaller than an oocyte (to make sure no instances of intersex males were missed). Primary oocytes of fathead minnows are 60-120 µM in diameter (Jensen et al. 2001). Five to ten 5-µm thick sections of testis were taken and adhered onto a glass microscope slide, then approximately 7 sections were cut and discarded (discarded tissue total thickness was 28-35 µM) and another 5-7 section slide was made. Sectioning was continued until the entire testes tissue was captured. All sides were left in a warming oven for at least 24 h before they were stained with hematoxylin and eosin, and had coverslips applied.

Female tissues were imaged at 50 x magnification (020-525.024 Microscope, Leica and QICAM Fast, QImaging) and stitched together using Northern Eclipse (Empix Imaging, Inc., Mississauga ON, CAN). The oocytes were categorized as primary, cortical alveolar, early vitellogenic, late vitellogenic or atretic and the number of each counted using ImageJ. All oocytes within one representative section for each sample were counted and the proportion of each stage calculated. For each fish about 250-320 oocytes were counted in total. An atresia grade was given to each ovary sample as follows: 0- no atretic eggs present and no interstitial vitellogenin or cell debris, 1 – one to five atretic eggs present and/or mild interstitial vitellogenin or cell debris, 2 –

five to ten atretic eggs present and/or moderate interstitial vitellogenin or cell debris, 3 – more than ten atretic eggs present and/or interstitial vitellogenin or cell debris throughout the tissue.

Male tissues were scanned at 200 x magnification for the presence of testis-ova (male testicular tissue with female oocytes present) and each sample staged as follows: stage 0 – underdeveloped, only immature stages present, stage 1 – early spermatogenic, primarily immature stages, some spermatozoa present, stage 2 – mid spermatogenic, all stages present in roughly equal proportion, stage 3 – late spermatogenic, all stages observed, predominantly spermatozoa. On average about 20-25 slides were assessed per male fish

Metformin measurements in exposure aquaria

Metformin hydrochloride, guanylurea phosphate, and ammonium formate were obtained from Sigma Aldrich (St Louis, MO, USA), and the isotope labelled internal standards, metformin-D_6 hydrochloride and guanylurea $^{15}N_4$ hydrochloride were obtained from Toronto Research Chemicals (North York, ON, Canada). Methanol and water were purchased from Fisher Scientific (Fairlawn. New Jersey, USA) and were Optima LCMS grade. Suprapur formic acid (98-100%) was obtained from EMD Millipore, Billerica, MA, USA.

Metformin was measured every two weeks in each of the 20 individual exposure aquaria. Samples of 20 mL exposure water samples were taken from each tank and filtered (to remove particles and fish waste) using a sterile syringe (Henk Sass Wolf Norm-ject, 4200-X00V0 20mL) with a glass microfiber disposable syringe filter (Whatman, Pore size 0.7µm, 6890-250725mm GD/X). Filtered samples were stored in 20 mL pre-cleaned and pre-fired glass tubes. An aliquot of 180 µL pre-filtered aquaria water was diluted with 20 µL of either a diluted labelled solution of

dimethyl D_6 metformin hydrochloride or $^{15}N_4$ guanylurea hydrochloride. The resulting sample was vortexed and 2 µL was directly injected onto the LC-MS-MS.

LC-MS-MS analyses were performed using a Waters Xevo TQS mass spectrometer coupled to an Acquity UPLC system. The mass spectrometer was operated using an electrospray (Zspray) ionization source in positive ion and multiple reaction monitoring (MRM) mode. An Agilent Eclipse Plus C18 RRD 1.8 µm, 2.1 x 100 mm analytical column was used for the analysis of metformin and guanylurea. The mobile phase consisted of water and methanol both containing 2 mM ammonium formate and 0.1 % formic acid. Column temperature was maintained at 25 °C. LC gradient conditions are presented in Supplementary Material 2 Table S1. The precursor to product transitions monitored for native and labelled metformin and guanylurea are presented in Supplementary Material 2 Table S2. Two ions were chosen so that a ratio between the two could be used as a further confirmation of compound identity (Supplementary Material 2 Table S2, Figure S1 and Figure S2).

All standards and working solutions for both analytes were prepared in initial mobile phase (water:methanol (90:10) gradient with 2 mM ammonium formate and 0.1% formic acid). A 13 point and 7 point calibration was generated for metformin and guanylurea respectively (Supplementary Material 2 Figure S3 and Figure S4).

Quantifications were performed using external calibration. R^2 values for the concentration range were 0.99 or better. Labelled standards were used as performance standards to monitor instrument stability. Waters TargetLynx software was used to integrate chromatograms, generate calibration curves and calculate sample concentrations.

The Limit of Detection (LOD) for metformin was 0.0088 µg/L, which was calculated as 3 x standard deviation of 32 blank water samples. The Limit of Quantification (LOQ) for metformin was 0.029 µg/L, which was calculated as 10 x standard deviation of the 32 blank water sample values. The LOD and LOQ for guanylurea, the main metformin breakdown product, were 1.3 and 5.4 µg/L based on the standard deviation of 8 blank water samples.

References

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