Supporting Information

Lifelong exposure to dioxin-like PCBs alters paternal offspring care behavior and reduces male fish reproductive success

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S1. Methodology

All sediment for *L. variegatus* cultures was collected from approximately the top 10 cm of sediment from submerged wetland habitats. Control and PCB-1 sediment was collected from Southern Illinois University's Touch of Nature pond facility. Sediment for PCB-1 *L. variegatus* cultures was spiked with Aroclor 1254 30-d prior to addition of *L. variegatus*, with the spiked sediment mixed for 5 min each week to ensure a homogenous mixture. *Lumbriculus variegatus* were grown in cultures for at least 60 d prior to being fed to fathead minnows. Monthly samples from *L. variegatus* culture tanks were collected and analyzed for PCB concentrations to determine variability in prey PCB concentrations provided to fish throughout the study. Each day during the experiment, all fish fed on treatment-specific *L. variegatus*. Because the daily biomass of *L. variegatus* was suitable for PCB dosing but insufficient for growth, fish were fed uncontaminated commercial fish pellets (Purina AquaMax Fry Starter; Purina Mills, Inc., St. Louis, MO, USA) in excess each day after consuming all *L. variegatus* provided.

Once mature, one adult male fathead minnow from each replicate aquarium was randomly selected to remain in the aquarium for spawning trials, with extra fish removed from the study. In control aquaria, all males had dark colored bodies (later confirmed with dissection), so one male per tank was randomly selected out of all dark colored individuals. In the two PCB treatments, males were not all darkly colored. In these treatments, females were easily identifiable and excluded due to extended abdomens from egg development. One fish per tank was randomly selected from the remaining individuals to be used in spawning trials. These fish were later confirmed to be males during dissection. See Table S1 for a comparison of sizes of male fish excluded from spawning trials.

PCB sample analysis

Overview

Male fathead minnow exposure to PCBs via *L. variegatus* was confirmed by the analysis of tissue extracts. Monthly samples from *L. variegatus* cultures (to assess variability in dosing fathead minnows through time) and three randomly selected male fathead minnows per treatment (entire fish except testes, brain, and pituitary), after spawning trials, were analyzed for PCB concentration. Polychlorinated biphenyls in samples were extracted[1,2] and cleaned up using sulfuric acid[3] prior to analysis by gas chromatography/mass spectroscopy in electron impact mode.[4] The PCB concentrations were normalized for lipid fraction on a wet mass basis, where lipid fraction was determined using extraction and spectrophotometry.[5]

Chemicals

The PCB standard contained 119 congeners from the combination of four custom PCB mixtures (30 µg/mL in nonane) that were purchased from AccuStandard, Inc. (New Haven, CT, USA). Recovery surrogates 4,4′-dibromooctafluorobiphenyl (DBOFB, 250 µg/mL in acetone) and PCB-186 (35 µg/mL in isooctane) were purchased from Supelco Inc. (Bellefonte, PA, USA) and AccuStandard. Stable isotope labeled internal standards $^{13}C_{12}$-PCB-15, $^{13}C_{12}$-PCB-52, $^{13}C_{12}$-PCB-141, and $^{13}C_{12}$-PCB-209 (each 40 µg/mL in nonane) were purchased from Cambridge Isotope Laboratory (Andover, MA, USA). Optima grade solvents (hexane, acetone, and methylene chloride), silica gel (60-200 mesh, grade 60), sea sand (washed), ACS grade concentrated sulfuric acid, and anhydrous sodium sulfate were purchased from Fisher Scientific

(Hampton, NH, USA). Nitrogen (purity: 99.998%) and helium (99.999%) were supplied by Airgas Inc. (Marion, IL, USA).

Sample Extraction and Cleanup

The PCBs were extracted from fathead minnow tissue by pressurized liquid extraction using a Dionex 200 Accelerated Solvent Extraction (ASE) System (Waltham, MA, USA). Prior to extraction, tissue samples were freeze dried (Labconco, Kansas City, MO, USA) and ground by hand using a mortar and pestle. Freeze-dried tissue (0.2-0.6 g) was placed in an ASE cell equipped with a filter and 5 g of silica gel (activated at 130 °C for 12 h) and filled with sea sand. Samples were extracted using a 1:1 dichloromethane:acetone solution at 100 °C and 1500 pounds per square inch (psi) which was held for two heat-static cycles of 10 min each. After extraction, extracts were solvent exchanged with 20 mL of hexane, and concentrated to 1 mL for cleanup.

Polychlorinated biphenyls were extracted from *L. variegatus* tissues by exhaustive chemical extraction. *L. variegatus* tissue (0.1-0.25 g) was placed in 10 mL of acetone and the tissue was extracted by a high-intensity sonicator (Sonics & Materials VCX400, Newtown, CT, USA) using three 10 s pulses. Next, 10 mL of hexane was added to the vial and the samples were incubated in a bath sonicator (Branson 3510, Branson Ultrasonic Corporation, Danbury, CT, USA) for 30 min. The extracts were evaporated to 1 to 2 mL.

Extracts were cleaned by adding 1 to 2 mL sulfuric acid to the extract and mixed by vortexer (3000 rotations per minute (rpm)) for 5 min.[3] The mixture was centrifuged at 3000 rpm for 5 min and the top organic layer containing the PCBs was removed. The aqueous bottom layer was washed three times using 1 mL of hexane and the top layers were combined and dried by

passing through a column containing anhydrous sodium sulfate (1 g). The final volume was adjusted to 1 mL with hexane.

To assess recovery, 50 ng of DBOFB and PCB-186 surrogates were added to each sample prior to extraction. In addition, for every batch of samples extracted, a set of four quality assurance samples were prepared and analyzed along with samples. These consisted of one to two matrix and/or solvent blanks, a matrix sample and a matrix duplicate sample spiked with 50 ng of each target analyte. Matrix samples were made using 50 *L. variegatus* and one fathead minnow, respectively, obtained from cultures free from target analytes.

Gas chromatography/mass spectrometry analysis

Polychlorinated biphenyls were quantified by gas chromatography/mass spectrometry (GC/MS) using an Agilent 6890 gas chromatograph 5973N mass spectrometer. Samples were injected (2 µL) in pulsed splitless mode at 265 °C and separated using an Agilent DB-XLB column (30 m × 180 µm, 0.18-µm film thickness). Oven temperature was initially set at 100 °C for 1 minute, and increased to 255 °C at a rate of 1.2 °C min^{-1}, followed by an increase to 280 °C at 25 °C min^{-1}, and held for 3.33 min. Analytes were separated using helium carrier gas with a 0.9 mL min^{-1} flow rate and detected in electron impact ionization (EI) mode. The MS temperatures were as follows: transfer line: 280 °C, ion source: 230 °C, quadrupole: 150 °C.[1,2]

Polychlorinated biphenyls were identified in extracts by comparison to standards using retention time (< 0.05% agreement), using selected ion monitoring with the molecular ion (M^+) as the target ion and qualified using at least one target ion/qualifier ion ($(M+2)^+$ and $(M-70)^+$) ratio (< 20% agreement). Each sample and standard was spiked to 20 ng mL^{-1} with an internal standard mixture consisting of $^{13}C_{12}$-4,4'dichlorobiphenyl,$^{13}C_{12}$-2,2',5,5'-tetrachlorobiphenyl,

$^{13}C_{12}$-2,2',3,4,5,5'-hexachlorobiphenyl, and $^{13}C_{12}$-decachlorobiphenyl. Analytes were quantified using internal standard calibration with an eight-point quadratic calibration curve (1 ng mL^{-1} to 400 ng mL^{-1}) using the 119-congener PCB standard mixture (AccuStandard). Instrument performance was monitored using a check standard injected once every eight samples. The reporting limit was set to the lowest calibration standard, 1 ng mL^{-1}, and the concentration of the congeners greater than the reporting limit were summed. The sum of PCBs was normalized by lipid mass.

The lipid content in *L. variegatus* and fathead minnow was analyzed using individuals from each test. For *L. variegatus,* one or two individuals from each experimental replicate were randomly chosen and blotted dry prior to extraction. For fathead minnows, freeze-dried tissue was subsampled from the homogenate of each replicate. Tissues (0.01-0.02 g) were extracted with chloroform and methanol (1:1, v/v) as previously described (Lu et al. 2008). A vanillin/phosphoric acid reagent was added and transmittance was read at 525 nm using a spectrophotometer (Spectronic 20 Genesys™; Spectronic Instruments, ThermoFisher Scientific, Waltham, MA, USA). A five-point calibration curve was constructed using dilutions of vegetable oil and treated the same as tissue samples. The transmittance readings were conducted in triplicate to obtain a mean and standard deviation for each sample.

Gene expression quantification

Gene expression was quantified using real-time polymerase chain reaction (qPCR). Total RNA was extracted from male fathead minnow brain with pituitary samples using QIAzol (Qiagen) followed by a cleanup using the RNeasy MinElute Cleanup Kit (Qiagen), as described previously.[6] A Nanodrop 1000 spectrophotometer was used to quantify RNA concentrations as

well as 260/280 and 260/230 ratios to ensure quality standards, and both ratios were between 1.8 and 2.1, signifying the lack of contamination with proteins or reagents, respectively.[7] Samples meeting quality standards were first treated with deoxyribonuclease (DNase) I (Fermentas, Hanover, MD) according to the manufacturer's instructions to eliminate any genomic DNA contamination. DNase-treated RNA was then reverse transcribed to complementary DNA (cDNA) using a high-capacity reverse transcription kit (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. Primer sequences for the subset of genes tested (Table S2) were designed based on previously published work and purchased from Integrated DNA and tested to determine their efficiency. Melt curves were developed to ensure amplicons were consistent with predicted size. qPCR reactions were performed in a Hard-Shell 96-well white plate (Bio-Rad) and sealed with a microseal plate sealing film adhesive (Bio-Rad). Each reaction contained 10 µl SYBR Green Supermix (Bio-Rad), 10 µM forward and reverse primers, 30 ng of cDNA and nuclease free water with a total volume of 20 µL per well, using a Bio-Rad CFX96 system (Hercules, CA). All reactions were performed in duplicate using the following qPCR cycle: template denaturation at 95 °C for 3 min, 40 cycles of 95 °C for 10 sec, primers annealing at 58 °C for 30 sec, extension at 72 °C for 30 sec and a final extension at 65 °C to 95 °C in increments of 0.5 °C for 5 sec. The expression of each target gene was normalized to a reference gene (ribosomal protein L8; *rp18*) to calculate relative gene expression (ΔCt). Expression of each target gene was quantified by estimating the average $2^{-\Delta\Delta Ct}$ of all biological replicates.[8]

Geometric morphometric analysis

Thirteen landmarks[9] (Table S3; Fig. S1) were digitized on each fish using tpsDig2 software, and MorphoJ software[10] was used to perform a Procrustes superimposition. Data were

checked for outliers and residuals from a regression between Procrustes coordinates and centroid sizes were calculated. Residuals were compared across treatments using a permutational multivariate analysis of variance (PERMANOVA; 4999 permutations, Euclidean distance).

Male physical characteristics

Fatpads on male fathead minnows were categorically scored based on the visual appearance of the fatpad on the body following a modification of Danylchuk and Tonn.[11] Fatpad score 1 represented males with no visible fatpad present; fatpad score 2 had a small fatpad visible; fatpad score 3 had a clearly visible fatpad just slightly above the surrounding body; fatpad score 4 had prominent fatpads present that clearly extended above, but not overhanging, the body surface; and fatpad score 5 had very prominent fatpads that extensively protruded beyond (i.e., overhanging) the surrounding body.

Nuptial tubercles were also categorically quantified for males into one of three categories representing increasing tubercle size using a previously established scoring system.[12,13] Tubercle score 1 represented tubercles that were present but small, with the tubercle height and radius were nearly equal. Tubercle score 2 reflected enlarged tubercles with a radial base, the presence of furrows, and a jagged vertical appearance. Tubercle score 3 represented pronounced, very large tubercles with a rounded appearance, large base, and deep furrows. Personnel were not blinded to the treatment associated with each fish while performing physical characteristic measurements.

Population modeling

179 The potential for long-term, population-level consequences to emerge from results
180 observed during spawning trials were assessed using a deterministic life-stage model (stagePop
181 package in R[14]). Probabilities of surviving each life stage for all simulations were 0.2 for the
182 larval stage, 0.5 for juveniles, and 0.05 for adults.[15-17] The embryo stage was determined to last 5
183 days, the larval stage 14 days, the juvenile stage 6 months, and the adult stage 2.5 years. Per
184 capita growth rate was modeled as $1.5(N)\cdot(1 - N/10000)$, where N is the number of adults in the
185 current time step (see S4 Appendix below).

186

187 **Data analysis**

188 Statistical analyses were performed in program R. Final sample sizes varied by response
189 variable (Table S4). Concentrations of PCB congeners from males were compared among
190 treatments using a generalized linear model (GLM; gamma family; log link), with post-hoc
191 Tukey's tests (Holm-adjusted p-values). Spawning behaviors, number of embryos laid,
192 proportion of nonviable embryos, and proportion of a clutch surviving to 30 days were compared
193 using Kruskal-Wallis tests, followed by pair-wise, Holm-adjusted Mann-Whitney tests.
194 Generalized mixed effect models (GLMM; gamma family; log link) compared length and mass
195 of 30-day-old offspring (random tank effect, covariate of number of offspring per tank).

196 Breeding males develop a unique body shape that can be influenced by exposure to
197 chemical pollutants[18] but may be poorly quantified using univariate measurements. Therefore,
198 photographs of males were used to compare multivariate measurements of morphology across
199 treatments using landmark-based geometric morphometric analysis. Holm-adjusted pairwise
200 PERMANOVAs identified specific treatment differences and a warped outline drawing was used
201 to visualize morphological differences across treatments.

202 Proportion of males with dark bodies and dark vertical banding were compared using
203 Fisher's exact tests. Male body length and mass were analyzed using analysis of variance
204 (ANOVA; *post-hoc* Tukey's tests). A PERMANOVA (4999 iterations; Bray-Curtis distance)
205 was used to compare categorical nuptial tubercle scores. Male fatpad score and total number of
206 nuptial tubercles were analyzed using a Kruskal-Wallis test and subsequent Mann-Whitney tests,
207 and ANOVA was used to compare fatpad index and GSI across treatments. Gene expression was
208 analyzed separately for each gene using a GLM (gamma family, log link). Germinal epithelium
209 thickness of testes was analyzed using ANOVA, and multivariate sperm cell counts were
210 compared across treatments using PERMANOVA (4999 iterations; Bray-Curtis distance). For
211 males measured for PCB concentration, linear regression was used to assess relationships
212 between PCB concentration (total PCBs, dioxin-like PCBs, and anti-androgenic PCBs) and
213 spawning behavior variables that were significantly different across PCB treatments (Holm-
214 adjusted).

215 **Table S1.** Mean (SE) size of male fathead minnows dosed with treatment-specific PCB

216 concentrations throughout life that were excluded from spawning trials.

Treatment	Mass (g)	Length (mm)
Control	2.2 (0.2)	56 (1)
PCB-1	2.3 (0.2)	58 (1)
PCB-2	2.2 (0.1)	57 (1)

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220 **Table S2.** Description of primers used in gene expression analyses from brain and pituitary

221 samples collected from male fathead minnows used in spawning trials.

Gene Name	Primer Sequence (5'-3')	GenBank Accession Number	Source
cyp19b	F: AGGGTGTATCCTGGCAACTG R: ATCTGCACCCGTTTCATTTC	AJ277866	He et al. 2012[19]
fshβ	F: AGCTGCATCACAATCGACAC R: AGGGCAGCCTTTAAACTCGT	DQ242616	He et al. 2012[19]
gnrhr	F: TGCAAAGCCAGTGAAAATTG R: TTGTCAAACTGGGACGTGAG		He et al. 2012[19]
lhβ	F: GTCGTTGCTCAAAGCTCCTT R: TGGAGAACGGGCTCTTGTAT	DQ242617	He et al. 2012[19]
rpl8	F: CTCCGTCTTCAAAGCCCATGT R: TCCTTCACGATCCCCTTGATG	AY919670	Johns et al. 2009[20]
tshβ	F: GGTGCAGCCTCTCTGAACCA R: CTTCTGCTTCTCCAGGGACAGT	DQ677879	Lema et al. 2009[21]

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Table S3. Description of landmark locations[9] used in geometric morphometric analysis of male fathead minnows used in spawning trials (10 males per treatment). Landmark numbers correspond to landmark positions in Figure S1.

Landmark	Description
1	Anterior point of the snout
2	Anterior extreme of bony orbit of the eye
3	Top of cranium at midpoint of eye
4	Top of cranium at posterior point of the bony opercle
5	Posterior point of the bony opercle
6	Dorsal insertion of pectoral fin
7	Anterior ventral point of bony opercle
8	Anterior insertion of dorsal fin
9	Dorsal origin of caudal fin membrane
10	Posterior border of the hypural bones at the lateral midline
11	Ventral origin of caudal fin membrane
12	Anterior insertion of anal fin
13	Anterior insertion of pelvic fin

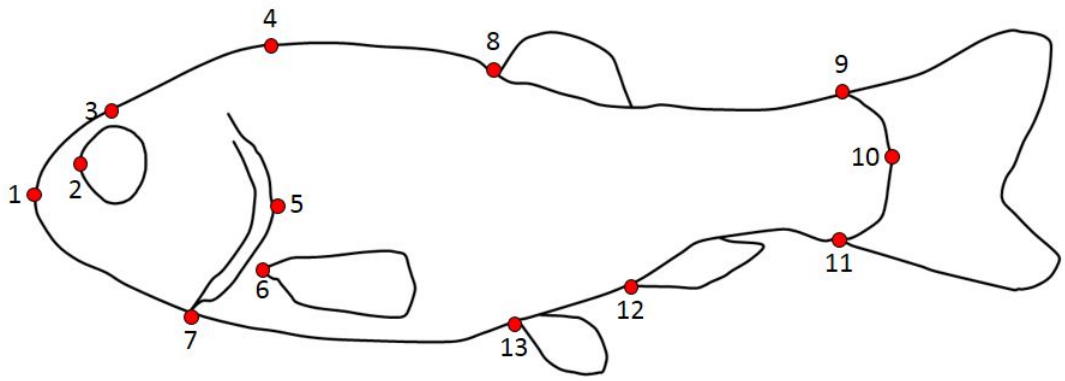

Figure S1. Landmark locations used in geometric morphometric analysis of male fathead minnows. Landmark descriptions are listed in Table S6.[9]

S2. Results

Three sediment samples were collected from each treatment's *Lumbriculus variegatus* culture tanks before *L. variegatus* were added in order to quantify sediment PCB concentrations. Total mean (SE) PCB concentrations were 8 (1) $ng \cdot g^{-1}$ dry weight for control sediment, 167 (4) $ng \cdot g^{-1}$ dry weight for PCB-1 sediment, and 2979 (813) $ng \cdot g^{-1}$ dry weight for PCB-2 sediment.

Table S4. Final sample sizes for response variable categories. Analyses for gene expression and histology did not yield usable samples for all 30 male fathead minnows, resulting in reduced sample sizes for these endpoints.

Response Variable	Sample Size
PCB concentrations	9
Gene expression	17
Histology	25
Physical characteristics	30
Spawning behavior and success	30
30-day-old offspring	90

Table S5. Mean (standard error) PCB concentrations from *Lumbriculus variegatus* samples collected monthly from treatment-specific culture tanks. Low abundance of *L. variegatus* in the PCB-2 culture tanks prohibited sample collection the final two months of the experiment. Data represent two replicate samples collected from culture tanks each month.

	All PCB congeners ($\mu g \cdot g^{-1}$ lipid)			Dioxin-like congeners ($\mu g \cdot g^{-1}$ lipid)		
Month	Control	PCB-1	PCB-2	Control	PCB-1	PCB-2
July	7 (4)	510 (209)	751 (115)	1 (1)	97 (39)	17 (4)
Aug	0 (0)	341 (34)	925 (143)	0 (0)	66 (8)	19 (1)
Sept	0 (0)	369 (67)	636 (276)	0 (0)	78 (12)	15 (6)
Oct	19 (10)	232 (16)	-	2 (2)	51 (0)	-
Nov	0 (0)	343 (-)	-	0 (0)	36 (-)	-

Table S6. Mean (SE) concentration of dioxin-like and anti-androgenic PCB congeners in male fathead minnows (n = 3 males per treatment).

Congener	Dioxin-like*	Anti-androgenic†,‡	Control ng·g^{-1} lipid	PCB-1 ng·g^{-1} lipid	PCB-2 ng·g^{-1} lipid
PCB-47		X	<RL	21 (7)	684 (540)
PCB-51		X	<RL	<RL	48 (48)
PCB-52		X	<RL	239 (82)	45 (24)
PCB-53		X	<RL	<RL	14 (14)
PCB-74		X	<RL	73 (21)	<RL
PCB-77	X		<RL	<RL	<RL
PCB-81	X		<RL	<RL	<RL
PCB-95		X	<RL	363 (113)	134 (68)
PCB-100		X	<RL	<RL	195 (145)
PCB-101		X	<RL	908 (243)	877 (494)
PCB-105	X		<RL	569 (145)	49 (15)
PCB-114	X		<RL	33 (18)	<RL
PCB-118	X	X	<RL	1225 (315)	217 (72)
PCB-123	X		<RL	112 (33)	40 (21)
PCB-126	X	X	<RL	<RL	<RL
PCB-128		X	<RL	186 (42)	134 (43)
PCB-136		X	<RL	43 (11)	107 (52)
PCB-137		X	<RL	5 (5)	<RL
PCB-138		X	18 (9)	792 (190)	1667 (605)
PCB-153		X	36 (13)	569 (137)	2917 (989)
PCB-156	X		<RL	150 (37)	214 (66)
PCB-157	X		<RL	45 (9)	<RL
PCB-167	X		<RL	33 (8)	87 (33)
PCB-169	X		<RL	<RL	<RL
PCB-170		X	<RL	51 (6)	1153 (319)
PCB-180		X	<RL	21 (4)	1162 (365)
PCB-189	X		<RL	<RL	88 (13)
PCB-190		X	<RL	25 (5)	330 (82)

RL: Reporting Limit; 2 ng·mL^{-1}

*[22]

†[23]

‡[24]

S3. References

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S4. Appendix

Script in program R used to model population dynamics.

```
#*************************************************************************
#BELOW SCRIPT FOR ESTIMATING POPULATION ABUNDANCE THROUGH TIME
#WAS DEVELOPED BY:
#Kettle H, Nutter D (2015) stagePop: modelling stage-structured populations in r. Methods in
#Ecology and Evolution 6:1484-1490
#*************************************************************************

#Vectors specified in increasing life stage: e.g. eggs to adults

library(stagePop)

trt.sim = "Control"  #Set to either "Control", "PCB-1", or "PCB-2"

solver.options=list(DDEsolver='deSolve', atol=1e-3, rtol=1e-3,
                    hbsize=1e4)

#Reproduction:
FHM_Functions <- list(
  reproFunc=function(x,time,species,strain){
    r=1.5
    K=10000  #Carrying capacity
    reprod=r*x$FHM['adults',1] * (1-(x$FHM['adults',1]/K)) #Population growth rate
    return(reprod)
  },

#Mortality:
  deathFunc=function(stage,x,time,species,strain){
    #Per capita mortality for each life stage: values fixed for non-embryo stages
      #Embryo values correspond to treatment-specific observed survival from experiments
    if(trt.sim=="Control"){a=c(1.44,42.5,1.4,0.05)} #Using Control data
    if(trt.sim=="PCB-1"){a=c(10.77,42.5,1.4,0.05)} #Using PCB-1 data
    if(trt.sim=="PCB-2"){a=c(2.17,42.5,1.4,0.05)} #Using PCB-2 data
```

```
    return(a[stage])
  },

#Duration of each life stage:
  durationFunc=function(stage,x,time,species,strain){
    a=c(0.014,0.038,0.5,2.5) #Duration in years
    return(a[stage])
  },

#Immigration & Emigration; closed system:
  immigrationFunc=function(stage,x,time,species,strain){return(0)},
  emigrationFunc=function(stage,x,time,species,strain){return(0)}
)

#Set initial population size
init.pop = matrix(0,nrow=4,ncol=1)
  init.pop[4,1] = 100 #Set starting number of adults

modelOutput = popModel(
  numSpecies=1,
  numStages=4, #Number of life stages
  ICs= list(init.pop),
  timeVec=seq(0,100,1), #Duration modeled in years
  timeDependLoss=TRUE,
  timeDependDuration=FALSE,
  rateFunctions=FHM_Functions,
  solverOptions=solver.options,
  stageNames=list(c('eggs','larvae','juveniles','adults')),
  speciesNames=c('FHM')
)

if(trt.sim=="Control"){write.csv(modelOutput,"Control Population Simulation.csv")}
if(trt.sim=="PCB-1"){write.csv(modelOutput,"PCB-1 Population Simulation.csv")}
if(trt.sim=="PCB-2"){write.csv(modelOutput,"PCB-2 Population Simulation.csv")}
```