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Assessing Impacts of Land-Applied Manure from Concentrated Animal Feeding Operations on Fish Populations and Communities

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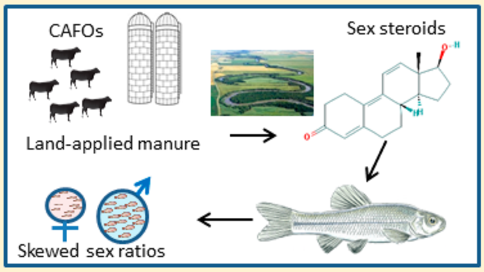
Supporting Information

ABSTRACT: Concentrated animal feeding operation (CAFO) manure is a cost-effective fertilizer. In the Midwest, networks of subsurface tile-drains expedite transport of animal hormones and nutrients from land-applied CAFO manure to adjacent waterways. The objective of this study was to evaluate impacts of land-applied CAFO manure on fish populations and communities. Water chemistry including hormone, pesticide, and nutrient concentrations was characterized from study sites along with fish assemblage structure, growth, and endocrine disruption assessed in selected fish species. Although most CAFO water samples had hormone concentrations <1 ng/L, equivalent concentrations for 17β-E2 and 17α-TB peaked at >30 ng/L each during the period of spawning, hatching, and development for resident fishes. CAFO sites had lower fish species richness, and fishes exhibited faster somatic growth and lower reproductive condition compared to individuals from the reference site. Fathead minnows (*Pimephales promelas*) exposed to CAFO ditchwater during early developmental stages exhibited significantly skewed sex ratios toward males. Maximum observed hormone concentrations were well above the lowest observable effect concentrations for these hormones; however, complexities at the field scale make it difficult to directly relate hormone concentration and impacts on fish. Complicating factors include the consistent presence of pesticides and nutrients, and the difference in temperature and stream architecture of the CAFO-impacted ditches compared to the reference site (e.g., channelization, bottom substrate, shallow pools, and riparian cover).



INTRODUCTION

Manure from concentrated animal feeding operations (CAFOs) is often applied to agricultural fields as a waste management strategy and a source of inexpensive fertilizer and/or irrigation water. Subsurface tile-drain networks are widely used in the Midwestern U.S. to lower the water table in agricultural fields. However, these networks also expedite the transport of nutrients, pesticides, and manure-borne constituents to receiving ditches and downstream waterways.[1] Manure can transfer synthetic and natural hormones to the environment.[2,3] Timing is critical for organisms exposed to hormones. Thus, even though hormones have short half-lives (days[4]), brief exposures during critical developmental periods can induce long-lasting effects.[5]

Few studies have investigated the effects of CAFO-impacted water on fish reproduction. Sellin et al.[6,7] observed a decrease in estrogen responsive genes in caged fathead minnows (*Pimephales promelas*) deployed for 7 days in a stream close to a beef CAFO. The only published field study of feral fish inhabiting streams receiving feedlot effluent demonstrated reduced reproductive fitness in male fathead minnows.[8] These studies suggest that fishes are affected by CAFO-impacted waters, although more explicit studies are needed to assess the potential biological and ecological impacts of land-applied CAFO manure.

Our objective was to further evaluate the impacts of land-applied CAFO manure on fishes. We hypothesize that sites near fields receiving CAFO manure have greater hormone and nutrient concentrations, lowered fish species richness and evidence of endocrine disruption, compared to a reference site. To test these hypotheses, we evaluated water quality including hormone, pesticide, and nutrient concentrations, fish abundance and species richness, and growth and reproductive characteristics of feral creek chub (*Semotilus atromaculatus*).

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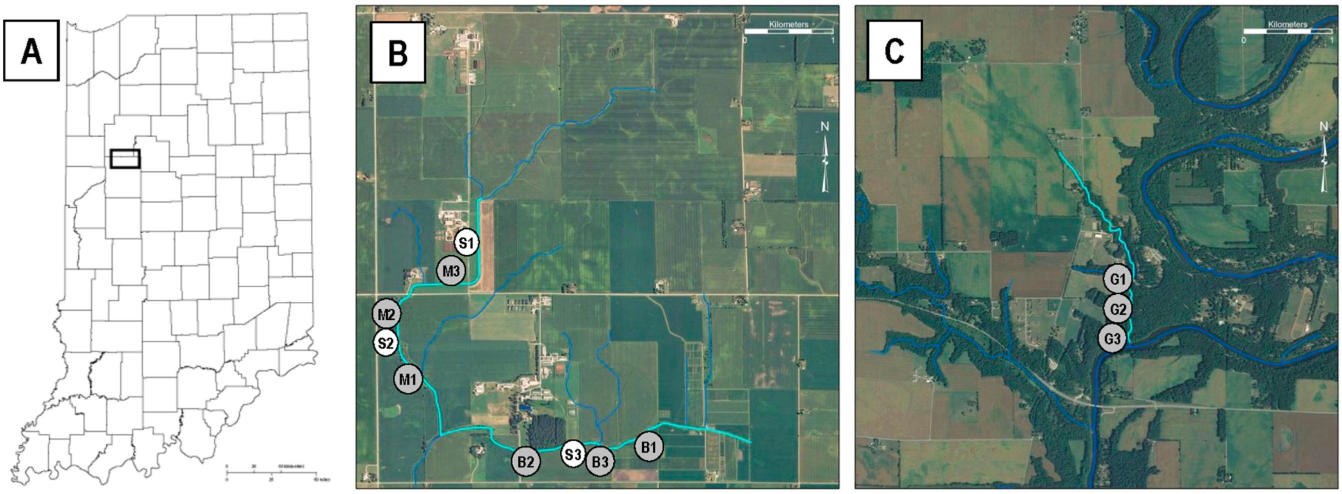

Figure 1. (A) Study sites in northwestern Indiana. (B) CAFO site at Purdue University Animal Science Research and Education Center. (C) Reference site located ~25 km NE of CAFO site, a small tributary of the Tippecanoe River (dark blue). Agriculture surrounding Marshall Ditch (M1−M3), Box Ditch (B1−B3), and water monitoring stations (S1−S3) is in contrast with forest surrounding Ghost Creek (G1−G3). Light blue = sampled waterways.

Table 1. Water Chemistry Data Showing Maximum Observed Concentrations (Max) and Potency of the Hormone Mixtures in the Ditchwater at Each Study Site during the Fish Community Study (April−October) Represented by Hormone Equivalents

		S1 (n = 261)	S2 (n = 260)	S3 (n = 218)	Ghost Creek (n = 7)
hormones	equivalency factor	max (ng/L)	max (ng/L)	max (ng/L)	max (ng/L)
17β-estradiol	1.0	6.54	20.94	5.18	0.50
estrone	0.28	13.68	40.02	9.00	0.30
estriol	0.05	6.28	12.39	<LOQ[a]	–[b]
total 17β-estradiol equivalents[c]		**10.76**	**32.95**	**7.74**	**0.59**
17α-trenbolone	1.0	9.72	19.13	1.73	–
17β-trenbolone	0.47	10.40	28.24	3.28	–
androstenedione	0.091	16.65	8.13	4.96	0.10
testosterone	0.078	50.49	15.45	8.84	–
total 17α-trenbolone equivalents[c]		**20.02**	**34.28**	**4.4**	**0.01**

[a]Below limit of quantification. [b]Below limit of detection. [c]Total equivalents = adjusted hormone concentration (ng/L). Relative Binding Affinities (RBAs) presented as percent (RBA of 100% = 1.0) used as equivalency factors. Estrogen RBAs are relative to 17β-estradiol (Denny et al. 2005). Androgen RBAs are relative to 17α-trenbolone (calculated using values from Wilson et al. 2007).

Finally, we conducted in situ exposures of fathead minnow embryos and caged adults to CAFO-impacted waters.

■ MATERIALS AND METHODS

Study Sites. Studies were conducted at two CAFO-impacted sites (Marshall and Box Ditches) and one reference site (Ghost Creek). Ditches receive subsurface tile drainage and runoff from adjacent agricultural fields located at Purdue University's Animal Science Research and Education Center, West Lafayette, IN (Figure 1B). Marshall Ditch receives subsurface tile drain discharge from fields applied with solid manure and lagoon effluent from beef and dairy CAFOs, and Box Ditch receives runoff from fields subsurface injected with swine manure and irrigated with effluent from swine and poultry waste lagoons (see Gall et al.[1] for details). Ghost Creek is a tributary to the Tippecanoe River ~25 km northeast of the CAFO sites near Brookston, IN (Figure 1C). A reference site that is agricultural but not CAFO-impacted is very difficult to identify in our study area since it is unknown what farms apply animal manure as fertilizers.

Marshall and Box Ditches are channelized agricultural streams with extensive bank erosion and sedimentation, few pools, and no riffle habitats. Base flow is low, and only a few shallow pools persist during late summer and early fall. Only one riparian canopy was present (transect M2). In contrast, Ghost Creek is a meandering stream surrounded by forest with riparian canopy throughout. Aside from a stone path used to cross the stream at transect G2, Ghost Creek has little bank erosion and sedimentation. Each Ghost Creek transect contained several pool-riffle sequences characterized by substrates ranging from sand to cobbles.

Water Quality and Chemical Analysis. Temperature (°C) and dissolved oxygen (DO, mg/L) were measured using YSI meters (Yellow Springs, OH). Water for chemical analysis was collected throughout 2009 by automated sampling stations S1, S2, and S3 (Figure 1B). These sites corresponded to Marshall Ditch transects M3 and M2 and Box Ditch transect B3, respectively. Hormones and pesticides were quantified using solid phase extraction, eluting with methanol, evaporating eluant, and reconstituted residues in methanol (0.5 mL) followed by high performance reverse-phase liquid chromatography tandem electrospray ionization mass spectrometry (HPLC/ESI-MS/MS) for the hormones (details provided in Gall et al.[1]) and GC/MS for the pesticides. Hormones included

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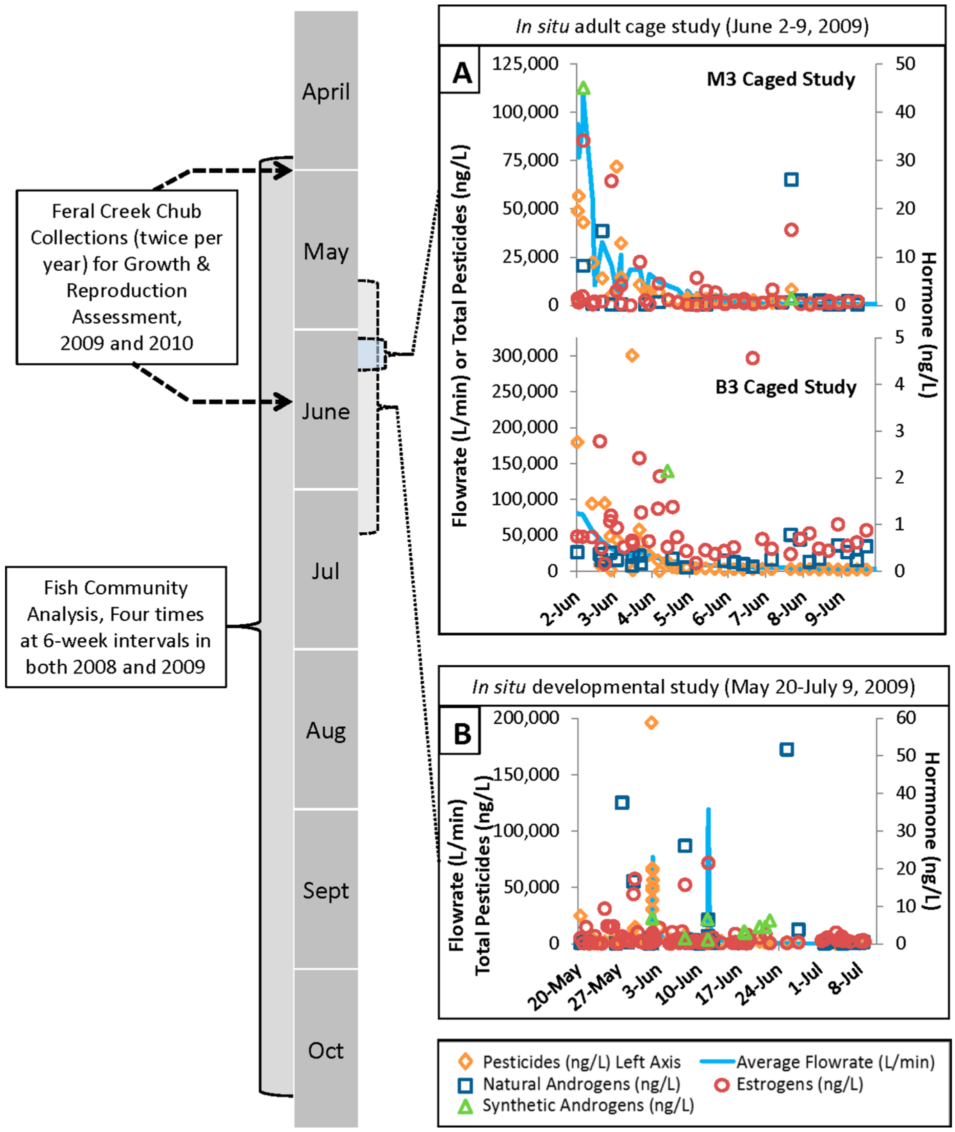

Figure 2. Timeline for studies and chemographs of total hormone and pesticide concentrations in water at CAFO sites for in situ adult cage study (A) and developmental study (B) (see Figure 1 for locations). Concentrations below limits of quantitation are not shown. Note the different concentrations units for the different chemicals.

estrone (E1), 17α-estradiol (17α-E2), 17β-estradiol (17β-E2), estriol (E3), testosterone (TST), androstenedione (AND), 17α-trenbolone (17α-TB), 17β-trenbolone (17β-TB), and trendione (TND). Pesticides included atrazine, deethyl-atrazine (DEA), and deisopropyl-atrazine (DIA). Total nitrogen (TN) and total phosphorus (TP) were determined from grab samples collected at all three sites during creek chub sampling by TN-persulfate (method 10072) and TP-PhosVer3 with acid persulfate digestion (method 8190) kits (Hach spectrophotometer, DR 2700, Loveland, CO).

Maximum observed hormone values were converted to equivalency values and summed to determine total estrogen and androgen potency at each site. Data for relative binding to fathead minnow estrogen (ER) and androgen (AR) receptors were used in these calculations because this species is closely related to cyprinids found at the study sites. Estrogen equivalency values were calculated based on binding of each estrogen relative to the binding affinity of 17β-E2 to fathead minnow ER, since 17β-E2 was the most potent estrogen detected (Table 1).[9] Androgens were converted to 17α-TB equivalents since this was the most potent androgen detected (Table 1).[10] Values for 17α-E2 and TND were not included because there is no published information on binding affinity for these hormones to fathead minnow ER and AR.

Fish Community Study. Fish abundance and diversity were assessed from three 50 m transects per site (Figure 1) using a backpack electrofisher (ABP-3, ETS, Madison, WI). Sites were sampled every six weeks from May − October 2008 and 2009 (eight sampling events). These data were used to calculate an Index of Biotic Integrity (IBI)[11] modified by Simon and Dufour[12] for the U.S. East Central corn belt.

Creek Chub Study. Creek chubs were found at all sites and thus used to examine CAFO impacts on fish growth and reproduction. Twelve creek chubs were collected from each site in late April/early May and mid-June (2009 and 2010), euthanized (300 mg/L MS-222), blotted dry, weighed (±0.01 g) and measured for total length (mm). Gonads were weighed (±0.01 g) to calculate gonadosomatic index (GSI, total gonad weight/total fish weight × 100). Sex was confirmed histologically using standard hematoxylin and eosin stain and

Table 2. Average Number of Fish Found at CAFO Sites (Marshall Ditch and Box Ditch) and a Reference Site (Ghost Creek) during 2008 and 2009

family	common name	genus species	Marshall Ditch Mean SE[a]	Box Ditch Mean SE	Ghost Creek Mean SE
Cyprinidae	central stoneroller	*Campostoma anomalum*	0.04 ± 0.01	0	7.67 ± 2.26
	spotfin shiner	*Cyprinella spiloptera*	0	0	1.83 ± 0.89
	common shiner	*Luxilus cornutus*	0.04 ± 0.04	1.58 ± 0.93	0.21 ± 0.12
	striped shiner	*Luxilus crysocephalus*	0	0	2.50 ± 1.04
	golden shiner	*Notemigonus crysoleucas*	0.67 ± 0.45	0.04 ± 0.04	0.04 ± 0.04
	sand shiner	*Notemigonus stramineus*	0	0	0.33 ± 0.21
	bluntnose minnow	*Pimephales notatus*	0.38 ± 0.19	0.58 ± 0.58	0.63 ± 0.32
	blacknose dace	*Rhinichthys atratulus*	0	0	3.04 ± 0.64
	creek chub	*Semotilus atromaculatus*	28.00 ± 7.44	8.00 ± 2.32	37.75 ± 3.81
Catostomidae	white sucker	*Catostomus commersoni*	0.67 ± 0.38	0.17 ± 0.10	1.00 ± 0.39
	creek chubsucker	*Erimyzon oblongus*	3.92 ± 0.87	1.79 ± 1.04	0.08 ± 0.08
	lake chubsucker	*Erimyzon sucetta*	3.71 ± 1.47	1.46 ± 0.87	0.17 ± 0
Catostomidae	northern hogsucker	*Hypentelium nigricans*	0	0	0.13 ± 0.09
Ictaluridae	brown bullhead	*Ameiurus melas*	0.21 ± 0.12	0.21 ± 0.17	0.08 ± 0.06
	black bullhead	*Ameiurus natalis*	0.33 ± 0.21	0.29 ± 0.13	0.25 ± 0.11
	yellow bullhead	*Ameiurus nebulosus*	0.13 ± 0.09	0.21 ± 0.13	1.13 ± 0.33
Percidae	rainbow darter	*Etheostoma caeruleum*	0	0	2.29 ± 1.03
	johnny darter	*Etheostoma nigrum*	0.96 ± 0.53	0.04 ± 0.04	0
	orangethroat darter	*Etheostoma spectabile*	1.75 ± 0.69	0.08 ± 0.08	0.46 ± 0.16
Centrarchidae	green sunfish	*Lepomis cyanellus*	1.38 ± 0.32	0.75 ± 0.37	0.29 ± 0.14
	pumpkinseed	*Lepomis gibbosus*	0.08 ± 0.06	0	0
	bluegill	*Lepomis macrochirus*	0.29 ± 0.15	0.38 ± 0.24	18 ± 0.04
	longear sunfish	*Lepomis megalotis*	0.08 ± 0.06	0.25 ± 0.17	0.04 ± 0.00
	smallmouth bass	*Micropterus dolomieu*	0	0	0.13 ± 0.09
	spotted bass	*Micropterus punctulatus*	0.17 ± 0.10	0	1.71 ± 0.76
Cottidae	mottled sculpin	*Cottus bairdi*	0	0	14.79 ± 2.74
Fundulidae	blackstripe topminnow	*Fundulus notatus*	0	0.13 ± 0.13	0.04 ± 0.04
Esocidae	grass pickerel	*Esox amaricanus*	1.13 ± 0.36	0.63 ± 0.19	0
species richness			**4.92 ± 0.43**[c]	**3.08 ± 0.54**[c]	**9.41 ± 0.66**
IBI total[b]			**17.83 ± 0.80**[c]	**17.58 ± 0.61**[c]	**29.50 ± 1.28**

[a]SE = Standard error. [b]IBI = Index of Biotic Integrity. [c]Denotes significant difference from Ghost Creek ($p < 0.05$).

reproductive stage determined (Supporting Information (SI) Figure S1).[13] Age was determined using polished otoliths mounted on glass slides.[14]

In Situ Adult Caged Study (7 Day). Adult fathead minnows (4–6 months) were caged and expression of hepatic vitellogenin (*vtg*) quantified. Fish were obtained from the U.S. Environmental Protection Agency (USEPA) Cincinnati, OH and were exposed in situ at the CAFO (nine males, nine females deployed at M3 and B3) and reference (eight males, nine females at G2) sites from June 2–9, 2009 (for locations see Figure 1). Fish were placed in plugged minnow cages according to sex (one male and one female cage/site). Cages were secured to the stream bottom using rebar to allow for natural feeding during the trial. Cages were retrieved after 7 days and fish were processed as previously described for creek chubs.

Prior to cage deployment, a random group of minnows were sampled and used as controls for gene expression and histology comparisons. To assess changes in *vtg* expression, total RNA was isolated from liver using TRI Reagent (Molecular Research Center, Cincinnati, OH). Reverse-transcription quantitative polymerase chain reaction (RT-QPCR) quantification procedures and *vtg* expression calculations were performed as described in Biales et al.[15]

In Situ Developmental Study (~6 weeks). Fathead minnow embryos (<24 h post fertilization, hpf) were exposed in situ to CAFO ditchwater followed by histological analyses. Minnows from USEPA Cincinnati, OH were spawned at the Baker Aquatic Research Laboratory (ARL), Purdue University. Water from Marshall Ditch was pumped into a flow-through system of 10 L tanks kept at 26 °C (±2 °C) in a water quality station (S1) adjacent to the ditch (Figure 1B) from May 20 to July 9, 2009. Well water was pumped into a corresponding

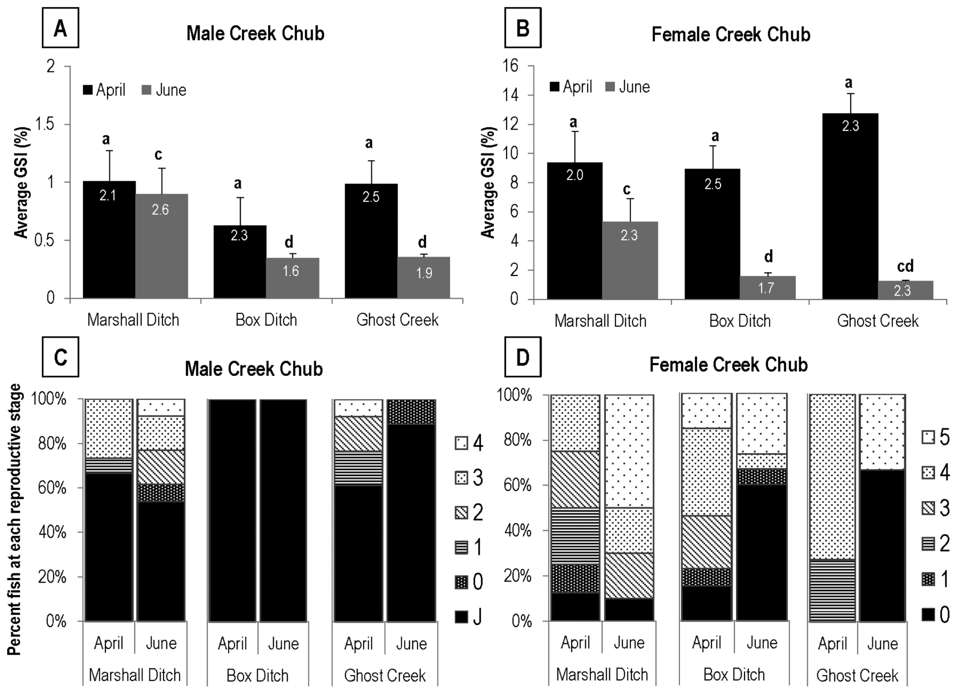

Figure 3. (A,B) Mean ± SE of gonadosomatic index for creek chubs collected late April/early May during spawning (April) and mid-June after spawning (June), with average ages of fish presented in bars. Significant differences in GSI within each month are noted by different letters (*t* test, $p < 0.05$); letters a,b for April, letters c,d for June. (C,D) Percentage of corresponding gonad stages for each collection.

flow-through system kept at 24 °C (±1 °C) inside the ARL. One clutch of eggs still attached to the breeding substrate was placed in each tank (control $N = 5$; exposed $N = 8$). All but 50 eggs were removed from the substrate after eggs eyed. After hatch, fish were fed freshly hatched *Artemia ad libitum* twice a day. At 40–45 day post fertilization (dpf) fish were euthanized and measured as previously described and the midsection fixed for histological sex determination (10–25 fish/tank, 250 fish total).[16]

Data Analysis. Data analyses were performed using JMP 8 and SAS 9–2. Fish species richness, IBI, GSI, and water quality variables were compared across sites using analysis of variance (ANOVA) followed by a post hoc analysis of significance (Tukey). Analysis of covariance (ANCOVA) was used to detect site effects on creek chub growth rate (age vs length). Correlation between creek chub GSI and gonad stage was analyzed by a generalized linear model. Sex ratios were compared across treatments using a chi-square test.

RESULTS AND DISCUSSION

Water Quality and Chemical Analysis. Water temperatures during collection of grab samples in April and June were significantly higher at Ghost Creek than the ditch sites ($P < 0.0001$), with Box Ditch also having higher temperatures ($P = 0.0023$) than Marshall Ditch in April (SI Table S2). DO was lower at Ghost Creek in April ($P = 0.0006$), but not in June.

Maximum hormone concentrations and hormone equivalency values for water samples collected during the fish community study (April to October 2009) are summarized in Table 1. Hormone concentrations in all water samples collected (2009) are summarized in SI Table S1. The highest hormone concentrations occurred in late spring/early summer, coinciding with fish spawning/early development (Figure 2). Particularly high concentrations occurred during storm events following land-application of CAFO manure. Hormones were detected in over 80% of ditchwater samples, with E1 detected most frequently and E3 the least. Natural androgens (TST and AND) were detected more frequently than synthetic ones (17*α*- and 17*β*-TB, SI Table S1), and estrogens and androgens detected in the highest concentrations were E1 and TST, respectively (Table 1). The highest hormone equivalency values for 17*β*-E2 (33.0 ng/L) and 17*α*-TB (34.3 ng/L) were observed at S2 on June 1. In contrast, Ghost Creek water samples contained very low concentrations of natural hormones, with AND, E1, and 17*β*-E2 detected only once and no synthetic androgens detected (Table 1). There was a trend of lower TN and TP concentrations measured from grab samples at Ghost Creek compared to the ditches, but it was not statistically significant (SI Table S2). Atrazine concentrations ranged from 0.37 ng/L at the reference site to >200,000 ng/L at the CAFO sites (SI Table S1).

See Figure 2 for a timeline overview and water chemistry results for the in situ experiments. In the following paragraphs, hormone concentrations in water are discussed in relation to their relative hormone equivalency values. No rain occurred during the 7 days in situ cage study and hormone concentrations were correspondingly low. At Marshall Ditch (M3), 17*β*-E2 equivalency spiked the first day of deployment to 10.6 ng/L with a second spike to 4.8 ng/L midway through exposure, although it was <1 ng/L all other days. 17*α*-TB equivalency was 31.7 ng/L the first day of deployment. At Box Ditch (B3), 17*β*-E_2 and 17*α*-TB equivalency was <1 ng/L all days, except one day during midexposure when 17*β*-E_2 equivalency reached 1.5 ng/L.

See Figure 2B for total hormone concentration summary at Marshall Ditch during the in situ developmental study; equivalency values discussed below. There were several spikes in hormone concentrations coinciding with critical days during gonad development (14–24 dpf) for the fathead minnow. The maximum 17*β*-E2 equivalency value was 8.3 ng/L between 0–

10 dpf depending on when egg clutches were added to tank replicates (May 26−30). Spikes in 17β-E2 equivalents to 6.9 and 9.3 ng/L occurred between 10−18 and 14−22 dpf, respectively (depending on tank replicate), but were ≤2 ng/L for all other days. The maximum 17α-TB equivalency was 9.7 ng/L between 0−12 dpf depending on tank replicate (May 29−June 1). With exception of spikes to 3.1 and 4.0 ng/L 17α-TB equivalents at 10−18 and 28−36 dpf, respectively, values were ≤1 ng/L all other days during this study.

Fish Community Study. Creek chub were the most abundant fish species at all sites. Various bullhead, sucker, and sunfish species were also common among all sites, although less consistently and in far lesser abundance than creek chub. Additional abundant species at Ghost Creek were bluegill (*Lepomis macrochirus*), mottled sculpin (*Cottus bairdii*), and central stoneroller (*Campostoma anomalum*). Many of the species present at Ghost Creek, including fluvial specialists like mottled sculpin and central stoneroller were absent at Marshall or Box Ditches (Table 2). Species richness and IBI were significantly higher at Ghost Creek compared to both ditches ($P < 0.0001$).

Creek Chub Study. Creek chubs from the CAFO sites grew at a faster rate than those from Ghost Creek ($P = 0.03$; SI Figure S2). Differences in male GSI across sites were only significant during June with higher values at Marshall Ditch than Box Ditch ($P = 0.04$) and Ghost Creek ($P = 0.03$) (Figure 3A). A similar trend was observed in females, with significantly higher GSI in Marshall Ditch ($P = 0.007$) (Figure 3B). Histological examination revealed that all males from Box Ditch, even those sampled during the spawning period (May) and over two years of age had immature testes (Figure 3C,D). There was a significant correlation between gonad stage and GSI ($P < 0.0001$).

In Situ Adult Cage Study (7 Days). Survival of minnows was lower in the ditches (Marshall males 67%, females 89%; Box males: 44%, females 89%) vs Ghost Creek (100%). At the end of the 7 day deployment, GSIs were low (i.e., < 2.0%) and comparable to day 0 for all three sites. In both exposed fish and day 0 fish female gonads were at stage 4 or 5, and males were stage 3 or 4.[13] No changes were observed in hepatic *vtg* expression in females, with a mean expression (normalized with 18S ± SE) of 64.2 ± 12.6 at day 0, and 53.6 ± 13.1, 73.4 ± 10.9, and 49.6 ± 15.3 at day 7 for Ghost Creek and Box and Marshall Ditches, respectively. No *vtg* induction was observed in any of the males.

In Situ Developmental Study (~6 Weeks). Survival (±SE) was 64.8 ± 6.0% in the exposed group ($N = 8$) and 74.8 ± 5.3% in the control group ($N = 5$). Mean (±SE) weight (exposed = 278 ± 98 mg, control = 148 ± 23 mg; $P = 0.001$) and length (exposed = 30.9 ± 0.6 mm, control = 25.3 ± 1.2 mm; $P = 0.0006$) were significantly higher in the exposed vs control group. Fathead minnow broods typically have a 1:1 sex ratio, but there was a significant skew toward males in minnows exposed to ditchwater during development (60.4 ± 3.3%; $P = 0.01$) compared to 48.7 ± 3.9% males in the controls.

Natural and synthetic hormones were present in most CAFO-impacted ditchwater samples at low concentrations (low ng/L) comparable to other similarly influenced surface waters.[17,18] However, total hormone equivalents at maximum observed concentrations reached 33.0 ng/L 17β-E2 and 34.4 ng/L 17α-TB equivalents. This 17β-E2 concentration is over 3 fold higher than the proposed LOEC in juvenile *Gobiocypris rarus* exposed to ≥25 ng/L 17β-E_2. The only study to date to evaluate the effects of 17α-TB in fish reported changes in plasma levels of VTG and TST along with the appearance of male secondary sex characteristics in female fathead minnows after 21 day exposure to 30 ng/L 17α-TB.[19] Zebrafish (*Danio rerio*, Cyprinidae) exposed as juveniles for 21 days to 9.7 ng/L 17β-TB (less potent but similar mechanism as the α isomer) resulted in 100% males.[20] Therefore, the equivalency values for total estrogens and androgens found in our CAFO-impacted ditches were above LOECs and present risk of potential endocrine disruption in fish. The only estrogens detected at Ghost Creek were E1 and 17β-E2, and concentrations were comparable to the few reports of naturally occurring hormone levels in surface waters (<1 ng/L).[1,3]

Atrazine concentrations in our study fell in the range seen in other surface waters.[21] Atrazine has been shown to disrupt neuroendocrine function.[22] However, a recent study observed no impacts on sexual development in zebrafish with atrazine exposure at environmentally relevant concentrations.[23] It is unclear how atrazine in mixture with other chemicals, as seen at our CAFO sites, may impact sexual development and reproduction.

We found evidence of reduced reproductive capacity in creek chubs at the CAFO sites. Fish from Marshall Ditch exhibited higher GSIs and more varied gonad stages during June compared to the other two sites. Since GSI is lower after spawning and the second fish collection occurred late in the spawning season for creek chubs, this indicates spawning may have been limited or not occurred in these fish. This, combined with evidence of increased somatic growth at the CAFO sites, suggests these fish may be directing excess energy to somatic and not gonadal growth and reproduction. Whether this was due to the presence of hormones, other chemicals (e.g., pesticides, nutrients), or simply to the lack of spawning habitat, remains unknown. Deegan[24] reported decreased expression of gonadal aromatase (*cyp19a*), the enzyme responsible for aromatizing androgens into estrogens in female chubs from agricultural ditchwaters in the Cedar Creek watershed in northeast Indiana. Deegan[24] also collected intersex creek chubs from agricultural ditches in this study.

Creek chubs reach sexual maturity at age 2 or 3.[25] Although age 2−3 males were sampled from Box Ditch during April, all had undeveloped testes. Orlando et al.[8] reported smaller testis size in feral fathead minnow collected from a stream receiving feedlot effluent. Gonadal recrudescence is known to be induced by increased water temperatures. Although water temperatures at Marshall and Box ditches were lower compared to Ghost Creek, they were sufficiently high during the first sampling event (>14 °C) to induce spawning in this species.[26]

Lower fish species richness and IBI at the CAFO sites was not unexpected given the difference in quality of habitat and environmental conditions. There are many differences in the physical features of the CAFO-impacted ditches compared to our reference site (e.g., channelization, bottom substrate, shallow pools, and riparian cover) that could have also impacted reproduction in ways not captured with our data collection and experimental design. Furthermore, Sullivan et al.[27] argued that channel morphology is the most influential habitat component on fish community assemblage (i.e., lower habitat heterogeneity = lower fish diversity). Our data follow this observation, as Ghost Creek is a nonchanneled stream that provides many diets, habitats, and spawning conditions suitable for a larger number of fish species to thrive compared to the agricultural ditches. Creek chubs are generalists that can feed on different diets and thus can thrive in various environmental conditions, lending it to do well in more pristine creeks (i.e., Ghost Creek) as well as disturbed streams (i.e., Marshall and Box Ditches). Mottled sculpins are sensitive to environmental degradation and need riffles and rocky stream substrates, which are present at the reference site. The central stoneroller also needs the more pristine conditions present at the reference creek, as they feed on algae on rocks and cobble substrates. Although a few darters were collected at the CAFO sites, they were species that are able to tolerate some habitat degradation. Rainbow darters (*Etheostoma caeruleum*), a species of darter known to be sensitive to habitat degradation,[11] was only found at Ghost Creek and not the CAFO sites. The agricultural ditches do not have many riffles, are inundated with fine particle substrates, and are far from pristine due to the history of human activity and disturbance, including channelization, bank erosion, and increased nutrient loading due to runoff from adjacent agricultural fields. Habitat conditions and hormone loads found at CAFO sites may therefore be cause for concern relative to apparent reduced fish reproduction and diversity.

Sellin et al.[6] found alterations in expression of genes related to endocrine function when fathead minnows were caged and deployed in streams receiving agrichemicals for 7 days. However, this study did not correlate these effects with compounds detected at the study site. Our study detected relatively low estrogen levels during the 7 day adult fathead minnow in situ exposures, with only a slight spike on the first day at Marshall Ditch, and no changes in *vtg* expression were observed. A simple interpretation of this data would conclude a lack of "estrogenicity" in the CAFO sites. Kolok et al.[17] found no changes in *vtg* expression after deployment at a CAFO-impacted site. This was thought to be due to the reproductive stage of the fish deployed, which was similar to that of the fish deployed in our study. Androgen levels also only spiked on the first day of deployment at Marshall Ditch reaching a 17α-TB equivalency of 31.7 ng/L. If CAFO associated contaminant mixtures are having a more "androgenic" effect on aquatic organisms, other biomarkers besides *vtg* expression are likely needed. However, no robust biomarkers of androgen exposure and effects are currently available.

Since little is known about sex determination and differentiation in fathead minnows, other environmental factors could have contributed to our findings of a male skewed sex ratio with in situ exposure at a CAFO-impacted site. For example, temperature is known to influence sex ratios in many teleosts,[28] and the temperatures in our exposure tanks were much more variable compared to the control tanks due to mechanical difficulties with the field flow-through system. However, a recent study conducted by Brian et al.[29] provides evidence that temperature may not be a driving factor in fathead minnow sex determination. The masculinizing potential of synthetic androgens found at the CAFO site during our developmental study could have played a role in the male skewed sex ratios observed. In order to better evaluate the impact of CAFO associated chemical mixtures on fish sex ratios, further studies are being conducted exposing fatheads (embryos to juveniles) to a mixture of hormones and nutrients similar to that found in the CAFO sites site under controlled laboratory conditions to eliminate effects of sediment, DO, or temperature spikes.

Higher temperatures may have influenced faster growth in minnows exposed to ditch water during development compared to the control group. However, temperature differences could not explain faster somatic growth seen in feral chubs at the CAFO site due to consistently higher temperatures at the reference site. The faster somatic growth paralleled in both studies suggests that other factors are likely contributing to this growth.

Implications and Study Limitations. Our study is only the second attempt to assess feral fish health in aquatic systems associated with CAFO manure. This study combines field and in situ approaches to assess the role of steroidal hormones from land-applied CAFO manure to aquatic systems. Fish communities at CAFO sites were less diverse and lacked the presence of intolerant fish species compared to the reference creek. Reproductive condition of feral creek chub from the CAFO sites was also reduced. Since multiple environmental factors can negatively impact fish reproduction and assemblage structure, the exact role of hormone mixtures may be playing in these observations remains unclear. Our study focused on comparing CAFO-impacted sites with a typical preagriculture reference site leading to considerable habitat differences, which likely played a large role in fish assemblage differences. Another challenge we faced was lack of information on fish movement in and out of the ditch sites, making it difficult to ascertain the exact exposure feral fish had to hormones and pesticides. Due to the complex nature of fieldwork, it is difficult to directly relate hormone concentration and impacts on fish. However, we hypothesize that the presence of increased hormone concentrations at our agricultural sites is aggravating the impacts of degraded habitat on fish assemblages and reproductive condition. Most importantly, the maximum hormone loads are taking place during the spring, which coincides with fish spawning, hatching, and developing.

More studies are needed that quantify sex ratios in feral fish populations inhabiting CAFO-impacted streams. Nevertheless, this project has shown that sex steroid hormones at CAFO sites can reach concentrations above LOECs for reproductive effects in teleosts. These concentrations occur during the spawning season and early life-stage development for most fish species in the Midwest, warranting further research on the risk of land-applied CAFO manure on aquatic ecosystems. Specifically, more studies are needed that investigate impacts of environmentally relevant mixtures of sex steroidal hormones on fish at various life stages, including effects on sex determination, sex differentiation, and reproduction.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Contains additional hormone and water quality data and fish growth and gonadal histology data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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