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Airborne particulate matter collected near beef cattle feedyards induces androgenic and estrogenic activity *in vitro*

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ABSTRACT

Steroid growth promoters are commonly administered to beef cattle residing on feedyards, and a portion of these compounds are excreted in manure along with endogenous steroids. Steroids associated with aerosolized particulate matter (PM) can be transported from feedyards *via* wind. To assess potential androgenic and estrogenic activity of PM extracts, total suspended particulate samples were collected upwind and downwind of feedyards in the Southern High Plains and subjected to *in vitro* transcriptional activation assays. Androgen-mediated transcriptional activation induced by exposure to extracts from PM collected downwind of feedyards was significantly higher than that induced by exposure to extracts of corresponding upwind samples, whereas estrogen-mediated transcriptional activation was detected after exposure to upwind and downwind PM sample extracts. Detection and quantitation of metabolites of the synthetic androgen trenbolone acetate downwind, and estradiol both upwind and downwind, suggest that synthetic growth promoters contribute to observed *in vitro* activity. No significant correlations were observed, however, between individual steroid concentrations or total androgen/estrogen concentration and *in vitro* activity, indicating the contributions of additional, unquantified compounds to observed androgenic and estrogenic activity. Results indicate that steroids affiliated with feedyard PM have the potential to elicit endocrine-modulating effects.

1. Introduction

Recent studies have identified and quantified anabolic steroids in airborne particulate matter (PM) emanating from beef cattle feedyards (Blackwell et al., 2011, 2013). Most commercial U.S. feedyards house over 1000 cattle (USDA, 2013), which leads to production of large amounts of manure, the primary component of feedyard PM (Huang et al., 2013). Estrogenic and androgenic steroids are present in manure following metabolism of endogenous steroids (*i.e.*, estradiol, testosterone) and exogenous steroids (*i.e.*, trenbolone acetate), which are administered to 84.8% of cattle on large (>1000 head; USDA, 2013) feedyards as growth promoters. Presence of these steroids in PM suggests that there is potential for humans and wildlife to be exposed to these powerful endocrine disrupting compounds near feedyards. Exposure to these types of steroids can induce a wide array of reproductive, behavioral, and other endocrine-mediated effects (*i.e.*,Leet et al., 2011; Frye et al., 2012).

Exposure to estrogenic compounds in the environment has been well studied in a variety of species. Male fish exposed to ethinylestradiol alone or in combination with other estrogens in wastewater treatment plant effluent during early life stages exhibit feminization, with increased incidence of ovarian tissue in testes and decreased reproductive success as adults (zebrafish, Nash et al., 2004; roach, Tyler and Jobling, 2008). Effects are not limited to reproductive endpoints, as zebrafish exposed to ethinylestradiol exhibit decreases in function of hepatic nucleotide excision repair, as indicated by decreases in nucleotide excision repair gene expression *in vivo* (Notch et al., 2007) and a decreased capacity to repair DNA adducts *in vitro* (Notch and Mayer, 2009). Male rats exposed to ethinylestradiol *in utero* and during lactation display decreased reproductive organ weights and sperm counts as adults (Howdeshell et al., 2008). Exposure to estrogens can also lead to neurobehavioral effects, altered social and exploratory behavior in male rats (Dugard et al., 2001; Colman et al., 2009) and altered courtship behaviors in male fathead minnows (Salierno and Kane, 2009). Similarly, exposure of female rats to the phytoestrogen genistein *in utero* can lead to abnormal estrous cycles and

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decreased fecundity as adults (Jefferson et al., 2005). Increased incidence of mammary cancers in a variety of species has also been linked to exposure to estrogenic compounds alone (*i.e.*, rats and bisphenol A, Jenkins et al., 2009; humans and pharmaceutical estrogens, Hulka and Stark, 1995) or in combination with other steroids (estrogens + progestins, Beaber et al., 2014). In humans, exposure to estrogenic compounds in the environment has been linked to a variety of other pathologies, including infertility and development of endometriosis in women (reviewed in Yoon et al., 2014) and alterations in timing of puberty in boys and girls (reviewed in Roy et al., 2009).

Androgenic compounds can likewise induce a variety of deleterious effects depending on gender and age at exposure. Females in both mammalian and fish species exposed to 17β-trenbolone during early life stages demonstrate masculinization, characterized by increased anogenital distance, decrease in nipple number, and increases in genital malformations among rats (Hotchkiss et al., 2007) and increases in ovotestis and male secondary sex characteristics in mosquitofish (Sone et al., 2005). Male medaka exposed during development to 11-ketotestosterone exhibited increases in thyroid follicular hypertrophy (León et al., 2007). Adult female medaka and fathead minnow exposed to 17β-trenbolone display increased development of male secondary sex characteristics (Seki et al., 2006) and decreased fecundity (Ankley et al., 2003). Males exposed to 17β-trenbolone as adults demonstrate altered circulating hormone concentrations (fathead minnow, Ankley et al., 2003) and increased reproductive tissue weights (rat, Wilson et al., 2002a). Effects in humans following androgen disruption have primarily been associated with anti-androgenic compounds (reviewed in Luccio-Camelo and Prins, 2011).

It is clear that exposure to steroids present in the environment can result in significant effects *in vivo*. However, the effects of inhalational exposure to estrogens and androgens are less well characterized than exposure *via* ingestion, injection, or that which occurs in aquatic systems. No published data address inhalational bioavailabilty of trenbolone, though endogenous androgens and estrogens appear to be bioavailable after inhalation (testosterone, Davison et al., 2005; estradiol, Wang et al., 1999). Based on their low volatility and hydrophobicity (*i.e.*, Ying et al., 2002), steroids emanating from feedyards would be expected to be associated with PM, not free in the aerosolized or vapor form, which complicates evaluation of exposure and effects. Steroidal association with organic matter in PM may decrease exposure potential and bioavailability, making it unclear whether inhalational exposure to the feedyard PM has potential to result in androgenic or estrogenic effects. To address this question, we collected PM upwind and downwind of feedyards and used transcriptional activation assays to assess the potential for feedyard-derived PM extracts to induce androgenic or estrogenic responses *in vitro* and used LC–MS/MS to confirm steroid occurrence.

2. Methods

2.1. Filter collection

Total suspended particulate samples were collected on 4″ glass fiber filters using high volume air samplers (CF-902, Hi-Q Environmental Products, San Diego, CA) upwind and downwind of five feedyards in the Southern High Plains. Feedyards sampled in this study were meant to be representative of feedyards throughout the region, and were selected based on vehicular access to upwind and downwind sampling sites. Number of cattle on feed at the time of sampling and growth promoter usage were not determined for sampled feedyards; estimated capacity of feedyards sampled was 35,000–55,000 head. Sampling dates (April–July 2012) were selected based on ideal weather conditions for PM around feedyards (Table 1), and sampling was conducted in the evening to correspond with the daily period of peak PM (Purdy et al., 2007). Samplers were located approximately 2 m off the ground <18 m from feedyard boundaries; depending on the arrangement of facilities within the feedyard, this corresponded to distances up to 185 m from the nearest cattle for downwind samples and up to 396 m from the nearest cattle for upwind samples. Fields (grass or cropland) were present upwind of all sampled feedyards. Downwind samples (n = 4 per feedyard, collected simultaneously) were collected first at each feedyard and upwind samples (n = 4 per feedyard, collected simultaneously) were collected immediately after downwind for the same amount of time; collection times ranged between 25 and 50 min (Table 1). Following collection, filters were placed on ice for return to the lab, where they were weighed to determine PM mass and stored at −80 °C until extraction.

2.2. Extraction

In addition to feedyard collected filters, two laboratory blank filters were included with the extractions. Filters were extracted by shaking with 30 mL of methanol for 30 min at 300 rpm. Tubes were then centrifuged to remove large filter debris, methanol was transferred to a clean tube, and extractions were repeated once. Methanol aliquots were combined, passed through a 0.45 μm

Table 1
Details of PM sampling.

Feedyard		Sampling flow rate (m^3/min)[a]	Sampling duration (min)	Temperature (°C)[b]	Relative humidity (%)[b]	Wind speed (kph)[b]	Wind direction[b,c]	Nearest cattle facility[d]	Feedyard area[e]	
									Total	Pens
1	Upwind	0.69	47	26.1	13.9	25.9	212.3	4.5 km N	1.6 km^2	1.2 km^2
	Downwind	0.37		30.2	10.6	25.1	235.8			
2	Upwind	0.66	50	25.4	12.5	18.2	198.1	9.5 km W	0.9 km^2	0.6 km^2
	Downwind	0.52		31.3	8.5	23.7	211.8			
3	Upwind	0.41	43	26.4	30.3	20.0	168.9	1.4 km E	1.7 km^2	0.9 km^2
	Downwind	0.43		30.7	23.2	24.8	173.6			
4	Upwind	0.35	25	30.4	19.3	14.5	161.2	12.4 km N	1.0 km^2	0.6 km^2
	Downwind	0.28		34.4	13.5	15.3	168.2			
5	Upwind	0.34	25	28.8	27.8	15.8	126.3	4.5 km NE	1.4 km^2	0.8 km^2
	Downwind	0.25		30.2	25.2	19.3	123.8			

[a] Mean of 4 samplers.
[b] Mean of data reported every 5 min during the sampling period from the closest West Texas Mesonet station (archived data obtained from www.mesonet.ttu.edu/dailysummary.html).
[c] Wind direction expressed in degrees, with 0 equaling north.
[d] Distance and direction to nearest feedyard or dairy.
[e] Areas of feedyards estimated from satellite images in Google Maps; total area includes lagoons, feed storage, etc. in addition to cattle pens.