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SHORT-CHAIN CARBOXYLIC ACIDS FROM GRAY CATBIRD
(*Dumetella carolinensis*) UROPYGIAL SECRETIONS VARY WITH TESTOSTERONE
LEVELS AND PHOTOPERIOD

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Abstract—The uropygial gland of birds produces secretions that are important in maintaining the health and structural integrity of feathers. Non-volatile components of uropygial secretions are believed to serve a number of functions including waterproofing and conditioning the feathers. Volatile components have been characterized in fewer species, but are particularly interesting because of their potential importance in olfactory interactions within and across species. We used solid-phase microextraction headspace sampling with gas chromatography-mass spectrometry to detect and identify volatiles in uropygial secretions of gray catbirds (*Dumetella carolinensis*), a North American migratory bird. We consistently detected the following carboxylic acids: acetic, propanoic, 2-methylpropanoic, butanoic, and 3-methylbutanoic. We tested for the effect of lengthened photoperiod and/or exogenous testosterone on volatile signal strength and found a negative effect of lengthened photoperiod on the signal strength of propanoic, 2-methylpropanoic, and butanoic acids, suggesting a trade-off between their production and heightened night-time activity associated with lengthened photoperiod. Signal strength of propanoic and 2-methylpropanoic acids was lower in birds treated with exogenous testosterone than in birds treated with placebos. Sex did not affect signal strength of any of the volatile compounds.

Key Words— carboxylic acids, *Dumetella carolinensis*, gas chromatography-mass spectrometry, gray catbird, solid-phase microextraction, static headspace sampling, uropygial gland, volatile organic compounds.

1. Introduction

Although researchers traditionally have assumed that chemically-mediated interactions in birds are negligible, recent studies have shown their importance within and among bird species (see reviews by Hagelin and Jones, 2007; Rajchard, 2007; Balthazart and Taziaux, 2009). In a variety of avian species, scent has been hypothesized to function in courtship and mate selection (Hagelin et al., 2003; Hagelin, 2007), defense against ectoparasites (Douglas et al., 2001) and mosquitoes (Douglas et al., 2005), defense against predators (Burger et al., 2004; Reneerkens et al., 2005), nest and partner location (Bonadonna and Nevitt, 2004), parenting behaviors (Whittaker et al., 2009), and individual recognition (Bonadonna et al., 2007).

In many bird species, odorants originate in the uropygial or preen gland (Jacobs and Ziswiler, 1982). Located at the base of the tail, this exocrine gland produces waxy secretions that are applied to the feathers during preening. The nonvolatile secretions have long been thought to condition and waterproof feathers (but see Jacobs and Ziswiler's 1982 review), as well as regulate microbes (Shawkey et al., 2003; Reneerkens et al., 2008) and ectoparasites (Jacobs and Ziswiler, 1982).

Uropygial gland secretions vary qualitatively and quantitatively across avian species (Haribal et al., 2005; Haribal et al., 2009) and through time within species (Haribal et al., 2005; Reneerkens et al., 2008). If secretion components are acquired passively through food, interspecies variation may simply result from variation in diet (Sandilands et al., 2004). Alternatively, they may be the product of selective pressures such as those exerted by arthropod or microbial ectosymbionts and

other ephemeral environmental factors that adversely affect feathers. Moreover, because secretions enhance feather condition—which is important in mate choice—sexual selection may also result in variation in secretions between sexes (Piersma et al., 1999).

Uropygial secretions also vary with season. Soini et al. (2007) found variation in some volatile components of uropygial secretions between dark-eyed juncos (*Junco hyemalis*) maintained in captivity under breeding and nonbreeding photoperiods. They suggested that increased production of linear alcohols might reflect seasonal changes in the need for microbial and predator defenses. They also found increased variation of volatiles between sexes and among individuals during the breeding season. Subsequent work by Whitaker et al. (2009) on juncos during the breeding season reveals a possible role of volatiles in parental care as well. Soini et al. speculated that linear alcohols detected in the junco may be a by-product of lipid biosynthesis, and in turn be used by conspecifics as signals of reproductive condition. Martín-Vivaldi et al. (2009) found strong seasonal variation in the uropygial secretions of European hoopoes (*Upupa epops*); in subsequent work (2010), they discovered that secretions from nestlings and breeding females included antimicrobial volatile components produced by symbiotic bacteria that provide protection against feather-degrading bacteria. Bisson et al. (2009) found that the plumage microbial community of nearctic migratory birds varies with phase of the annual cycle. They suggested that, among other factors, this difference may be due to seasonal changes in the composition of uropygial secretions.

In this study, we identify short-chain carboxylic acids, the most abundant volatiles in captive gray catbird uropygial secretions, for use in future bioassays of interspecific interactions with arthropods. We also examine the influence of two seasonal variables, hormones and photoperiod, on the abundance of these compounds. The gray catbird, (*Dumetella carolinensis*) is a Nearctic-Neotropical migratory songbird that provides a useful model for the study of the effect of seasonal factors. Migratory activity in captive catbirds can be initiated by manipulation of photoperiod (Gwinner 1986; Berthold 2001) and exogenous hormone implants have been used to elevate testosterone levels in males to that observed in free-ranging breeding birds (Ketterson et al., 1991). We predicted that if volatiles function in intraspecific communication or in protection from predators or pests, they may be costly to produce, and therefore should vary seasonally with changes in sex hormone levels and migratory activity associated with lengthened photoperiod. We also tested for the effect of sex on volatile production to test the prediction that, if important in sexual selection, other forms of natural selection, or aspects of behavior, volatiles will vary between males and females.

2. Materials and Methods

2.1 Bird Handling

We captured catbirds in July and August 2006 at three sites in north-central Ohio: Carlisle Reservation, Lorain County Metro Parks (41°17'N, 82°8'W), with woodland, old field and recreated wetland habitats, located in LaGrange, Ohio; Firelands Scout Reservation (41°16'N, 82°20'W), with old field and woodlands, located in Lorain County five miles west of Oberlin, Ohio; and Killbuck Marsh Wildlife Area (40°41'N, 81°58'W), in Wayne County, Ohio. Birds were captured in mist nets, banded with a unique color band combination, weighed, and

examined for ectoparasites. All birds were housed in individual cages in the Oberlin College Animal Care Facility and fed *ad libitum* as described below.

Catbirds were transported to University of Southern Mississippi in September 2006 and housed in the University Animal Research Facility as part of a separate study on the influence of migration and testosterone on latent viral infections. For purposes of that experiment, birds were randomly assigned to experimental groups ($N = 10/\text{group}$). We then randomly selected individuals from each group for analysis of uropygial secretions (sample size in parentheses). The groups were defined as follows, where designation “migration” refers to the groups experiencing lengthened photoperiod and “testosterone” refers to groups receiving exogenous testosterone via implant: male, testosterone migration ($N = 5$); male, testosterone non-migration ($N = 5$); male, placebo migration ($N = 4$); male, placebo non-migration ($N = 6$); male, non-implanted non-migration ($N = 4$); and female, non-implanted non-migration ($N = 7$). In January 2007, birds in the migratory group were photoadvanced 30 minutes each day for 8 days until they were at a 16:8 light:dark (L:D) photoperiod. The ‘non-migratory’ group was kept at a 12:12 L:D photoperiod. The resulting activity was measured by infrared motion sensors via a data logger (JoAC Elektronik) and activity analysis software (NI LabVIEW, National Instruments, Inc.). For the testosterone treatment, males were implanted with silastic tubing (ID 1.47mm, OD 1.96mm, length 25mm) packed with crystalline testosterone (Androsten-17B-ol-3-one, Sigma Chemical, St. Louis, MO), and sealed with silicone gel. This amount of testosterone elevates plasma testosterone to levels comparable to free-ranging, breeding male catbirds (Owen, unpublished data). Control birds received a placebo implant. All implants were inserted into the birds under sterile conditions. Hormonal assays are described in Owen et al. (2010). Birds were housed in individual cages equipped with infrared sensors to monitor migratory activity and fed *ad libitum* a semi-synthetic diet consisting of meal worms, blackberries, blueberries, wheat and malted barley cereal, moistened ZuPreem monkey biscuits, cottage cheese, freeze-dried crickets, and a vitamin supplement. Body condition was monitored bimonthly by weighing and by assessing the extent of fat (Helms and Drury, 1960) and muscle (Bairlein et al., 1995) stores. Birds were euthanized with carbon dioxide and necropsied in March 2007, at which time uropygial glands were collected and frozen at -80°C until sampling.

2.2 Sample Collection

For each analysis, a uropygial gland was removed from cold storage and a sterile blade was used to dissect the uropygial lobe from the gland. The lobe, which contains the secretory tissue, was identified by its yellow color and oily appearance. Forceps were used to press the gland fragment across the inner walls of a 1.8-ml vial that had been baked at 110°C along with its septum cap for at least 1 hr before sampling. Approximately 6 mg of uropygial secretion was collected from each gland by this method (mean \pm SD = 0.006 ± 0.002 g, $N = 31$). Sealed vials containing secretion were placed into a heating block maintained at 44°C (the body temperature of gray catbirds) to equilibrate volatile chemicals in the headspace. A SPME fiber (75 μm with Carboxen-PDMS coating) was exposed to the headspace for 90 min, after which the fiber was retracted and the adsorbed chemicals were analyzed by GC-MS.

To confirm that the secretions collected as described above are representative of those applied to bird feathers, we compared them to secretions obtained from 1) the uropygial gland of a live, captive catbird and 2) an intact dissected gland of a euthanized catbird. In both cases, the gland

was exposed and gently massaged until a drop of fluid was observed on the end of the papilla. A 1-ml tuberculin syringe was then used to transfer the secretion to a vial.

2.3 Chemicals and Supplies

The following chemicals were used as GC-MS standards: formic acid (96%), 2-methylpropanoic acid (99%), butanoic acid (99+%), 2-methylbutanoic acid (98%), 3-methylbutanoic acid (reagent grade), 2,2-dimethylpropanoic acid (99%), 2-methylpentanoic acid (97%), 3-methylpentanoic acid (99%), 4-methylpentanoic acid (99%), hexanoic acid (99.5+%) and octanoic acid (98+%) (Aldrich Chemical, Milwaukee, WI); acetic acid (glacial) (Acros Organics, Geel, Belgium); propanoic acid (certified) (Fisher Scientific, Pittsburgh, PA); pentanoic acid (technical) (Eastman Chemical, Kingsport, TN); heptanoic acid (99+%) (Fluka Chemical, Milwaukee, WI). Solid-phase microextraction holders and fiber assemblies were from Supelco (Bellefonte, PA).

2.4 Chromatographic Methods

Separation and identification of volatiles from uropygial samples used a Trace GC gas chromatograph equipped with a Polaris Q quadrupole ion trap mass analyzer (Thermo Finnigan, Waltham, MA). Thermogreen LB-2 septa from Supelco were used in the injection port. Fibers were exposed for 10 s in a 225°C injection port to desorb volatiles. Injection was in splitless mode with a 1 ml/min flow of helium. The gas chromatograph contained a polar AT-WAX column (15 m length, 0.25 mm ID, 0.50 µm film thickness; Alltech, Deerfield, IL.) The column was held at 50°C for 1 min then ramped at 15°C/min to a final temperature of 200°C. Detection was performed in full-scan, positive ion mode over a mass-to-charge ratio (*m/z*) range of 33 to 350. Initial identifications were made by searching a built-in NIST MS library. Retention times and mass fragmentation patterns were then confirmed by SPME/GC-MS analysis of the pure compounds. Integrated peak areas from the total ion chromatograms were subjected to the statistical analyses used for inter-group comparisons.

After each analysis, the SPME fiber was cleaned by exposing it in the heated GC injection port for at least 10 min. Prior to incubation of the fiber with any sample or standard, a blank injection was done to confirm that material from previous analyses was not carried over.

2.5 Statistics

Hormone data were analyzed using mixed between-within-subjects ANOVA. Within-subject variables (repeated measure) were: days of sampling, pre-implant, 3 days post-implantation (DPI), 9 DPI, and 14 DPI. The between-subject blocking factor was group (testosterone or placebo). We met all critical assumptions for these tests. A *P*-value of ≤ 0.05 was considered significant. All analyses were performed using SPSS 15.0 (SPSS 2004).

We conducted a Kruskal Wallance ANOVA to examine the overall effect of migration and testosterone on relative abundance of all carboxylic acids detected. We then tested for the effect of sex, lengthened photoperiod, and exogenous testosterone with a Mann-Whitney-U (SPSS 15.0, 2004). Values were considered statistically different at $P \leq 0.05$.

3. Results

Through SPME sampling and GC-MS we identified the following volatile carboxylic acids: acetic, propanoic, 2-methylpropanoic, butanoic, and 3-methylbutanoic (Fig. 1). In chromatograms with high signal-to-noise ratios, we also identified pentanoic, 2-methylpentanoic, and hexanoic acids. Longer exposure of the SPME fiber in the injection port consistently enabled the identification of these less volatile acids. Although desorbing analytes for 60 s enhanced signal intensity, it also broadened the chromatographic peaks so that propanoic acid and 2-methylpropanoic acid were not resolved; butanoic acid, 3-methylbutanoic acid, and pentanoic acid were also unresolved. Secretions collected from the uropygial gland of a live catbird and secretions pressed out of an intact dissected gland contained the same carboxylic acid profile as secretions collected from dissected sections of uropygial lobe. The identities of compounds detected in the uropygial secretions were confirmed by comparing their retention times and mass spectral fragmentation patterns to those of pure carboxylic acids. Retention times agreed to within ± 0.02 min. The pattern of features seen in the total ion chromatogram of uropygial secretions was not duplicated in SPME sampling of gray catbird brain, spleen, liver, or kidney tissues, confirming that the sampled volatiles are present in the uropygial secretions and are not an artifact of the sampling or analysis process.

In the experimental groups from which catbirds used in this study were randomly selected, testosterone levels in males receiving implants were significantly higher 3 DPI (mean = 9.34 ng/ml) than before implantation (mean = 0.24 ng/ml; $df = 8$, $t = -5.36$, $P = 0.001$). Testosterone levels in placebo-implanted males did not differ between pre-implant (mean = 0.80 ng/ml) and 3 DPI (mean = 2.12 ng/ml; $df = 8$, $t = -0.85$, $P = 0.420$). All birds in the lengthened photoperiod group displayed migratory restlessness. In addition, we found no effect of lengthened photoperiod on catbird testosterone levels across time ($F_{1,15} = 3.02$, $P = 0.103$).

When photoperiod treatment and testosterone treatment were examined together, we found a significant overall effect of treatment on propanoic and 2-methylpropanoic acids (Fig. 2). During subsequent pair-wise comparisons, we found that catbirds experiencing lengthened photoperiod had reduced signal strength of propanoic, 2-methylpropanoic, and butanoic acids (Table 1). The signal strengths of propanoic and 2-methylpropanoic acids were also significantly lower in catbirds that received testosterone implants than placebos (Table 2). Despite these results, we found no additive effect of migration and testosterone. We also found no effect of sex on signal strength of any of the five carboxylic acids (Table 3).

4. Discussion

We found that lengthened photoperiod and the resulting activity was associated with reduced signal strength of propanoic, 2-methylpropanoic, and butanoic acids. This observed reduction in the level of three of the five carboxylic acids is notable; although the explanatory mechanism is beyond the scope of this study, we propose two opposing mechanisms to explain these observations. Short-chain carboxylic acids are common in nature and often produced as metabolic by-products of bacteria; Martín-Vivaldi et al. (2010) demonstrated that butanoic acid is produced by symbiotic bacteria of the European hoopoe uropygial gland. If the above acids from catbird uropygial glands were also produced by symbiotic bacteria, perhaps the physiological changes associated with increased photoperiod inhibited metabolism in these bacteria, thereby resulting in the reduced production of their carboxylic acid metabolic by-

products. Moreover, if these products are antimicrobial, their altered production during migration could explain the variation in diversity of feather-degrading bacteria in nearctic migratory songbirds at different points in their annual cycle as reported by Bisson et al. (2009). Indeed, Martín-Vivaldi et al. demonstrated that butanoic acid inhibits growth of several bacterial indicator strains. Propanoic acid was also detected in the uropygial gland of the green woodhoopoe (Burger et al., 2004) and appears to be a highly effective antimicrobial agent (Berrang et al., 2006) at high concentrations. In addition, 2-methylpropanoic acid is believed to be a defensive compound in *Triatoma infestans* (Hemiptera: Reduviidae) (Cruz et al., 1995) and trace amounts of butanoic acid have been found in other Hemiptera defensive compounds (Blum, 1981). The degree to which symbiotic bacteria of the uropygial gland produce these acids in the gray catbird and their subsequent function as antimicrobials is the focus of current work.

The observed decline in volatile carboxylic acids that we report is in contrast to Soini et al. (2007) who found increased levels of linear alcohols and some longer chain fatty acids with increased photoperiod in dark-eyed juncos. They reasoned that the alcohols were the by-product of lipid biosynthesis and might function to communicate condition to conspecifics during mate choice. Why the abundance of short-chain carboxylic acids in our study does not follow this predicted pattern during lipid biosynthesis is not clear.

Alternatively, if production of these carboxylic acids is in fact costly for birds, reduced production could reflect a strategic reallocation of resources away from acid production, consistent with the concept of life history trade-offs (Sheldon and Verhulst, 1996). Although the reallocation of carbon in such a scenario is likely to be small, even the slightest reallocation of resources can be critical for a migratory songbird given the extreme energy demands of migration and associated depletion of fat reserves. In extreme cases, such a deficit can result in metabolism of muscle tissue (Berthold, 1975; Guglielmo et al. 2001). Furthermore, during migration immune function in passerines is found to be compromised (Owen and Moore 2006, 2008). Bonneaud et al. (2006) linked major histocompatibility complex (MHC) genes, which code for molecules involved in resistance to pathogens, with mate choice in house sparrows; females appear to choose males based on MHC compatibility and diversity. They go on to suggest that although the cues on which female choice is based are not fully understood, the use of odor has been documented in fish and mammals (Brown and Eklund, 1994 and Reusch et al., 2001; Wedekind et al., 1995) and might be important for birds as well. Soini et al. (2007) suggest that odorous products of MHC genes and their evolutionary precursors, CD1 genes, might be used by birds to indicate condition of potential mates. Allocation of resources away from immune function and the expression of MHC gene products during migration could also result in the reduced production of volatiles that we observed. Despite the absence of a definitive explanation, the observed decline of short-chain carboxylic acids with increased photoperiod is noteworthy and represents interesting avenues of future research.

Reduced signal strength of propanoic and 2-methylpropanoic acids in response to exogenous testosterone is more difficult to interpret. Our observations are consistent with reports that gland secretions of other bird species vary seasonally (Soini et al., 2007; Douglas et al., 2008; Reneerkens et al., 2008) in response to changes in the annual cycle, such as breeding and migration (Sandilands et al., 2004). However, Soini et al. reported increased secretion of even-numbered longer-chain fatty acids, the interpretation of which was confounded by large

individual variation. Perhaps the physiological costs of elevated testosterone impose constraints on the production of uropygial volatiles. Elevated testosterone in birds has been associated with immunosuppression (Folstad and Karter, 1992) and decreased resistance to oxidative stress (Alonso-Alvarez et al., 2007). If propanoic and 2-methylpropanoic acids are in fact produced by symbiotic bacteria in catbird uropygial glands, these symbionts might also incur a cost. Diet might also influence the components of uropygial secretions (Sandilands et al., 2004). However, because diet, and environmental conditions other than photoperiod, were constant throughout our laboratory experiment, we can exclude it as a factor contributing to the variation that we observed in this study unless testosterone treatment impaired or modified foraging behavior, a parameter not measured.

We suggest that the short-chain, low molecular weight—and consequently highly volatile—acids detected in this study may be involved in olfactory communication. Each has been found in intact uropygial glands from necropsied birds, uropygial lobe secretory tissue of necropsied birds, and in the gland secretions of live birds, strongly suggesting that they are delivered to the surface of the gland and distributed over the feathers during preening. Moreover, each acid had a distinctive pungent odor. Although analysis of these volatiles via bioassay, currently underway, is necessary to determine their biological significance in intra and interspecies interactions, the arthropod responses that they elicit in other contexts are compelling.

The relatively small number of compounds identified in this study is attributable to our sampling and analysis strategy. The goal of the present investigation was to identify volatiles secreted at high levels from catbird uropygial glands for use in future bioassays of interspecific interactions. More concentrated volatile compounds, detectable at a distance by arthropods such as mosquitoes, are of greater relevance than semi-volatiles and non-volatiles. Our use of static headspace SPME sampling (Zhang and Pawliszyn, 1993) was a result of this motivation. Headspace sampling identifies only the most volatile compounds, whereas volatiles, semi-volatiles, and non-volatiles may be detected using extraction methods. In addition, the polar GC stationary phase is most effective for polar compounds, such as carboxylic acids; analysis of the SPME adsorbates on a nonpolar DB5-MS column (Alltech, Deerfield, IL) gave very poorly resolved acid features and no other compounds of comparable abundance. The profile of chemical composition presented here is not intended to be exhaustive. Current investigations focus on more inclusive sampling methods and sampling from free-ranging catbirds at important points in their annual cycle.

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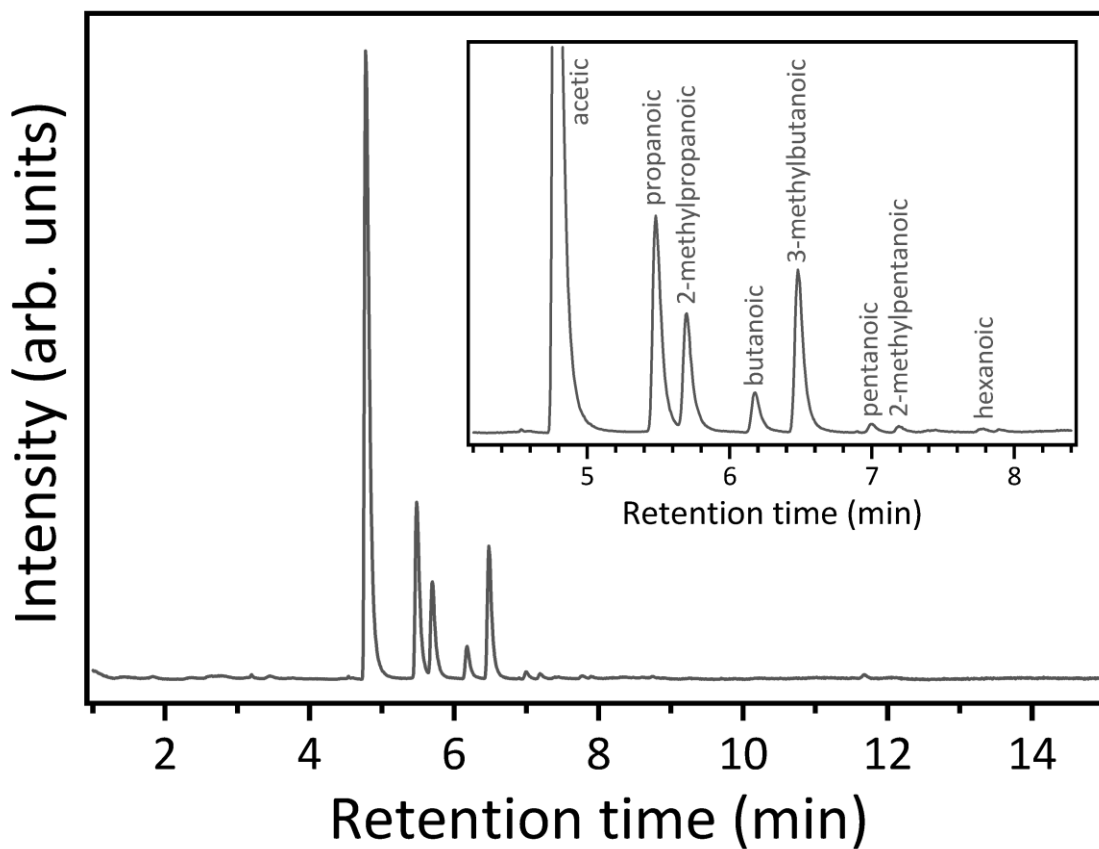


Figure 1. Representative total ion chromatogram from the SPME/GC-MS analysis of ~5 mg gray catbird uropygial secretion. Inset represents enlarged section of chromatogram containing identified compounds.

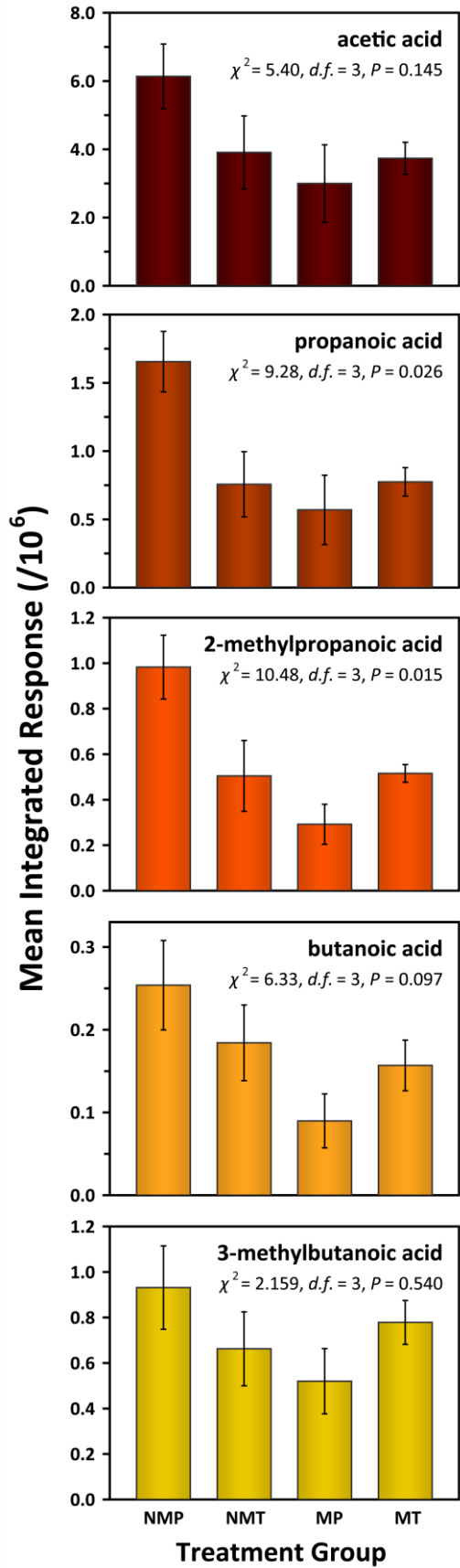


Figure 2. Overall effect of treatments (nonmigratory placebo [NMP], nonmigratory testosterone [NMT], migratory placebo [MP], and migratory testosterone [MT]) on each of the five carboxylic acids detected in uropygial secretions of male gray catbirds: acetic acid, propanoic acid, 2-methylpropanoic acid, butanoic acid, and 3-methylbutanoic acid. Values are integrated peak areas extracted from the total ion chromatograms. Error bars = +/- 1 SE.

Table 1. Effect of lengthened photoperiod on relative abundance (area mean \pm SE) of carboxylic acids in uropygial secretions of male gray catbirds with placebo implants

Compound	Migratory ($N = 4$) mean (\pm SE) /10 ⁶	Nonmigratory ($N = 6$) mean (\pm SE) /10 ⁶	Z	P-value
acetic acid	3.0 (\pm 1.1)	6.1 (\pm 0.9)	-1.919	0.055
propanoic acid	0.6 (\pm 0.3)	1.7 (\pm 0.2)	-2.345	0.019
2-methylpropanoic acid	0.29 (\pm 0.09)	0.98 (\pm 0.14)	-2.345	0.019
butanoic acid	0.09 (\pm 0.03)	0.25 (\pm 0.05)	-2.132	0.033
3-methylbutanoic acid	0.52 (\pm 0.14)	0.93 (\pm 0.18)	-0.853	0.394

Table 2. Effect of exogenous testosterone on relative abundance (area mean \pm SE) of carboxylic acids in uropygial secretions of male gray catbirds not experiencing lengthened photoperiod treatment.

Compound	Testosterone ($N = 5$) mean (\pm SE) /10 ⁶	Placebo ($N = 6$) mean (\pm SE) /10 ⁶	Z	P-value
acetic acid	3.9 (\pm 1.1)	6.1 (\pm 0.9)	-1.643	0.100
propanoic acid	0.8 (\pm 0.2)	1.7 (\pm 0.2)	-2.191	0.028
2-methylpropanoic acid	0.50 (\pm 0.16)	0.98 (\pm 0.14)	-2.191	0.028
butanoic acid	0.18 (\pm 0.05)	0.25 (\pm 0.05)	-1.278	0.201
3-methylbutanoic acid	0.66 (\pm 0.16)	0.93 (\pm 0.18)	-0.913	0.361

Table 3. Effect of sex on relative abundance (area mean \pm SE) of carboxylic acids in uropygial secretions of male and female gray catbirds not experiencing lengthened photoperiod treatment.

Compound	Males ($N = 4$) mean (\pm SE) /10 ⁶	Females ($N = 7$) mean (\pm SE) /10 ⁶	Z	P-value
acetic acid	2.1 (\pm 0.4)	3.5 (\pm 0.9)	-1.134	0.257
propanoic acid	0.60 (\pm 0.11)	0.9 (\pm 0.2)	-0.378	0.705
2-methylpropanoic acid	0.38 (\pm 0.12)	0.51 (\pm 0.16)	-0.189	0.850
butanoic acid	0.10 (\pm 0.02)	0.14 (\pm 0.04)	-0.567	0.571
3-methylbutanoic acid	0.47 (\pm 0.06)	0.8 (\pm 0.3)	-0.189	0.850