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Metabolite Profiling of Fish Skin Mucus: A Novel Approach for Minimally-Invasive Environmental Exposure Monitoring and Surveillance

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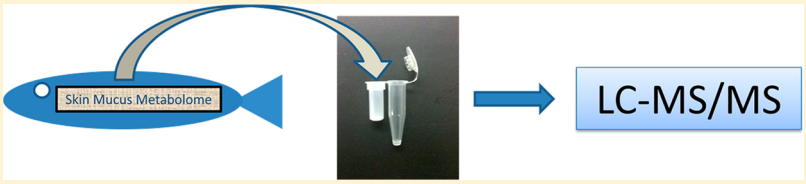
Supporting Information



ABSTRACT: The application of 'omics tools to biologically based monitoring and surveillance of aquatic environments shows considerable promise for complementing chemical monitoring in ecological risk assessments. However, few of the current approaches offer the ability to sample ecologically relevant species (e.g., fish) in a way that produces minimal impact on the health of the organism(s) under study. In the current study we employ liquid chromatography tandem mass spectrometry (LC-MS/MS) to assess the potential for skin mucus-based metabolomics for minimally invasive sampling of the fathead minnow (FHM; *Pimephales promelas*). Using this approach we were able to detect 204 distinct metabolites in the FHM skin mucus metabolome representing a large number of metabolite classes. An analysis of the sex specificity of the skin mucus metabolome showed it to be highly sexually dimorphic with 72 of the detected metabolites showing a statistically significant bias with regard to sex. Finally, in a proof-of-concept fashion we report on the use of skin mucus-based metabolomics to assess exposures in male and female fathead minnows to an environmentally relevant concentration of bisphenol A, a nearly ubiquitous environmental contaminant and an established endocrine active chemical.

1. INTRODUCTION

Aquatic ecosystems routinely receive complex mixtures of contaminants from anthropogenic sources, including effluent from wastewater treatment plants (WWTPs), agricultural operations, industrial facilities, and various nonpoint sources.[1] WWTP effluents in particular often carry a wide variety of contaminants including pharmaceuticals, industrial solvents, and personal care products. Risks posed by the introduction of such contaminants have historically been assessed primarily through the use of chemical monitoring programs, from which ecosystem health risks can be evaluated on the basis of the concentrations and measures of toxicities of individual contaminants. Unfortunately, this strategy has significant limitations, due in part to the often unpredictable toxicities such complex mixtures may pose to resident biota (e.g., fish). Moreover, nonchemical stressors (temperature changes, anoxia, salinity, etc.) often co-occur with chemical contaminants and may also present a threat to the health of impacted ecosystems. Thus, assessment of risk(s) from such complex mixtures of stressors would benefit from the use of end points able to integrate a variety of impacts and provide more meaningful data to risk assessors than available from contaminant measurements alone. In response, biologically based tools (e.g., histopathology, cell-based bioassays, biomarkers, morphology, 'omics, etc.) are increasingly being employed as a way to complement chemical monitoring efforts.[2]

Typically, small quantities of various biofluids (e.g., blood) or tissues (e.g., liver, gonad, etc.) are collected and analyzed when conducting biologically based exposure assessments with fish. However, collections of such matrices are often quite injurious or even fatal. This is an obvious drawback when carrying out large ecological studies that may involve the sampling of many individuals (and the resulting reduction of local populations), when repeated sampling of individuals over a time course is required, or when sampling the status of endangered species. Use of a biological matrix that can be collected in a minimally invasive manner, while simultaneously providing relevant information regarding stressors, is desirable. With this in mind, some researchers have explored the potential use of skin

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mucus from fish for assessing responses to various stressors. For example, Smith and Ramos[3] reported on the release of hemoglobin into skin mucus in a variety of fish species as a result of stress due to simulated capture. In a subsequent study, they reported changes in a limited number of clinical health parameters (e.g., enzymes, metals, triglycerides) in fish skin mucus that were induced by anoxia.[4] More recently, researchers have shown that fish skin mucus can be used to track adverse responses upon exposure to estrogenic compounds such as 17β-estradiol and nonylphenol via the measurement of increased concentrations of vitellogenin (the precursor for the primary egg yolk proteins) and zona radiata proteins. As a result, skin mucus has been proposed as a potential matrix for conducting minimally invasive environmental monitoring in fish.[5−7]

The ability of fish skin mucus to reflect the effects of various stresses is perhaps not surprising given the number of roles it has been shown or suggested to play in fish biology. These include the following: respiration, ionic and osmotic regulation, reproduction, excretion, disease resistance, communication, feeding, and nest building and protection.[8] The capacity to support such a wide variety of life processes requires a corresponding variety in the types of biological compounds present in the mucus. Among those compounds reported in fish skin mucus include glycoproteins (mucins),[9−11] glycosaminoglycans,[12] immunoglobulins,[13] lectins,[14] pheromones,[15] proteolytic enzymes,[16] and a number of others.[8]

Given this variety in composition and suggested roles in fish biology, we hypothesized that a rich skin mucus metabolome might be detectable and useful for chemical exposure assessment. Metabolomics, the comprehensive, nontargeted measurement of endogenous metabolites in tissues or biofluids to determine responses to various stressors or disease, has indeed proven useful for detecting and understanding a variety of biological responses in fish both in laboratory experiments and in the field.[17−21] The current study represents initial application of metabolomics to characterize fish skin mucus collected from the fathead minnow (FHM, *Pimephales promelas*). We describe a simple and inexpensive method for a minimally invasive collection of skin mucus and report on the metabolites detected using a liquid-chromatography−mass-spectrometry-based metabolomics approach. In addition, we report on the sex-specificity of the skin mucus metabolome and consequently the potential for using it to screen for endocrine disruption. Finally, as a proof-of-concept, we present the results of a laboratory exposure to an environmentally relevant level of bisphenol A (BPA),[22] a nearly ubiquitous contaminant in surface waters and a recognized xenoestrogen.[23]

2. MATERIALS AND METHODS

2.1. Experimental Design. Sexually mature (ca. 5−6 month old) FHMs from an on-site culture facility at the Duluth EPA lab were held in a mass culture prior to the exposures. To initiate the experiment, four males and four females were placed together in glass aquaria holding 10 L of Lake Superior water (4 μM filtered, UV sterilized) or BPA dissolved in Lake Superior water (4 μM filtered, UV sterilized), which was continuously renewed at a rate of about 45 mL/min. Three replicate tanks were used for each treatment condition, yielding n = 12 for each sex and treatment. The animals were held at 25 °C under a 16:8 L:D photoperiod, and were fed thawed adult brine shrimp to satiation twice per day.

A solvent-free solution of BPA (>99% pure, Sigma-Aldrich) was prepared as a concentrated stock in filtered Lake Superior water. A continuous 4 day exposure to a nominal concentration of 10 μg BPA/L was conducted, resulting in the following four treatment classes: control males, control females, 10 μg BPA/L exposed males, and 10 μg BPA/L exposed females. Concentrations of BPA in the test tanks were monitored over the course of the exposure as previously described.[24] Measured concentrations in the water agreed well with target values (average deviation less than 10%). Mean recoveries (±SD, n) of BPA in spiked Lake Superior water samples were 98% (3.34, n = 3) and the agreement among duplicate samples was 97% (1.31, n = 4). There was no BPA detected in any of the control tanks (method detection limit = 1 μg/L).

2.2. Sample Collection and Processing. There were no treatment- or nontreatment-related mortalities. At the conclusion of the 4 d exposure, the fish were anesthetized with buffered tricaine methanesulfonate (MS-222; 100 mg/L buffered with 200 mg $NaHCO_3$/L; Finquel, Argent, Redmond, WA). Fish were weighed (see Table S1 in the Supporting Information for weight data), and skin mucus was sampled by placing each fish on its side and then laying a small piece (20 mm × 5 mm) of precut glass fiber filter paper (GE Healthcare Life Sciences, Piscataway, NJ) on the side of the fish, from the caudal peduncle to the head and blotting gently to avoid contact with the epithelium (note that we have not seen any significant impacts on fish health after repeated sampling of individual fathead minnows over the course of several days when using this technique). The filter paper was then placed into a 2.0 mL microfuge tube (Eppendorf AG, Hamburg, Germany), flash frozen in liquid nitrogen, and stored at -80 °C until extracted. Blood was collected from the caudal artery/vein with a microhematocrit tubule, and plasma was isolated by centrifugation for 3 min at 15 000 rcf. All laboratory procedures involving animals were reviewed and approved by the United States Environmental Protection Agency Mid-Continent Ecology Division Animal Care and Use Committee in accordance with Animal Welfare Act regulations and Interagency Research Animal Committee guidelines.

Metabolites were extracted from glass fiber filter strips by adding 200 μL of ice-cold methanol/water (8:1) to each Eppendorf tube containing a filter strip and vortexing at 2000 rpm for 5 min. The extraction solution was transferred to an AcroPrep 96-well 0.2 μm PTFE filter plate (Pall Corporation, Port Washington, NY) and spun at 3000g for 10 min at 4 °C. The collected flow-through was transferred to a 2 mL glass HPLC-vial with a fused 300 μL conical insert and dried using a vacuum concentrator (Thermo Scientific, Waltham, MA). Samples were processed in a randomized fashion to minimize technical bias. Further sample manipulations carried out prior to LC-MS/MS analysis, the LC-MS/MS methods used, and the procedures used to determine putative annotations of detected metabolites are described in Supporting Information.

2.3. Chemometrics Analyses. Prior to multivariate analysis, tricaine methanesulfonate (the anesthetic used at the time of sample collection) and BPA (from the proof of concept exposure) were removed from the data set. We also removed four metabolites that were putatively annotated as silicic acid, selenium sulfide, *N*-acetylhomotaurine, and dideoxycytidine because we did not have confidence that they were endogenous. All remaining metabolite intensities for each fish were then normalized to unit total integrated intensity, and this normalized data set was imported into SIMCA-P+ version 13.0

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Table 1. Metabolites Putatively Annotated[a] in Fathead Minnow Skin Mucus Using LC-MS/MS

HMDB classes and constituents[b]	sex bias (M or F)[c]
Alkylamines	
butylamine[f]	–
carnitine	–
3-dehydroxycarnitine	M
pantothenic acid	M
Amino Acids and Derivatives	
2-aminooctanoic acid	–
2-aminoadipic acid	M
arginine	–
aspartic acid	–
asymmetric dimethylarginine	M
β-alanine	F
citrulline[f]	–
creatine	F
glutamic acid	F
glutamine	F
leucine[f]	–
lysine	F
methionine	F
*N*2-acetylornithine	–
N-acetylalanine	–
N-acetylarginine	M
N-carboxyethyl-*γ*-aminobutyric acid	M
N-heptanoylglycine	–
orotic acid	–
phenylalanine	F
proline	F
*N*6,*N*6,*N*6-trimethyllysine	M
tyrosine	F
valine	–
Benzoquinones	
benzoquinoneacetic acid	–
Benzoic Acid and Derivatives	
p-aminobenzoic acid	–
Carboxylic Acids and Derivatives	
azelaic acid	–
cis-2-methylaconitate[f]	–
citric acid	–
malic acid	–
N-acetylputrescine	M
Diazines	
cytosine	–
uracil	–
Dihydrofurans	
ascorbic acid	–
Fatty Acid Esters	
propionylcarnitine	–
Fatty Acids and Conjugates	
2,3-methyleneglutaric acid	F
fumaric acid	–
hydroxyisocaproic acid	–
Flavonoids	
DHDMMD[d]	–
Furans	
3-furoic acid	–
Hydroxy Acids and Derivatives	
hydroxyoctanoic acid	–
glycolic acid	–
hydroxydecanoic acid	–
hydroxyadipic acid	–
hydroxynonanoic acid	–
Imidazopyrimidines	
guanine	–
hypoxanthine	F
uric acid	–
xanthine[f]	–
Indolequinones	
indole-5,6-quinone	–
Isothiocyanates	
1-isothiocyanatobutane[f]	–
Lactams	
creatinine	M
Monosaccharides	
glucose	–
threonic acid[f]	–
Nonmetal Oxoanionic Compounds	
phosphoric acid	F
Organic Phosphoric Acids and Derivatives	
N-methylethanolaminium phosphate	F
O-phosphoethanolamine	–
phosphodimethylethanolamine	–
Oxolanes	
succinic anhydride	F
Peptides	
oxidized glutathione	F
Phenols and Derivatives	
3,4-dihydroxyphenylvaleric acid	–
butylated hydroxyanisole	F
Piperidines	
piperidine	F
Purine and Nucleosides and Analogues	
guanosine	–
inosine	F
Purine Nucleotides	
adenosine monophosphate	–
guanosine monophosphate	M
inosinic acid	F
Pyridines and Derivatives	
niacinamide	F
Pyrimidine Nucleosides and Analogues	
uridine[f]	M
Pyrimidine Nucleotides	
cytidine monophosphate	M
uridine monophosphate	M
Pyrrolidines	
2-pyrrolidone	M
proline betaine	M
pyrrolidonecarboxylic acid	–
Sugar Acids and Derivatives	
CMP *N*-acetylneuraminic acid[e]	F
N-acetylneuraminic acid	–
2-Hydroxyethanesulfonate	
taurine	–
Thienoimidazolidines	
biotin amide	–
N/A	
hydroxyheptanoic acid	–
neurine	M
No Hit in HMDB	
N-acetyl-glucosamine	M

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Table 1. continued

[a]See Materials and Methods section for description of procedure used to putatively annotate detected metabolites. [b]HMDB (Human Metabolome Database) conventions were used to identify the metabolite classes that the metabolites belonged to. [c]The sex which showed the highest statistically significant (*t*-test, $p < 0.01$) relative abundance for a given metabolite is indicated (M = control male, *F* = control female). Metabolites for which no statistically significant difference between control males and control females was detected (*t*-test, $p \geq 0.01$) are indicated by --. The sex biases reported apply to control fish only, and do not have any relationship with the responses observed for the BPA exposures. [d]DHDMMD = 5,4′-dihydroxy-3,3′-dimethoxy-6:7-methylenedioxyflavone. [e]CMP *N*-acetylneuraminic acid = cytidine monophosphate *N*-acetylneuraminic acid. [f]Metabolites for which two putative annotations were determined (see Table S2 in Supporting Information).

(Umetrics, Umea, Sweden) for multivariate data analysis. Data were mean-centered and scaled to unit variance. Principal component analysis (PCA) and partial least-squares-discriminant analysis (PLS-DA) models were constructed. No outliers were observed on the basis of a Hotellings T^2 test at the 99% confidence interval using the preliminary PCA model. The PLS-DA model (R^2(cum) = 0.803, Q^2(cum) = 0.533) was validated using 7-fold cross-validation and permutation testing. Furthermore, the optimized PLS-DA model was tested for significance using ANOVA on the cross-validated (CV) residuals, using a CV-ANOVA *p*-value of 0.05 as the threshold (specifically, the optimized model presented here was found to exhibit a CV-ANOVA *p*-value of 2.03×10^{-9}).[25] To assess sex bias in metabolite changes, we used a Student's *t*-test that compared individual metabolite responses of control males and control females. To assess the effect of BPA exposure on metabolite responses for a given sex, we also used Student *t*-tests to compare control and BPA-exposed individuals, with separate *t*-tests conducted for each metabolite and sex. Microsoft Excel 2007 (Microsoft Corporation, Redmond, WA) was used for univariate data analysis by Student's *t*-test (two tailed) assuming significance at $p < 0.01$.

2.4. Vitellogenin Analyses. Vitellogenin (VTG) concentrations were determined in blood plasma using an enzyme linked immunosorbent assay (ELISA) with a polyclonal antibody to fathead minnow VTG.[26,27] Purified fathead minnow VTG protein was used as a standard. Differences between treatments were assessed using ANOVA followed by Tukey's multiple comparisons test. Analyses were performed with SYSTAT 9.0 for Windows, and differences were considered significant at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Skin Mucus Metabolome. The diversity of functions associated with skin mucus (either demonstrated or theorized) suggests that it is a biochemically complex matrix. Indeed, as noted in the Introduction, an assortment of molecular components has been reported, ranging from glycoproteins to immunoglobulins to pheromones. Using liquid chromatography−tandem mass spectrometry (LC-MS/MS), we were able to detect and putatively annotate a large number of low molecular weight molecules (i.e., a metabolome) in skin mucus. (The term "putatively annotate" is used here to refer to metabolite identification level 2 as described in the Metabolomics Standards Intitiative.[28] See the Supporting Information for additional details.) Specifically, 204 distinct metabolites were detected in fathead minnow skin mucus using both positive (+ESI) and negative (−ESI) electrospray ionization. Of these 204, we were able to putatively annotate 84 using both the accurate mass of the parent ion (MS) and the fragmentation pattern of its daughter ions (MS/MS) in conjunction with fragmentation information available from libraries (Human Metabolome Database and LipidMaps; Table 1). The results provide a striking glimpse into the complexity of this biological matrix and present further evidence for the role of skin mucus in a variety of biological processes. While it is outside the scope of the current paper to provide a comprehensive discussion of the functional significance of the entire skin mucus metabolome, a brief commentary on some of the more abundant or noteworthy metabolites or metabolite classes putatively annotated and their potential functions in skin mucus is presented below.

Amino Acids. Twenty-six percent of all putatively annotated metabolites in the skin mucus metabolome were free amino acids and amino acid derivatives (Table 1). This finding is in agreement with earlier reports on the presence of free amino acids in rainbow trout (*Oncorhynchus mykiss*) and goldfish (*Carassius auratus*) skin mucus samples.[29,30] The results of previous studies demonstrated that these amino acids play a role in chemical communication. For example, Tucker and Suzuki[31] found amino acids in the chemical composition of the alarm substance, Schreckstoff, used by white catfish (*Ictalurus catus*). Furthermore, Bryant et al.[32] reported detecting a change in the amino acid composition of the skin mucus of brown bullhead (*Ictalurus nebulosus*) after a territorial fight. The particular amino acid profile of the epidermal mucus has also been suggested to play a role in odorant-based schooling behavior. Saglio and Fauconneau[30] found diet to contribute considerably to the free amino acid content of epidermal mucus in goldfish, suggesting a possible role in body odor, which would be expected to be similar among individuals within a school due to comparable feeding habits. Thus, these free amino acids may serve an important role as pheromones in FHM as well.

In addition to the common free amino acids, several other amino acids or closely related compounds were putatively annotated in the mucus, including those that are not normally found in proteins, but which nonetheless play important biological roles. These include trimethyllysine, β-alanine, orotic acid, asymmetric dimethylarginine, aminoadipic acid, *N*-heptanoylglycine, creatine, and acetylated versions of ornithine, alanine, and arginine. Indeed, these less-classical amino acids or amino acid derivatives add considerable metabolic complexity to fish epidermal mucus, reflecting perhaps internal or external neurological activity (e.g., aminoadipic acid),[33] pyrimidine synthesis (e.g., orotic acid),[34] and energy shuttling (e.g., creatine).

Purines, Pyrimidines, Nucleosides, and Nucleotides. Another significant proportion of the metabolites that were tentatively identified represent precursors, intermediates, or end products of purine, pyrimidine, nucleoside, and nucleotide metabolism. Almost 20% of the putatively annotated metabolites are either directly involved or closely associated with these metabolite classes. Although measured here in the skin mucus, the primary source of these metabolites may well be the skin. There is certainly evidence in mammalian skin for

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the metabolism of all four of these types of compounds. This is regarded as being the result of active synthesis and breakdown of nucleic acids, a consequence of ongoing cellular division within the epidermis.[35] Such active metabolism likely occurs within the skin of fishes as well and, therefore, may contribute to the levels observed in the current study.

There is also evidence that skin mucus biochemistry in fishes, at least in part, correlates with that of the blood. Specifically, Schultz et al.[36] reported a correlation between 11-ketotestosterone levels in blood plasma with levels found in skin mucus in Koi (*Cyprinus carpio*), and Barkowski and Haukenes reported significant correlations between VTG levels in blood plasma and skin mucus samples collected from white bass (*Morone chrysops*) during their annual spawning migration.[37] The detection of various purines, pyrimidines, nucleotides, and nucleosides in the blood of teleosts at significant levels[38] suggests that blood plasma is at least a partial source of some of these metabolites in the skin mucus. Establishing further correlations between the blood and mucus metabolomes would be advantageous as it might indicate that skin mucus could, at least in part, serve as a surrogate for blood, which is more difficult to collect and produces greater stress on fish.

Additional Functions of Annotated Metabolites. Although not as highly represented as the classes discussed above, several other putatively annotated metabolites are noteworthy. For example, a number of these metabolites have previously been associated with antimicrobial properties. These include azelaic acid (inhibits protein synthesis in bacteria),[39] *N*-acetylneuraminic acid and *N*-acetylglucosamine (products of the activity of lysozyme), and hydroxyisocaproic acid (effective against both fungal and microbial infections).[40] This is not surprising given the importance skin mucus plays in the immune system. For example, Rajan et al.[41] reported that roughly 28% of the proteins in Atlantic Cod mucus are associated with the immune system.

Lipids are another class of metabolites that may serve a protective role in the skin mucus of fish.[42] Several free fatty acids were detected in the skin mucus metabolome in the current study, including the following: hydroxyheptanoic acid, hydroxynonanoic acid, hydroxyadipic acid, hydroxyoctanoic acid, and hydroxydecanoic acid (Table 1). In addition, azelaic acid (a dicarboxylic acid, as mentioned above), aminooctanoic acid (an amino fatty acid), and 2,3-methyleneglutaric acid (a branched fatty acid) were detected. Beyond providing a protective barrier, it has also been proposed that lipids (phospholipids and cholesterol specifically) may play a role in mucus viscosity or more generally in maintaining the internal structure of the mucus through interactions with glycoproteins.[42]

Additional studies will no doubt shed further light on the functional context of many of the metabolites that were putatively annotated, and suggest their utility as effective biomarkers of health status. Regardless, identification of such a diverse mixture of metabolite classes is encouraging as it suggests that the skin mucus metabolome can provide information on a variety of physiological functions, making it potentially useful for measuring responses to a wide array of environmental stressors.

3.2. Sex Specificity of the Skin Mucus Metabolome. The ability to detect sex specific responses to environmental exposures is critical for accurately assessing ecological risk. An obvious example is for the assessment of endocrine active compounds, which often pose greater risks to one particular sex (e.g., feminization of male fish exposed to 17α-ethynylestradiol) and can lead to population-level impacts at relatively low water concentrations.[43] Thus, we assessed the sex specificity of the skin mucus metabolome. Using principal component analysis (PCA), we observed a complete separation between control male and control female fish along component one (p-value = 6.71×10^{-10} for a two-tailed t-test of component one score values) with intrasex variation primarily captured along the second component (Supporting Information Figure S1). The putatively annotated metabolites responsible for this sexual dimorphism are listed in Table 1. Of the 204 detected metabolites, 72 displayed statistically significant differences based on pairwise t-tests (p-value <0.01) between control males and control females. A cursory assessment of metabolites found to be discriminatory shows some alignment with the results of previous studies examining sex differences in human blood serum. For example, carnitine is a metabolite widely recognized for playing a primary role in energy production via the transport of acyl groups from long-chain fatty acids into the mitochondrial matrix for β-oxidation.[44] We found that carnitine was increased in the skin mucus of male fish by 46% relative to that of females; however, the p-value for this increase (1.6×10^{-02}) was slightly above our predetermine threshold of significance (1.0×10^{-2}). β-Oxidation of fatty acids is a highly active process in skeletal muscle tissue and thus is typically associated with higher levels in males of multiple species. Indeed, various studies in humans have reported measuring higher levels of carnitine in the blood serum of males.[45−47] The various modified forms of carnitine have also been shown to be increased in males. For example, Mittelstrass et al.[48] reported serum levels of various acylcarnitines in humans to be significantly higher in males. The acylcarnitine, 3-dehydroxycarnitine, was determined to be discriminatory in the current study, supporting a similar sex discriminatory role for acylcarnitines in fish. This, in combination with its high level of statistical significance in the current study (p-value $=3.49 \times 10^{-11}$), suggests that it has considerable biological relevance with regard to sex.

Another metabolite of note, creatinine, was also identified as discriminatory in our analysis (p-value = 5.79×10^{-7}). Similar to carnitine, higher levels of creatinine are also associated with greater muscle mass and thus biased toward males.[49] James et al.[50] looked at creatinine levels in humans as a function of sex, reporting higher levels in both blood serum (21%) and urine (33%) of males. Thus, it appears that the discriminatory quality of creatinine in the current study likely fits within the context of what has been reported for mammalian systems. Further analysis of the skin mucus metabolome will undoubtedly provide additional insights into its sexual dimorphic nature and likely aid in the identification of metabolite markers of high diagnostic utility for tracking sex specific responses to environmental stressors. Indeed, the striking sexual dimorphism observed in the skin mucus of FHM suggests that this approach may have other applications as well, including the nondestructive determination of sex in nonsexually dimorphic species or for differentiating sexes at early life stages prior to the appearance of secondary sexual characteristics.

3.3. Impact of BPA Exposure on the Skin Mucus Metabolome. Responses to a waterborne BPA exposure (10 μg/L) were assessed to determine the sensitivity of the skin mucus metabolome to a chemical exposure. A validated four class PLS-DA model with control females, BPA-exposed females, control males, and BPA-exposed males shows a

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measurable response to BPA exposure for both male and female fish (Figure 1). Having observed that there was indeed a

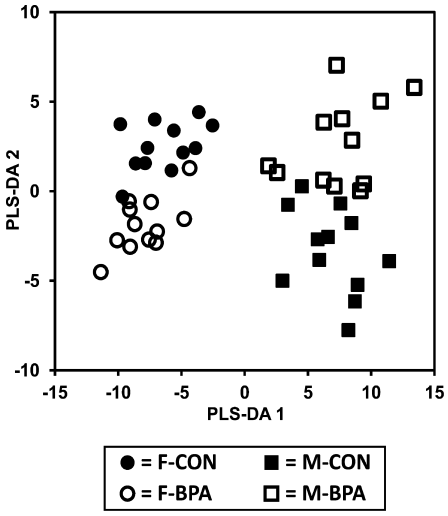

Figure 1. Scores plot from the validated partial least-squares-discriminant analysis (PLS-DA) model (CV ANOVA p-value = 2.03 × 10^{-9}) of all classes. Female controls (F-CON) are indicated by closed circles (●). Male controls (M-CON) are indicated by closed squares (■). Female BPA-exposed (F-BPA) are indicated by open circles (○). Male BPA-exposed are indicated by open squares (□).

response to the exposures, we then employed *t*-tests to determine the statistically significant metabolite changes (Supporting Information Table S2). Of the 204 metabolites detected in skin mucus, 11 showed altered levels in response to BPA exposure (p-value < 0.01) in the males, of which we were able to putatively annotate eight (Figure 2). Submission of these eight metabolites to the Pathway Analysis (MetPA)

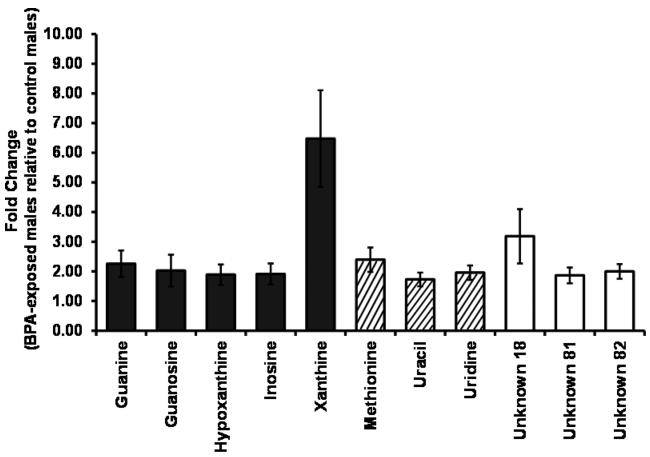

Figure 2. Fold change (mean ± SE) of statistically significant (*t*-test, p < 0.01) metabolites observed as changing in male fathead minnows in response to exposure to 10 μg/L BPA for 4 days (i.e., BPA-exposed males vs control males). Metabolites that serve as intermediates in the purine degradation pathway are shaded dark gray. Metabolites not associated with the purine degradation pathway are indicated by hashed bars. Unknown metabolites (labeled according to scheme used in Supporting Information Table S2) are indicated by white bars. Database searches to determine putative annotations found pseudouridine and 6,8-dihydropurine at the same measured masses as uridine and xanthine, respectively.

module within MetaboAnalyst 2.0 (using the *Danio rerio* (zebrafish) pathway database and default pathway analysis algorithms[51]) suggested a primary impact of BPA exposure on purine metabolism in males. Specifically, five of the eight putatively annotated metabolites that changed are represented in the purine degradation pathway (Figure 3). Moreover, these five represent half of all members in this pathway and show a concerted increase relative to controls. Interestingly, this response is in agreement with a previous metabolomics study that reported increased purine degradation as one of the primary responses measured in rat urine after exposure to BPA at 50 mg/kg/day.[52] In that report, the authors suggested hyperactivation of the purine degradation pathway by BPA exposure, and the resulting increase in its intermediates, could result in the oxidative stress response that has been reported previously in both rat liver and testes after exposure to BPA.[53,54] Increased urinary excretion of these degradates as reported by Chen et al. in 2014[52] may represent an effective compensatory response to BPA exposure in the rats by lowering the overall body burden. Oxidative stress as a result of BPA exposure is also a recognized adverse outcome in fish.[55] As the skin also offers a secretory route in fish,[56] the increased levels of these metabolites measured in the mucus suggests that a similar compensatory response may be occurring in the FHM in response to BPA exposure.

With regard to the females, we measured 10 metabolites as significantly changing in response to BPA exposure. Of these 10, we were able to putatively annotate six (Supporting Information Table S2). Pathway analysis using MetPA did not suggest any specific pathways being significantly represented by these six metabolites. While we could not ascribe the metabolite changes observed in the females to any particular pathways, it is interesting to note that the response to the BPA exposure was quite different from that seen for the males, as is evident from the inverse responses seen in the PLS-DA scores plot (Figure 1). This may be a result of any one of the myriad of biological activities that have been ascribed to BPA in vertebrate studies (e.g., ER agonist, thyroid receptor antagonist, estrogen-related receptor-γ ligand, glucocorticoid receptor agonist, and aryl hydrocarbon receptor ligand).[23] However, quite likely it is due to the endocrine active nature of BPA (e.g., estrogenicity) which could be expected to produce highly different responses in the sexes.

Indeed, the pronounced purine response to BPA exposure in the males raises the question of whether this was a result of the estrogenic character of BPA or some other sex-specific response (among the purines, only inosine was observed as changing in response to BPA exposure in the females). From previous reports, it appears that the purine response may have indeed been estrogen-mediated. For example, Ruggeri et al. 2008[57] reported an impact of 17β-estradiol on purine metabolism gene expression (in addition to oxidative stress) in male zebrafish. Furthermore, other studies have reported impacts of various estrogens on specific enzymes within purine metabolic pathways.[58,59] Thus, the impact of exposure on purine metabolism in male fish in the current study is plausibly a result of the estrogenic activity of BPA, and warrants further investigation into the potential utility of purine metabolites in fish skin mucus as bioindicators of an estrogenic response. Indeed, this demonstration of the ability of the skin mucus metabolome to reveal responses to environmentally relevant chemical exposures opens up a number of possibilities for rapid

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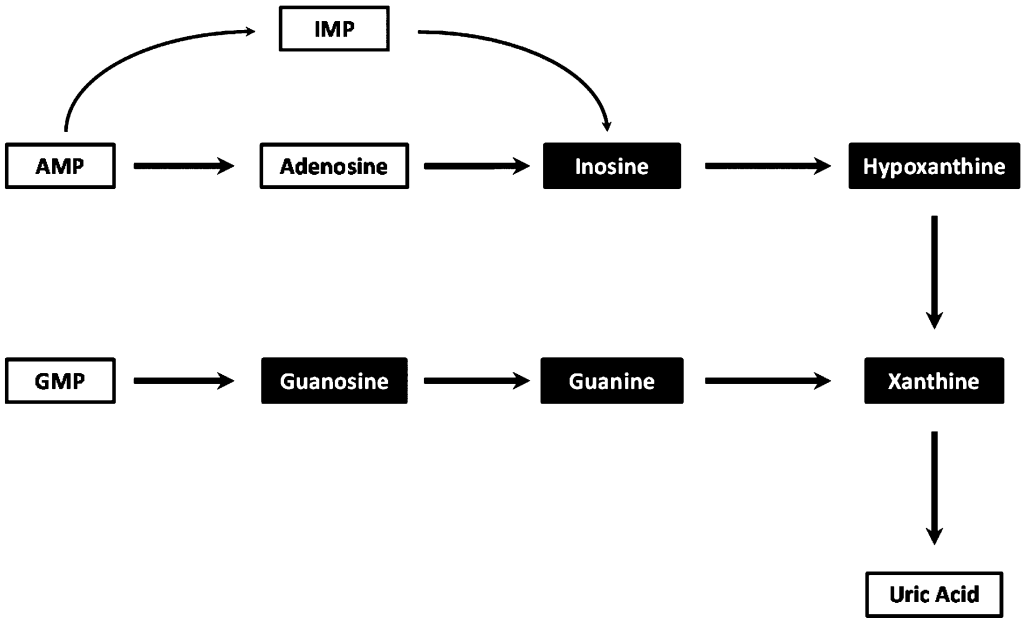

Figure 3. Purine degradation pathway. Metabolites that increased in the skin mucus of male fish after 4 days of exposure to BPA (10 μg/L) are shown in black shaded boxes. Adapted from Mathews and Van Holde, 1996.[65]

and minimally invasive sampling of fish exposed to environmental contaminants both in the laboratory and in the field.

3.4. Vitellogenin as an Indicator of Estrogenicity. A common method for assessing the estrogenicity of contaminants is through the measurement of blood plasma levels of the egg yolk precursor vitellogenin (VTG) in fish.[60] Given the recognized estrogenicity of BPA, we measured VTG in blood plasma to assess if BPA was producing an estrogenic response at the concentration tested. Past experience with exposure to BPA at this level (10 μg/L) has shown minimal to no induction of VTG in the blood plasma of male fathead minnows.[24] The current study further confirms this, as we saw no statistically significant VTG induction (a small, but not statistically significant increase was measured in the males) in any of the exposed fish (data not shown). This result is in contrast to the response in the skin mucus metabolome described above, which shows a significant and gender specific response to BPA exposure. Thus, the skin mucus-based metabolome may provide a highly sensitive and untargeted indicator of contaminant exposure that could be used in a discovery driven (i.e., open-ended) fashion, in conjunction with more targeted measures such as VTG. Along with this, the minimally invasive nature of skin mucus collection establishes great potential for this approach for use in ecological risk assessments.

3.5. Variation in the FHM Skin Mucus Metabolome. Sources of uninformative variation within a data set should always be considered when conducting metabolomics studies. Biological variation is frequently the largest contributor; however, technical (or analytical) variation must be considered as well.[61] From the standpoint of biological variation, biofluids (urine, blood, etc.) generally show higher levels of variation than tissues, which are typically under a greater degree of homeostatic control.[61] Thus, biofluids usually have the advantage of being collected in a nonlethal manner, but greater variation can generally be expected. Skin mucus, much like blood plasma, is likely under some level of homeostatic control related to the numerous functions it provides. However, to what extent this limits the interindividual variation among FHMs was uncertain. Thus, we sought to assess the level of variation for skin mucus to further evaluate its suitability for metabolomics applications. Relative standard deviation (RSD) has been shown to be a useful approach for conducting such assessments. As such, median %RSDs (standard deviation/mean × 100) were determined for each class (Supporting Information Table S3). Median %RSDs ranged from 45.0 (control females) to 50.1 (BPA-exposed males). These numbers are somewhat lower than those reported by Parsons et al. for both FHM blood plasma (58.4) and urine (52.9), which was measured using ^{1}H nuclear magnetic resonance (NMR) spectroscopy. Although measured with a different analytical technique (which limits direct comparison), it is encouraging that skin mucus was found here to be in the range of variability of these other biofluids (if not a bit lower), albeit considerably higher than has been reported for most tissues (<30).[61] Furthermore, as it is likely that the composition of the skin mucus metabolome varies depending on fish-body sampling location (e.g., gill vs tail region); we anticipate that this variation will decrease as our sampling expertise increases and a more homogeneous mucus sample is collected from each fish.

3.6. Potential for Monitoring and Surveillance. The number and complexity of chemicals continually being introduced into surface waters has created the demand for increasingly sophisticated tools for environmental monitoring and surveillance. In response, the scientific community has increased efforts to develop 'omics-based tools for assessing environmental exposures.[62] These tools are showing great promise for integrating responses to complex mixtures,[63] thus paving the way for greater application in the future. Moreover, the sensitivity afforded by the 'omics techniques allows one to collect a great deal of molecular information with relatively small amounts of a biological sample. However, collection of even small amounts of sample from an individual often requires dissection and is thus typically lethal. The amenability of metabolomics to the study of biofluids (e.g., urine) which can be collected with minimal impact on the organism has opened

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up significant avenues for conducting nonlethal assessments of ecologically relevant organisms.[64] The use of skin mucus-based metabolomics opens up yet another avenue for environmental scientists in this regard. As reported here, the skin mucus metabolome is biochemically complex, integrating a wide variety of biological processes of relevance to fish health. Moreover, the large degree of sexual dimorphism detected in the skin mucus metabolome is quite encouraging, given the growing list of environmental contaminants that are endocrine active. In addition, its sensitivity to chemical exposure (i.e., BPA) suggests that it may prove useful in an "early warning" capacity, giving risk assessors an indication that an ecosystem is stressed in time to take action before greater adverse impacts manifest themselves (e.g., population decline). Finally, the open-ended nature of metabolomics (and other 'omics techniques) is attractive for environmental monitoring and surveillance in the sense that risk assessors can never be certain that they are aware of all environmental stressors present and the potential adverse outcomes that might occur in resident populations.[2] Further development of skin mucus-based metabolomics could provide a tool for noninvasively collecting an unbiased assessment of fish health, enabling scientists to sample large populations of fish rapidly and inexpensively while not restricting their assessments to only those end points that have been identified as relevant in the past.

■ ASSOCIATED CONTENT

Supporting Information

Additional experimental details. 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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