Hyperpigmented melanistic skin lesions of smallmouth bass *Micropterus dolomieu* from the Chesapeake Bay watershed

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ABSTRACT: Hyperpigmented melanistic skin lesions (HPMLs) of smallmouth bass *Micropterus dolomieu* are observed in the Potomac and Susquehanna rivers, Chesapeake Bay watershed, USA. Routine, nonlethal population surveys were conducted at 8 sites on the mainstem Susquehanna River and 9 on the Juniata River, a tributary of the Susquehanna River, between 2012 and 2018, and the prevalence of HPMLs was documented. A total of 4078 smallmouth bass were collected from the mainstem Susquehanna River and 6478 from the Juniata River. Lesions were primarily seen in bass greater than 200 mm, and prevalence in the Susquehanna River (8%) was higher (p < 0.001) than in the Juniata River (2%). As part of ongoing fish health monitoring projects, smallmouth bass were collected at additional sites, primarily tributaries of the Susquehanna (n = 758) and Potomac (n = 545) rivers between 2013 and 2018. Prevalence in the Susquehanna River (13%) was higher (p < 0.001) than the Potomac (3%). Microscopically, HPMLs were characterized by an increased number of melanocytes in the epidermis or within the dermis and epidermis. RNAseq analyses of normal and melanistic skin identified 3 unique sequences in HPMLs. Two were unidentified and the third was a viral helicase (E1). Transcript abundance in 16 normal skin samples and 16 HPMLs showed upregulation of genes associated with melanogenesis and cell proliferation in HPMLs. The E1 transcript was detected in 12 of the 16 melanistic areas but in no samples from normal skin. Further research will be necessary to identify the putative new virus and determine its role in melanocyte proliferation.

KEY WORDS: Melanistic skin · Smallmouth bass · Histopathology · Transcript abundance

1. INTRODUCTION

Smallmouth bass *Micropterus dolomieu* are an important sportfish in the Chesapeake Bay watershed and elsewhere, generating millions of dollars in revenue each year (Shull & Pulket 2015). They are also a top predator and can have significant effects on their prey populations and on habitat complexity and trophic structure (Jackson 2002). Population declines in portions of the Susquehanna river watershed associated with disease (coinfections of multiple bacteria, myxozoan and trematode parasites, largemouth bass virus) in young-of-year smallmouth bass (Arway & Smith 2013, Smith et al. 2015, Walsh et al. 2018) and mortality events, visible skin lesions and similar coinfections of adults in the Potomac watershed (Blazer et al. 2010) have raised concern among the fishing public and resource managers. In addition to these mortality events, visible skin lesions, including melanistic lesions, are commonly observed.

Fish, unlike mammals, have a variety of chromatophores, including erythrophores, xanthophores, cyano-

phores, leucophores, iridophores and melanophores/melanocytes. Color changes in fish are either physiological, due to motile responses of chromatophores, or morphological, due to changes in pigment cell morphology and density (Sugimoto 2002). While melanophore and melanocyte have both been used to describe fish chromatophores that synthesize melanin, it has been suggested for consistency that the term melanocyte be used due to the increasing evidence of conserved genetics in melanocyte biology (Schartl et al. 2016).

Melanosis, melanistic areas or hyperpigmentation has most commonly been reported in marine fish species including Pacific rockfish *Sebastes* spp. (Okihiro et al. 1993), North Sea dab *Limanda limanda* (Noguera et al. 2013), Hawaiian goldring surgeonfish *Ctenochaetus strigosus* (Work & Aeby 2014), axillary seabream *Pagellus acarne*, coral trout *Plectropomus leopardus* and wire netting cod *Epinephelus quoyanus* (Sweet et al. 2012, Ramos et al. 2013, Diggles et al. 2018). However, these skin abnormalities are also observed in freshwater fishes including salmonids (Brocklebank & Armstrong 1994), brown bullhead *Ameiurus nebulosus* (Blazer et al. 2007), and black bass species.

Locally known as the 'blotchy bass syndrome', reports of melanistic areas on otherwise normal-appearing smallmouth and largemouth *M. salmoides* bass collected in New York and Pennsylvania emerged in the 1980s (Carlson 1989, Skinner et al. 1994). Although the syndrome, characterized by irregular, black pigmented areas of varying size on the body surface, fins and mouth, has been recognized for many years, the aetiology is currently unknown. Within the Chesapeake Bay watershed there is a public perception of increasing prevalence, but this has not been substantiated. To better understand the prevalence, mechanisms of development and potential risk factors associated with melanistic lesions in smallmouth bass we (1) compared prevalence of hyperplastic melanistic skin lesions (HPMLs) among sites located in the Susquehanna and Potomac River drainages, (2) described the lesions microscopically, and (3) compared gene expression (transcript abundance) between normal and affected skin samples.

2. MATERIALS AND METHODS

2.1. Sites and field collections

Smallmouth bass were surveyed at 8 sites on the mainstem Susquehanna River and 9 on the Juniata River (Fig. 1, Table 1) between 2012 and 2018 (sampling frequency and sample size are included in Table 2) as part of Pennsylvania Fish & Boat Commission's routine nonlethal population monitoring surveys. The prevalence of gross anomalies, including HPMLs, total fish length in mm and water temperature were documented. Fish from 75 to 525 mm total length (TL) were targeted. Additionally, as part of ongoing fish health monitoring projects, smallmouth bass were collected at 22 sites, primarily tributaries of the Susquehanna and Potomac Rivers (Fig. 1, Table 1) between 2013 and 2018. During the fish health monitoring, attempts were made to collect 20 sexually mature adults (ages 2 yr and older; TL > 200 mm) from each site during each sampling period by boat, barge or backpack electroshocking. Actual sample size and sampling times are included in Table 3. Fish were euthanized by immersion in Finquel (MS-222, tricaine methanosulfate 350 mg l^{-1}; Argent Labs) following procedures approved by the Leetown Science Center's Institutional Animal Care and Use Committee. Weight (g) and total length (mm) were measured, abnormalities documented and a complete necropsy performed. Sections of melanistic lesions were placed in 10% Z-fix (Anatech) or PAXgene® fixative (Qiagen) for histological analysis. Small sections (ca. 5 mm) of skin, or skin scrapings, from HPMLs and normal skin of a subset of fish from the Susquehanna River were collected and preserved in RNAlater® (Life Technologies). Lag time between euthanasia and tissue fixation was less than 10 min.

2.2. Histological examination

A total of 41 pieces of HPMLs and surrounding normal skin, plus normal skin from 6 bass without melanistic areas were collected from 6 sites in 2013–2017. All but 3 were collected during spring sampling events. Preserved skin samples were decalcified in 0.25 M EDTA (ethylenediaminetetraacetic acid disodium salt; Sigma-Aldrich), pH 7.2 for 2–4 d, trimmed into cassettes and routinely processed. Tissues were embedded in paraffin, sectioned at 6 μm, stained with hematoxylin and eosin (H&E), and coverslipped. A subset of sections was bleached for melanin pigment removal by placing in 0.20% aqueous potassium permanganate solution for 45 min (Luna 1992) and then stained with H&E to enhance observations of the cellular structure. Some sections were also stained with Masson's Trichrome (American Mastertech Scientific) following manufacturer's protocol to selectively stain collagen, connective tissue and muscle.
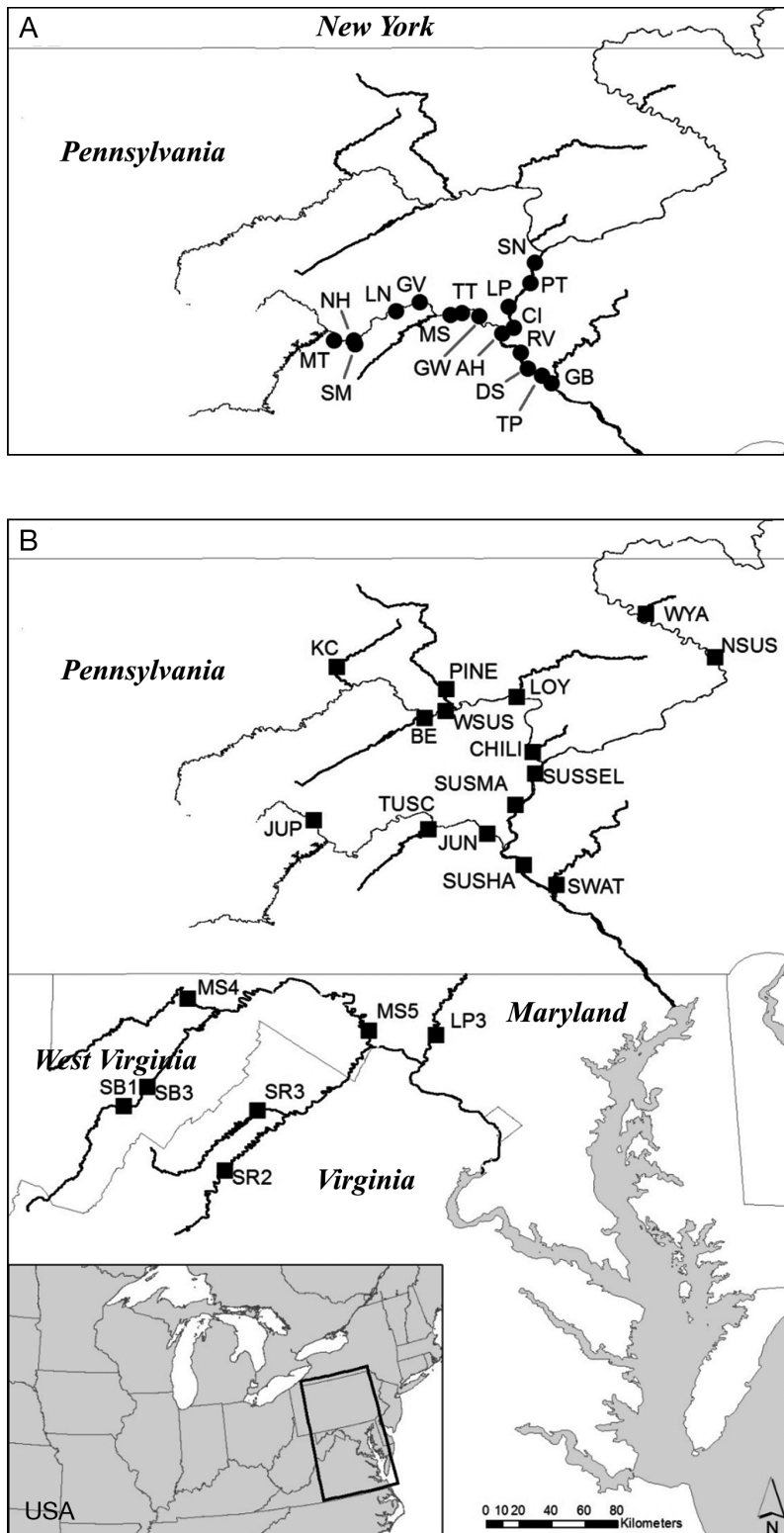
Fig. 1. (A) Sites at which the targeted sampling occurred on the Juniata and mainstem Susquehanna rivers. (B) Fish health assessment site locations within the Susquehanna and Potomac river watersheds. See Table 1 for full site names

2.3. RNA extractions and sequencing

Total RNA was extracted using the E.Z.N.A © Total RNA Kit I (Omega Bio-tek) following manufacturer's protocol. RNA integrity (RIN) was determined for each sample with the Agilent RNA 6000 Nano Kit on the Agilent 2100 Bioanalzyer (Agilent Technologies). RNA (RIN > 6) from 6 HPMLs and 6 normal skin samples was submitted to the Penn State Genomics Core Facility, University Park, PA, for 2 runs of 150 nucleotide single end read sequencing on an Illumina HiSeq2500 platform (Illumina). Libraries were prepared with the TruSeq Stranded mRNA Library Preparation Kit (Illumina). RNA from additional skin samples (n = 32) collected in April or November, 2017, were stored at −80°C for nCounter® (Nanostring Technologies) analysis.

2.4. Custom CodeSet Nanostring nCounter® analysis assay

A partial transcriptome was assembled as described in Young (2018). Sequence data is available in BioProject PRJNA530557 (NCBI). Coding regions of genes of interest were identified using Geneious (version R10.1) and verified via BLASTx (Table S1 in the Supplement; www.int-res.com/articles/suppl/d139p199_supp.pdf). A custom CodeSet was designed by Nanostring Technologies to evaluate transcript abundance of genes associated with melanogenesis, pathogen immune response, and contaminant responses. These probes were selected based on the RNAseq data and the literature. In the annotated set of contigs, only 3 unique sequences were expressed in the HPMLs. Two were unidentified and one was identified as a putative papillomavirus E1 helicase-like gene (GenBank accession no. MT010627). Probes designed to target the E1 gene as well as the major capsid protein of largemouth

bass virus were included in the Code-Set to target a total of 55 genes (Table 4). Transcript abundance was determined in 16 samples of normal skin and 16 HPMLs (from a total of 27 individual bass) collected in the Susquehanna drainage in 2017. From 4 affected fish a sample of both normal and melanistic skin were compared. Total RNA was quantified using Qubit (Thermo Fisher Scientific), and 50 ng was used in the nCounter® analysis assay conducted per manufacturer protocol.

Quality control of gene expression data was performed with nSolver™ Analysis Software v4.0. The geometric mean plus 3 standard deviations of the negative control probes were subtracted from each sample to account for background. Normalization of the raw counts was performed using the positive spike controls and housekeeping genes included in the custom CodeSet. Housekeeping genes included TATA box binding protein (TBP), β-actin (βACT), 40s ribosomal protein S12 (RPS12) and ribosomal protein L8 (RPL8), which were found to be stable using Normfinder (Andersen et al. 2004).

2.5. Statistical analysis

The Bioconductor package Nano-StringDiff version 1.16.0 (Wang et al. 2018) was used for differential expression analysis. Raw count data was normalized using the spike-in positive controls, negative controls, and all 5 housekeeping genes from the CodeSet. Differentially expressed genes were determined using the generalized linear model (GLM) likelihood ratio. To reduce type 1 error, p-values were adjusted using the Benjamini and Hochberg method (Benjamini et al. 2006). Genes with an adjusted p-value < 0.05 were considered differentially expressed. Prevalence data was compared using the chi-square statistic with Yates correction or the Fisher's Exact test (GraphPad Prism version 8.4.1, GraphPad Software).

Table 1. Site descriptions for the surveys (2012–2018) in the Susquehanna drainage, including the Juniata, and the fish health studies (2013–2018) in the Potomac and Susquehanna drainages

River	Site	Site ID	Latitude (°N)	Longitude (°W)
Surveys (2012–2018)				
Susquehanna	Shady Nook	SN	40.8220	76.8374
	Port Trevorton	PT	40.7099	76.8599
	Liverpool	LP	40.5780	76.9779
	Clemson Island	CI	40.4632	76.9502
	Rockville	RV	40.3276	76.9128
	Dock Street	DS	40.2409	76.8738
	Turnpike	TP	40.1994	76.7969
	Goldsboro	GB	40.1598	76.7439
Juniata	Mapleton	MT	40.3930	77.9369
	Shawmut	SM	40.3738	77.8188
	Newton Hamilton	NH	40.3942	77.8262
	Lewistown Narrows	LN	40.6050	77.4669
	Granville	GV	40.5563	77.5964
	Muskrat Springs	MS	40.5343	77.2992
	Thompsontown	TT	40.5433	77.2334
	Greenwood	GW	40.5265	77.1406
	Amity Hall	AH	40.4307	77.0148
Fish health studies (2013–2018)				
Potomac	South Branch @ Petersburg	SB1	38.9991	79.0862
	South Branch @ Old Fields	SB3	39.1040	78.9577
	North Branch @ Cumberland	MS4	39.3877	77.3798
	Monocacy River	LP3	39.5869	78.7375
	Antietam Creek	MS5	39.4143	77.7463
	North Fork Shenandoah	SR3	38.9743	78.3551
	South Fork Shenandoah	SR2	38.6460	78.5345
Susquehanna	Upper Juniata River	JUP	40.5645	78.0469
	Tuscarora Creek	TUSC	40.5155	77.4195
	Juniata @ Howe Township	JUN	40.4920	77.0980
	Swatara Creek	SWAT	40.2123	76.7210
	Susquehanna @ Harrisburg	SUSHA	40.3186	76.8988
	West Branch Mahantango Creek	SUSMA	40.6478	76.9430
	Susquehanna @ Selinsgrove	SUSSEL	40.6513	76.9232
	Chillisquaque Creek	CHILI	40.9416	76.8500
	Loyalsock Creek	LOY	41.2409	76.9377
	Pine Creek	PINE	41.2829	77.3215
	West Branch Susquehanna	WSUS	41.1628	77.3272
	Bald Eagle Creek	BE	41.1242	77.4413
	Kettle Creek	KC	41.4039	77.9212
	North Branch @ Fall	NSUS	41.4569	75.8515
	Wyalusing Creek	WYA	41.6974	76.2309

3. RESULTS

3.1. Gross observations

Melanistic areas were generally irregular, flat areas of black to dark brown pigmentation. Rarely these areas were slightly raised. Size, location and number of areas on an individual fish varied. The lesions ranged from very small (2–4 mm), round to

Table 2. Percentage (sample size) of smallmouth bass (≥200 mm) captured in the fall (September through November) with hyperpigmented melanistic lesions. –: no sampling event occurred

Site	2012	2013	2014	2015	2016	2017
Susquehanna mainstem						
Shady Nook	–	–	15.7 (191)	11.0 (82)	–	14.1 (99)
Port Trevorton	15.6 (45)	–	–	–	–	–
Liverpool	9.7 (31)	–	–	–	–	–
Clemson Island	–	0.0 (73)	7.0 (57)	7.0 (43)	0.8 (118)	1.7 (113)
Rockville	6.0 (50)	14.3 (84)[a]	14.3 (147)[a]	10.0 (70)[a]	6.9 (363)[b]	2.2 (137)[a]
Dock Street	–	0.0 (47)	4.8 (62)	0.0 (19)	0.0 (77)	0.0 (17)
Turnpike	0.0 (6)	0.0 (44)[a]	20.0 (30)	11.4 (44)	–	4.4 (45)
Goldsboro	–	3.5 (57)	7.3 (123)	10.9 (55)	6.2 (146)	9.7 (72)
All sites	9.8 (132)	4.8 (294)	8.9 (610)	9.6 (313)	5.0 (704)	5.8 (483)
Juniata River						
Mapleton	3.2 (31)	0.0 (82)	2.6 (152)[a]	3.8 (52)	5.0 (60)	1.2 (81)
Shawmut	–	–	–	–	–	3.7 (216)
Newton Hamilton	1.0 (98)	0.0 (71)	2.3 (130)[a]	2.8 (108)	4.6 (131)	1.4 (138)
Lewistown Narrows	0.0 (113)	0.0 (161)	2.5 (122)	–	0.0 (316)	–
Granville	–	–	–	–	2.5 (81)	1.3 (79)
Muskrat Springs	3.7 (27)	4.8 (21)	–	–	–	0.0 (59)
Thompsontown	0.0 (32)	0.0 (14)	0.0 (32)	0.0 (28)	2.1 (140)	0.0 (101)
Greenwood	0.0 (24)	0.0 (17)	0.0 (32)	7.1 (28)	0.0 (47)	6.1 (66)
Amity Hall	0.0 (49)	5.9 (68)	–	3.7 (54)	2.5 (122)	–
All sites	0.8 (374)	1.2 (418)	2.1 (468)	3.3 (270)	1.9 (897)	2.2 (740)

[a]Combined data from sampling events in September and October
[b]Combined data from sampling events in October and November

Table 3. Percentage of smallmouth bass with hyperpigmented melanistic lesions collected at sites within the Susquehanna and Potomac River drainages during fish health studies, 2013–2018. Sample size is 20 unless indicated in parentheses

Site	2013 Spring	2014 Spring	2015 Spring	2016 Spring	2016 Fall	2017 Spring	2017 Fall	2018 Spring	2018 Fall
Susquehanna River drainage									
PINE	–	0.0 (13)	4.3 (23)	10.0	0.0	0.0	0.0	0.0	5.6 (10)
SUSMA	15.0	–	40.0	55.0	0.0	25.0	5.0	0.0	15.0
CHILI	10.0	10.0	20.0	30.0	–	55.0	–	–	–
WYA	0.0	–	5.0	7.1 (14)	–	–	–	–	–
JUN	–	0.0	35.0	–	–	–	–	–	–
SUSSEL	5.0	–	–	–	–	–	55.0	–	–
LOY	20.0	6.7 (15)	–	–	–	–	–	–	–
WSUS	–	0.0 (10)	0.0	–	–	–	–	–	–
BE	–	0.0 (13)	20.0	–	–	–	–	–	–
SUSHA	35.0	–	–	–	–	–	–	–	–
SWAT	–	0.0 (18)	–	–	–	–	–	–	–
TUSC	–	0.0	–	–	–	–	–	–	–
KC	–	–	5.9 (17)	–	–	–	–	–	–
NSUS	–	–	5.9 (17)	–	–	–	–	–	–
JUP	–	–	–	10.0	–	–	–	–	–
All sites	14.2	2.3	15.3	23.4	0.0	26.7	20.0	0.0	10.5
Total samples	(120)	(129)	(177)	(94)	(40)	(60)	(60)	(40)	(38)
Potomac River drainage									
SB3	10.0	0.0	7.5a (40)	0.0	–	0.0	7.1 (14)	0.0	10.0
MS5	0.0	–	0.0	0.0	5.0	5.0	–	0.0	10.0
SB1	5.0	–	–	–	–	–	–	–	–
MS4	0.0	–	–	–	–	–	–	–	–
LP3	0.0	0.0	–	–	–	–	–	–	–
SR3	–	0.0	–	0.0	–	–	–	–	–
SR2	–	0.0	–	0.0	–	–	–	–	–
All sites	2.1	0.0	3.8	0.0	5.0	2.5	7.1	0.0	10.0
Total samples	(140)	(93)	(80)	(78)	(20)	(40)	(14)	(40)	(40)

[a]Includes sampling events in April and May

Table 4. Gene transcripts included in the Nanostring nCounter codeset for transcript abundance

Designation	Gene
Pathogen-related genes	
E1	Papillomavirus helicase
LMBV MCP	Largemouth bass virus major capsid protein
Melanogenesis/melanocyte-related genes	
DCT	L-dopachrome tautomerase
PMEL	Melanocyte protein PMEL-like
TYR	Tyrosinase
TYRP1	Tyrosinase-related protein 1a
POMC	Pro-opiomelanocortin
MC1R	Melanocortin receptor 1
MC2R	Melanocortin-2 receptor
MC5R	Melanocortin receptor 5
KILG	Kit ligand
KITA	Mast/stem cell growth factor receptor kita
MITF	Micropthalmia-associated transcription factor
Rab38	Ras-related protein Rab-38
Endocrine-related genes	
ARA	Androgen receptor alpha
ARB	Androgen receptor beta
GR	Glucocorticoid receptor
GPER	G couple estrogen receptor
THRB	Thyroid hormone receptor beta
Contaminant-related genes	
MT1	Metallothionein
CAT	Catalase
GST	Glutathione S-transferase theta-1
SOD	Superoxide dismutase
CYP1A	Cytochrome P450, family 1
CYP2	Cytochrome P450, family 2
CYP3A	Cytochrome P450, family 3
ARG1	Arginase
HSP70	Heat shock protein 70
HSP71	Heat shock protein 71
HSP90B	Heat shock protein 90b
Immune/inflammatory-related genes	
CCL4	C-C motif chemokine 4-like (MIP-b) (CCL4)
CXCL11	C-X-C motif chemokine 11-like (I-TAC) (CXCL11)
CD2	T-cell surface antigen CD2 (CD2)
CD4	T-helper cell glycoprotein (CD4)
EPXL	Eosinophil peroxidase-like (EPXL)
HLAA	MHC class I antigen (HLAA)
IFNAR1	Interferon alpha/beta receptor 1(IFNAR1)
IL12A	Interleukin-12 (IL12A)
IL17	Interleukin-17 (IL17)
IL1b	Interleukin-1 beta (IL1b)
IL6	Interleukin-6 (IL6)
TNF	Tumor necrosis factor alpha (TNF)
CD8A	CD8 alpha chain (CD8A)
TLR3	Toll-like receptor 3(TLR3)
TGFB	Transforming growth factor b (tgfb)
AKT1	RAC-alpha serine threonine-kinase
Oncogenes/tumor suppressor genes	
BCL-2	B-cell lymphoma 2
TP53BP1	Apoptosis-stimulating of p53 protein 1-like isoform X1
MAPK1	Mitogen-activated kinase 1
BRAF	Serine/threonine-protein kinase B-raf
RERG	Ras-related and estrogen-regulated growth-related inhibitor
Other	
KRT8	Keratin type II cytoskeletal 8-like
CASP3	Caspase 3
EGFR	Epidermal growth factor receptor
TRIM16L	Tripartite motif-containing protein 16-like
WNT11	Protein Wnt-11

irregular areas on fins and body surface (Fig. 2A) to irregular areas covering much of the body surface (Fig. 2B). In some fish these hyperpigmented areas were noted on the lips (Fig. 2C) and mouth. Two of the bass had areas of depigmentation along the periphery of the melanistic area (Fig. 2D). Commonly, scrapings of the melanistic areas on the body surface resulted in removal of the black tissue which was primarily present in the epidermis covering the scales (Fig. 2E).

3.2. Spatial and temporal prevalence

A total of 4078 smallmouth bass ranging in length from 75 to 525 mm collected from the Susquehanna River between Harrisburg and Selinsgrove were assessed for melanistic lesions between 2012 and 2017 (Table 2). Melanistic areas were observed on 5% of the fish and were only observed on bass 175 mm TL or longer. A total of 6478 bass from the Juniata River between Newport and Mapleton were assessed, and 1% of the fish had melanistic areas. In this watershed melanistic areas were observed on bass 125 mm and longer. Bass collected in the population monitoring surveys accounted for a broader range of lengths than those collected for fish health assessments. As such, fish under 200 mm TL were removed from further analysis at all sites. Only 8 fish, 4 from each drainage, with HPMLs fell within these smaller size categories. Prevalence in fish greater than 200 mm TL was 8% in the Susquehanna River, which was higher (p < 0.001) than the 2% observed in the Juniata River (Table 2).

At the Rockville site, sampling events took place in 2 consecutive months (September/October; October/November) each year from 2013–2017. In 2013 the prevalence of bass with melanistic areas was significantly higher (p = 0.001) in October vs. September and in 2016 the prevalence
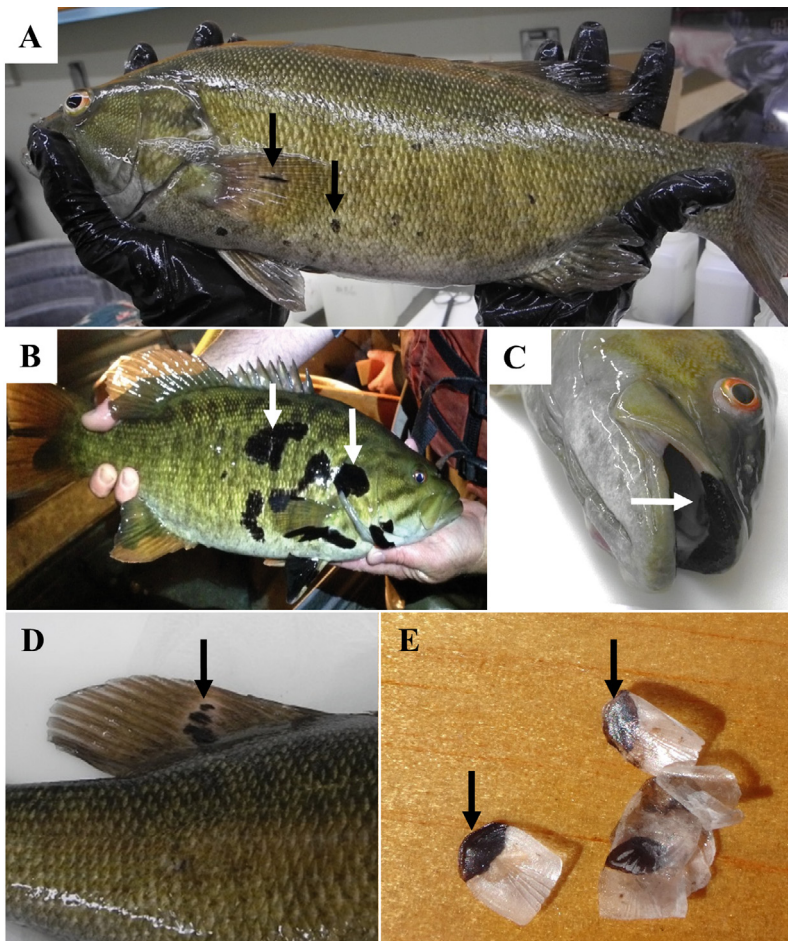
Fig. 2. Visible melanistic areas on smallmouth bass. (A) Small round to irregular black areas on the body surface and fins (arrows). (B) Multiple irregular black areas on the lateral body surface and opercle (arrows). (C) Melanistic area on the upper lip (arrow). (D) Melanistic area on the fin surrounded by a depigmented, pale border (arrow). (E) Scales scraped from a melanistic area on the body surface illustrating the black epidermis (arrows)

was higher (p = 0.0014) in November vs. October. While the prevalence was elevated in October vs. September of 2014 (p = 0.1293) and 2015 (0.0960), it was not significantly different. In 2016 the prevalence was higher (p = 0.0014) in November than October. In 2017, there was no difference (p = 0.4627) between September and October (Fig. 3A). The higher number of bass with HPML was associated with lower temperatures, except in 2017 (Fig. 3B).

In total, 1303 smallmouth bass from 22 sites were collected for fish health assessment studies (Table 3). In the Susquehanna River, 758 smallmouth bass were collected at 15 sites of which 13% exhibited HPMLs. In the Potomac River, 545 smallmouth bass were collected at 7 sites and 3% had HPMLs, which was lower (p < 0.001) than the prevalence noted in the Susquehanna River. Both temporal and spa-

tial variation in the occurrence of HPMLs was noted with no apparent pattern (Table 3).

3.3. Histopathology

Normal skin of smallmouth bass varied in thickness and number of mucous cells in the epidermis, thickness of the dermis and hypodermis depending on body location, season and site. Distinct layers of the epidermis were observed with columnar epidermal cells in the basal region, cuboidal cells in the intermediary region and squamous cells on the surface (Fig. 4A). Normal epidermis contained mucous cells but rarely melanocytes. Epidermis and dermis were separated by a basement membrane of collagen (Fig. 4B). Immediately below this membrane was a layer of melanocytes, loose connective tissue and the scale pocket within the dermis (Fig. 4).

The microscopic appearance of HPMLs varied among individual fish and among individual HPMLs on the same fish. Small melanistic areas were characterized by accumulations of small, compact melanocytes in the epidermis. This was rarely accompanied by a loss of melanocytes at the epidermal/dermal boundary, hemorrhage and inflammation (Fig. 5A). The epidermis was generally not thickened compared to adjacent normal epidermis, and the transition between normal and melanistic skin was evident with melanocytes present in the epidermis (Fig. 5B). Epidermal melanocytes within the larger HPMLs contained pleomorphic cells of varying size. Large round melanocytes, with dense melanin granules around the periphery of the cell, as well as smaller, stellate melanocytes, were present in the epidermis. In some HPMLs a layer of melanocytes was present in the dermis (Fig. 5C), while in other lesions this layer was not evident (Fig. 5D). Cystic spaces containing numerous melanocytes and cells interpreted as epithelial cells were observed (Fig. 5E). Occasionally, HPMLs contained fewer melanocytes in the epidermis and increased numbers in the dermis (Fig. 5F). Inflammatory cells including eosinophilic
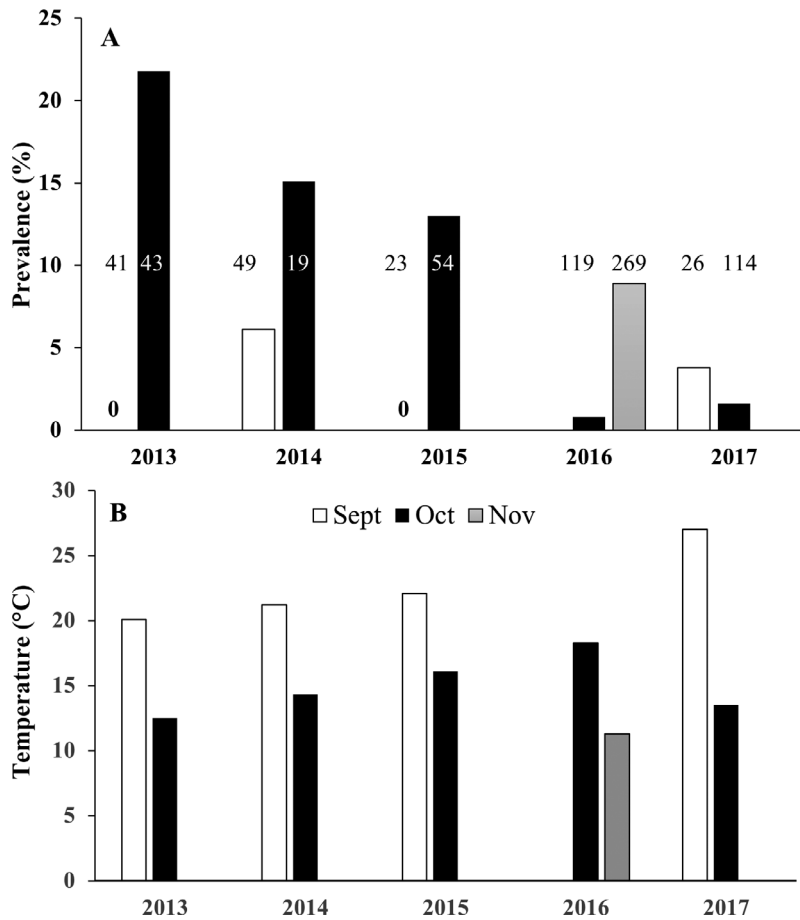
Fig. 3. Melanistic lesion prevalence and temperature observations at the Rockville site. (A) Prevalence between months in years 2013 through 2017. Values above or in the bars are sample sizes, 0 indicates no melanistic lesions observed. (B) Surface water temperature (°C) at the time of collection

granular cells and lymphocytes were evident in some lesions (Fig. 5E,F). No obvious inclusion bodies were observed in epithelial cells or melanocytes.

3.4. Transcript abundance

Of the 55 genes chosen for transcript abundance using the Nanostring nCounter custom codeset, 11 genes were differentially expressed between HPMLs and normal skin samples collected in 2017 (Table 5). Upregulated genes in the melanistic areas included L-dopachrome tautomerase (DCT), melanocortin receptor 5 (MC5R), micropthalmia-associated transcription factor (MITF), melanocyte protein PMEL-like (PMEL), tyrosinase (TYR), tyrosinase-related protein 1 (TYRP1) and Ras-related protein Rab-38 (Rab38), which are associated with melanogenesis. Additionally, RAC-alpha serine threonine-kinase (Akt1), a kinase which regulates many processes including proliferation, cell survival, growth and angiogenesis, was found to be significantly upregulated in melanistic areas. Two genes, CXC chemokine 11-like and the Wnt 11 protein (WNT11) were downregu-
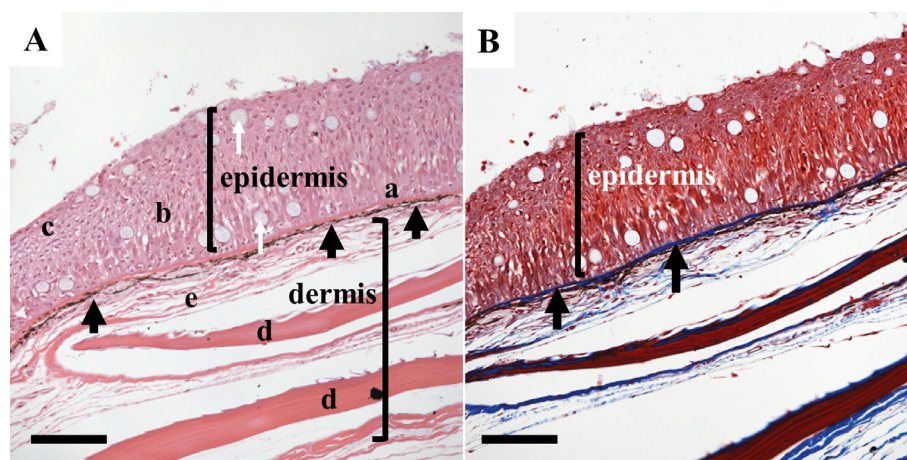

Fig. 4. Microscopic appearance of normal smallmouth bass skin. (A) Epidermis, between upper brackets, illustrating basal columnar epidermal cells (a), cuboidal cells in the intermediate portion (b) and squamous cells on the surface (c). Mucous cells (white arrows) are present in the epidermis. Below the basement membrane of the epidermis is a layer of melanocytes (black arrows). The dermis (between lower bracket) contains the scales (d) and loose connective tissue (e). H&E stain. Scale bar = 50 um. (B) Epidermis containing mucous cells (clear cells) and collagen (blue) of the basement membrane (arrows) between epidermis and dermis. Masson's trichrome stain. Scale bar = 50 µm
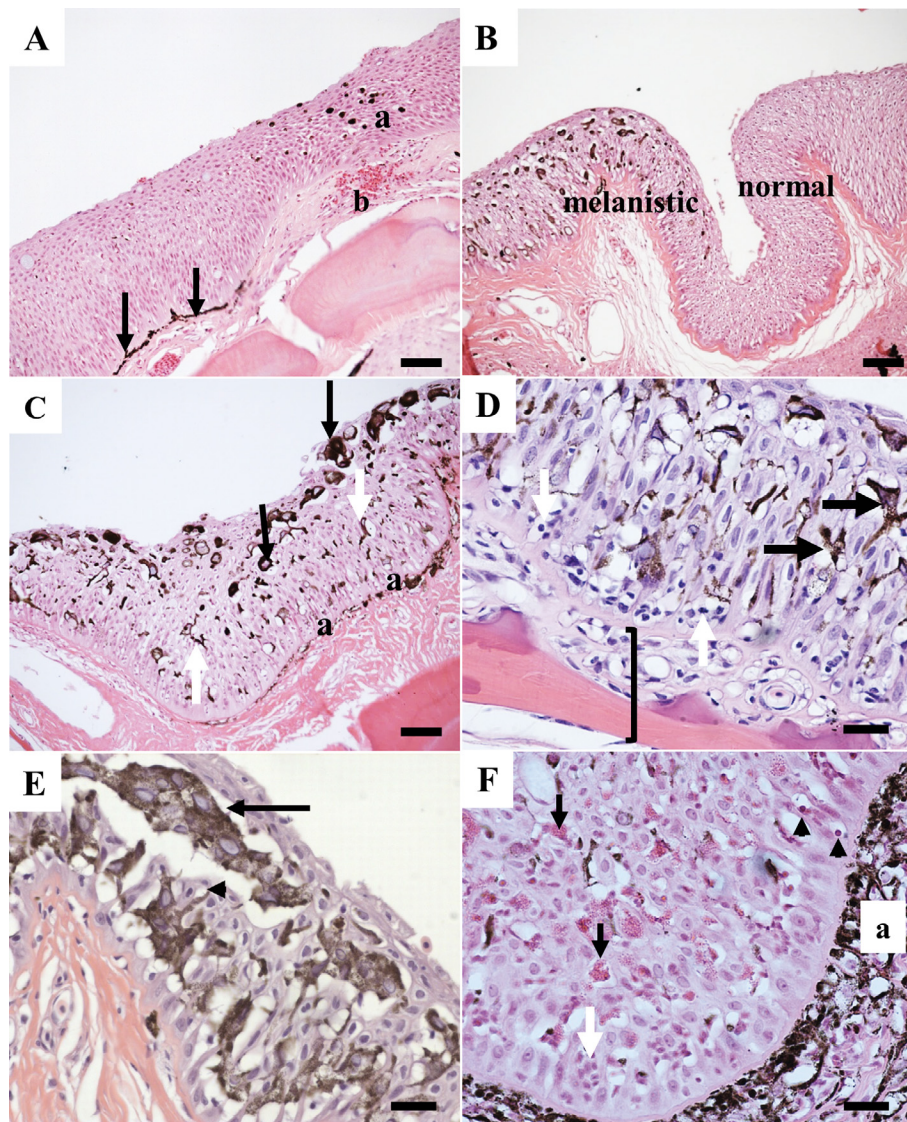
Fig. 5. Microscopic appearance of hyperpigmented melanistic lesions of smallmouth bass. (A) Section from a small melanistic area (as shown in Fig. 2A). A focus of melanocytes (a) within the epidermis, with an area of hemorrhage (b) immediately below within the dermis which is devoid of melanocytes. A layer of melanocytes (arrows) is present below normal epidermis (arrows). Scale bar = 50 μm. (B) Transition between melanistic and normal areas of skin. Pleomorphic melanocytes are present through-out the epidermis of the melanistic area. Scale bar = 50 μm. (C) Melanistic skin section containing large, rounded cells with an accumulation of melanosomes in the cytoplasm (black arrows) and more pleomorphic, dendritic melanocytes (white arrows). A layer of melanocytes (a) is evident along the basement membrane of the epidermis. Scale bar = 50 μm. (D) Melanistic area with numerous dendritic cells (black arrows) and lymphocytes (white arrows) within the epidermis. Dermis (brackets) is devoid of melanocytes. Scale bar = 20 μm. (E) Melanistic area with cystic spaces containing melanocytes and possibly epidermal cells (black arrow) and numerous dendritic melanocytes, some (arrowhead) appearing to surround epidermal cells. Scale bar = 20 μm. (F) Melanistic area with an increased number of melanocytes within the dermis (a) and eosinophilic granular cells (black arrows), pyknotic or apoptotic nuclei (arrowheads) and lymphocytes (white arrow) within the epidermis. Scale bar = 20 μm

lated in melanistic lesions. The viral helicase E1 transcript was detected in 12 of the 16 melanistic areas. No samples from normal skin had detectable levels of this viral transcript (Table 5).

Transcript abundance was measured in HPMLs and normal skin from 4 individual bass. In one indi-vidual none of the 11 genes were significantly differ-ent between normal and HPML samples, which may indicate insufficient melanistic skin was included in the sample. In the other 3 individuals there were dif-ferences between the 2 sections of skin. Fish 1 was collected in the West Branch Mahantango Creek, a

Table 5. Transcript abundance (median and range) of differentially expressed genes in 16 normal skin and 16 hyperpigmented melanistic areas of smallmouth bass collected in the Susquehanna River drainage in 2017 and difference between normal and melanistic skin. BD: below detection

| Gene | — Normal skin — | | – Melanistic areas – | | Significance |
	Median	Range	Median	Range	
E1	BD	BD–BD	98.6	BD–644	0.0002
PMEL	40.4	20–162	722.8	BD–2162	<0.0001
TYR	15.8	BD–109	283.2	BD–1079	<0.0001
TYRP1	10.9	BD–52	101.1	BD–782	0.0011
DCT	17.3	BD–49	283.1	BD–1240	<0.0001
MC5R	27.3	16.1–94	102.4	BD–328	0.0011
AKT1	362.8	257–501	434.4	302–603	0.0075
MITF	47.9	BD–91	139.6	36–510	<0.0001
Rab38	22.1	BD–78	64.3	BD–272	0.0096
WNT11	23.2	BD–123	BD	BD–BD	0.0387
CXCL11	474.0	28–356	92.2	BD–461	<0.0001

tributary, while Fish 2 and 3 were collected in the mainstem Susquehanna River. Fish 1 was collected in the spring while Fish 2 and 3 were collected in the Fall. In all 3 the E1 transcripts were below detection in normal skin and detectable in HPMLs. In addition, TYR and MITF transcripts were also upregulated in all HPMLs compared to normal skin. Transcripts of PMEL, DCT, TYR and TYRP1 were upregulated in Fish 2 and 3. It is currently not known if this is site or season-related or related to stage of infection (Fig. 6).

4. DISCUSSION

External abnormalities of fishes are used worldwide as indicators of environmental health (Baumann 1992, Sanders et al. 1999, Stentiford et al. 2009, Vethaak et al. 2009). Pigment cell hyperplasia and neoplasia in wild fishes have been associated with environmental factors such as UV radiation (Sweet et al. 2012), X-rays (Smith 1932) and chemical contaminants (Kimura et al. 1984, Kinae et al. 1990, Okihiro et al. 1993), although in most wild fish reports no definitive causes have been identified. Hyperpigmentation in North Sea dab, an important sentinel species for assessing contaminant effects, is first noted in 2 or 3 yr old dab (similar to smallmouth bass observations), depending on the site (Stentiford et al. 2010, Grütjen et al. 2013). The higher prevalence noted in older individuals was suggested to indicate a complex etiology (Noguera et al. 2013). The microscopic appearance of dab lesions, characterized by hyperplasia of iridophores, architectural distortion, lymphocyte infiltration and melanosome dispersion into dendritic extensions of melanocytes in the dermal layer, was different than smallmouth bass HPMLs.

In some dab lesions, clusters of melanin-containing cells were observed in the epidermis (Noguera et al. 2013). The prevalence of the dab lesions varied spatially and in certain areas of the North Sea increased from 5% to >40% between 1988 and 2009 (Grütjen et al. 2013). While the prevalence of a number of other fish health indicators (liver tumors, lymphocystis) has declined, providing evidence for a generally improving habitat, the prevalence of hyperpigmentation has continued to increase in most areas of the North Sea (Lang et al. 2017).

Microscopically, HPMLs of smallmouth bass were primarily characterized by increased accumulation of melanocytes in the epidermis and less commonly at the epidermal–dermal interface. The size, number, and shape of melanocytes varied in individual fish. Melanocytes with dendritic extensions were observed in the epidermis of the HPMLs. Some areas were accompanied by infiltration of varying degrees of inflammatory cells (eosinophilic granular cells, lymphocytes) and epidermal hyperplasia. These differences in microscopic appearance may indicate a progression in development of melanotic hyperpigmentation. It is unclear from the current study whether melanocyte hyperplasia in the dermal–epidermal interface occurs prior to migration into the epidermis. In some sections it appeared that as melanocytes migrated to the epidermis, these cells were absent from the dermis, while in other sections there were melanocytes in both dermis and epidermis (Fig. 5).

Based on reports in other species, we hypothesized the HPMLs of smallmouth bass may be related to environmental factors and used the transcript abundance to provide molecular insights into potential causes and mechanisms of development. Red seabream were shown to have increased size and abundance of melanocytes in the epidermis after UV exposure compared to shaded seabream (Adachi et al. 2005). Melanin is a biopolymer with many physiological properties that function to protect the cell. The pigment molecules are free radical scavengers that protect against reactive oxygen species (ROS) which can occur from UV radiation or chemical substances (Otreba et al. 2014). Other molecular defenses against ROS include the upregulation of heat shock proteins (HSP70, HSP 71, and HSP90B), superoxide dismutase (SOD), glutathione-S-transferase (GST), glucocorticoid receptor (GR), and catalase (CAT) (Rodriguez-Ariza et al. 1993, Lushchak 2011). None of
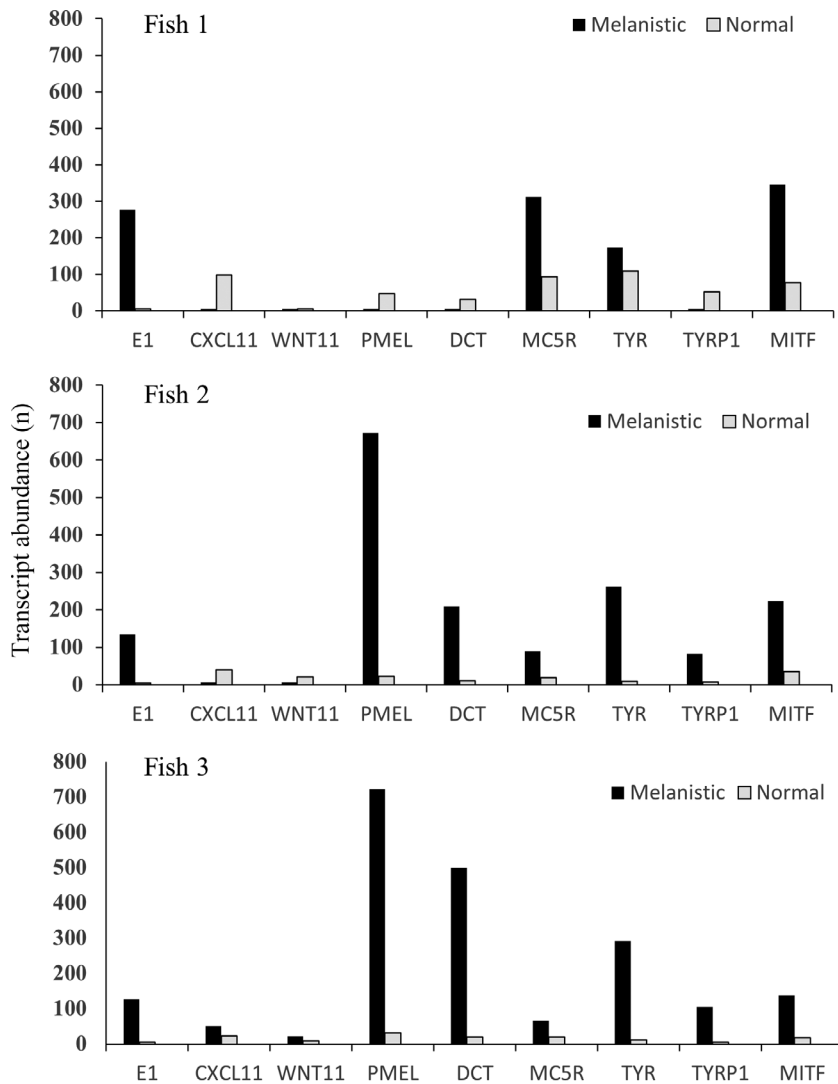
Fig. 6. Comparison of transcript abundance in melanistic and normal skin sections from 3 individual smallmouth bass. Fish 1 was collected in the West Branch Mahantango Creek, a tributary of the Susquehanna River in April 2017. Fish 2 and Fish 3 were collected in the mainstem Susquehanna River at Selinsgrove in November 2017. See Table 4 for full gene names

these genes were differentially regulated in melanistic skin areas when compared to normal skin. Nor was there upregulation of cytochrome P450 enzymes (CYP1A, CYP2, and CYP3A) associated with exposure to contaminants such as polycyclic aromatic hydrocarbons and polycyclic chlorinated biphenyls, linked to hyperpigmentation in mammals, fish, and the African clawed frog *Xenopus laevus* (Kimura et al. 1984, Okihiro et al. 1993, Baumann & Okihiro 2000, Fisher et al. 2003, Shimada & Fujii-Kuriyama 2004, Gräns et al. 2015). A number of proto-oncogenes and tumor suppressor genes shown to be dysregulated in melanomas of zebrafish (Patton et al.

2005) were included in the transcript abundance analyses. Mitogen-activated kinase 1 (MAPK1), serine/threonine-protein kinase B-raf (BRAF), ras-related and estrogen-regulated inhibitor (RERG) and apoptosis-stimulating of p53 protein (TP53BP) were not found to be differentially expressed between normal and melanistic skin.

The transcript abundance analyses in this study did indicate that the HPMLs of smallmouth bass are associated with changes in melanocyte density and upregulation of melanin biogenesis. A premelanosome protein (PMEL) is necessary for melanosome structure formation and maturation (Jiang et al. 2014). In zebrafish, melanocyte stem cells give rise to MITF+ melanoblasts which in turn give rise to the DCT+, TYR+, TYRP1+ differentiating or differentiated melanocytes/melanophores (Mort et al. 2015). Micropthalmia-associated transcription factor (MITF) is considered the master regulator of genes necessary for melanogenesis, cell growth, and survival. Melanogenesis involves sequential steps, the first and most rate limiting step being the oxidation of L-tyrosine by TYR to dopaquinone, while the next steps are catalyzed by DCT and TYRP-1 (Mason 1948, Riley 1997). Members of the Rab family, particularly Rab38, also play a key role in melanosome biogenesis (Coppola et al. 2016). Upregulation of DCT, TYR, TYRP-1, MC5R, MITF, PMEL, and Rab38 occurred in HPMLs when compared to normal skin of smallmouth bass.

The viral helicase (E1) initially identified, one of 8 early open reading frames (ORF) is the primary replication initiator protein for papilloma virus (Zheng & Baker 2006). It is a protein essential for viral replication and was one of 3 unique sequences identified in HPMLs. The sequence is most similar (32.79% identical) to the E01 helicase of the recently described guitarfish *Rhynchobatus djiddensis* adomavirus (Dill et al. 2018), belonging to a new viral family, the *Adomaviridae* (Welch et al. 2018). Similar to the current study, the conserved helicase domain was initially the only part of the adomavirus genome identified from guitarfish lesions by next generation sequencing and blast searches of avail-

able databases. The infected guitarfish had both pigmented and nonpigmented villus-like skin lesions with nuclear inclusions containing viral particles which regressed over a 6 mo period (Camus et al. 2016).

There are a number of other observations that support a viral etiology for the smallmouth bass HPMLs. Transcript abundance analyses found E1 helicase transcripts to be below detection in all of the normal skin samples, yet present in most HPMLs (Table 5). Young et al. (2019) cohabitated affected and nonaffected individuals in tanks with ambient (11.3 to 13.9°C) or heated (20.3°C) spring water. Some fish which initially had HPMLs gained surface area (increased number and/or size of melanistic areas) at both ambient (46%) and elevated (28%) temperatures, while others lost surface area. Six of the 11 normal-appearing bass developed HPMLs during the experiment, 3 at each temperature, suggesting an association with a transmittable agent. Transcript abundance analysis also showed upregulation of Akt1 in HPMLs. The Akt1 gene has pivotal roles in apoptosis, differentiation, and proliferation of cells (Dï-Poi et al. 2002). In mammals, Akt1 is part of the critical phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTor) cascade pathway that is activated during a host response to viral infection (Diehl & Schaal 2013). The PI3K/Akt/mTor cascade initiated by viral infection regulates autophagy of infected cells to degrade intracellular pathogens. In order for survival, viruses have evaded autophagy by increasing Akt regulation to prevent apoptosis. Studies have shown that the human papillomavirus is capable of activating Akt1 and consequently downregulate apoptotic proteins. This tactic causes hyperproliferation and inhibition of differentiation of infected cells, which in turn increases viral survival rate and time for replication to occur in keratinocytes (Charette & McCance 2007, Surviladze et al. 2013).

Further sequence-based investigations are in progress to definitively identify and fully characterize the virus. Additional studies will be necessary to determine if the virus alone or in combination with certain environmental stressors is responsible for HPMLs of bass. Our results do not suggest there is an increasing prevalence of the melanistic lesions in the Susquehanna river watershed. Rather it appears that there is annual variation that could be associated with environmental factors. For instance, the overall prevalence in the Susquehanna mainstem during targeted sampling was 9% or above in 2012, 2014 and 2015 but 5% or less in 2013 and 2016. In the Juniata the prevalence was 3% in 2015 but below 3% in all other years (Table 2). The Rockville site was sampled in all years from 2012 through 2017. Between 2013 and 2017 an overall decline was noted (Fig. 3). The prevalence of HPMLs on smallmouth bass also varied spatially. Routine population monitoring demonstrated the presence of affected bass at all sites in the Susquehanna and Juniata rivers at least once during the fall sampling from 2012–2017. In all years more melanistic bass were observed in the Susquehanna than in the Juniata (Table 2). A higher prevalence was also observed in the Susquehanna River watershed (13%) when compared to sites from the Potomac River watershed (3%) when all years/sites/seasons were combined from fish health monitoring data. Sites in the Potomac drainage all had a prevalence of 10% or less, while at some sites in the Susquehanna (i.e. Susquehanna at Selinsgrove, Susquehanna at Harrisburg, Juniata, Chillisquaque Creek and West Branch Mahantango Creek), 30% or greater prevalence was noted at least once during the years sampled (Table 3). A caveat with the fish health monitoring data set is that only 20 fish per site/per sampling time were sampled, and many sites were only assessed once or twice between 2013 and 2018.

The current study did not specifically address seasonal or temperature effects. Data from the Rockville site would suggest an increase in HPMLs as the temperature decreases, with the exception of 2017 (Fig. 3). There was also no clear seasonal trend at 4 fish health monitoring sites sampled in both fall and spring 2016–2018. In the Potomac drainage when sampling occurred in both seasons more melanistic bass were observed in the fall. In the Susquehanna, Pine and West Branch Mahantango creeks more melanistic bass were collected in the spring in 2016 and 2017, but more in the fall in 2018. Further monitoring throughout the year, concurrently documenting water temperature and other parameters will be necessary to determine the role of temperature and other environmental stressors on this infection.

To our knowledge no previous studies have found an association between hyperpigmented areas in fish skin and viral pathogens, although pigmented lesions in humans have been associated with human papilloma virus (Egawa et al. 1998). Raised, papillmatous lesions, both pigmented and nonpigmented did contain viral particles in the epithelial cells of guitarfish (Camus et al. 2016). There is increasing evidence for the role of melanocytes in response to infectious diseases and tissue damage in mammalian (Gasque & Jaffa-Bandjee 2015) and piscine (Lévesque et al. 2013) hosts. This disease offers a unique opportunity to better understand the role of melanocytes in disease resistance of fish and other organisms. Studies in ze-

brafish found hyperpigmentation in response to tissue damage was due to recruitment of both melanoblasts and melanocytes to the area, and this recruitment depended on innate immune cells. Interestingly, in these studies, melanocytes varied in size, were pleomorphic and in some cases clustered around and surrounded implanted beads (Lévesque et al. 2013). In many HPMLs of smallmouth bass, pleomorphic epidermal melanocytes were often observed with dendritic projection around vacuolated and apoptotic cells.

In conclusion, the HPMLs of smallmouth bass may be associated with a new viral disease leading to the proliferation of melanocytes. Further studies will be necessary to (1) characterize the viral organism and definitely demonstrate its role in the melanistic lesions; (2) determine if the virus infects the melanocytes themselves or if the proliferation is a response to the presence of virus-infected cells; (3) understand the environmental influences associated with infection and (4) identify modes of transmission and infection. Development of image-based methods such as *in situ* hybridization will likely be necessary to identify infected cell types.

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