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Method paper

Skin Transcriptomes of common bottlenose dolphins (*Tursiops truncatus*) from the northern Gulf of Mexico and southeastern U.S. Atlantic coasts

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ABSTRACT

Common bottlenose dolphins serve as sentinels for the health of their coastal environments as they are susceptible to health impacts from anthropogenic inputs through both direct exposure and food web magnification. Remote biopsy samples have been widely used to reveal contaminant burdens in free-ranging bottlenose dolphins, but do not address the health consequences of this exposure. To gain insight into whether remote biopsies can also identify health impacts associated with contaminant burdens, we employed RNA sequencing (RNA-seq) to interrogate the transcriptomes of remote skin biopsies from 116 bottlenose dolphins from the northern Gulf of Mexico and southeastern U.S. Atlantic coasts. Gene expression was analyzed using principal component analysis, differential expression testing, and gene co-expression networks, and the results correlated to season, location, and contaminant burden. Season had a significant impact, with over 60% of genes differentially expressed between spring/summer and winter months. Geographic location exhibited lesser effects on the transcriptome, with 23.5% of genes differentially expressed between the northern Gulf of Mexico and the southeastern U.S. Atlantic locations. Despite a large overlap between the seasonal and geographical gene sets, the pathways altered in the observed gene expression profiles were somewhat distinct. Co-regulated gene modules and differential expression analysis both identified epidermal development and cellular architecture pathways to be expressed at lower levels in animals from the northern Gulf of Mexico. Although contaminant burdens measured were not significantly different between regions, some correlation with contaminant loads in individuals was observed among co-expressed gene modules, but these did not include classical detoxification pathways. Instead, this study identified other, possibly downstream pathways, including those involved in cellular architecture, immune response, and oxidative stress, that may prove to be contaminant responsive markers in bottlenose dolphin skin.

1. Introduction

Common bottlenose dolphins (*Tursiops truncatus*) are year-round inhabitants and top predators in waters along the southeastern U.S. Atlantic (SE) and Gulf of Mexico (GoM) coasts (Waring et al., 2015) and, as such, are exposed to anthropogenic chemicals, particularly those that biomagnify through the food web (Aguilar et al., 1999; Wells et al., 2004). Persistent organic pollutants (POPs), such as polychlorinated biphenyl (PCB), legacy pesticides, etc. are particularly an issue because they magnify and bioaccumulate in the blubber. Remote

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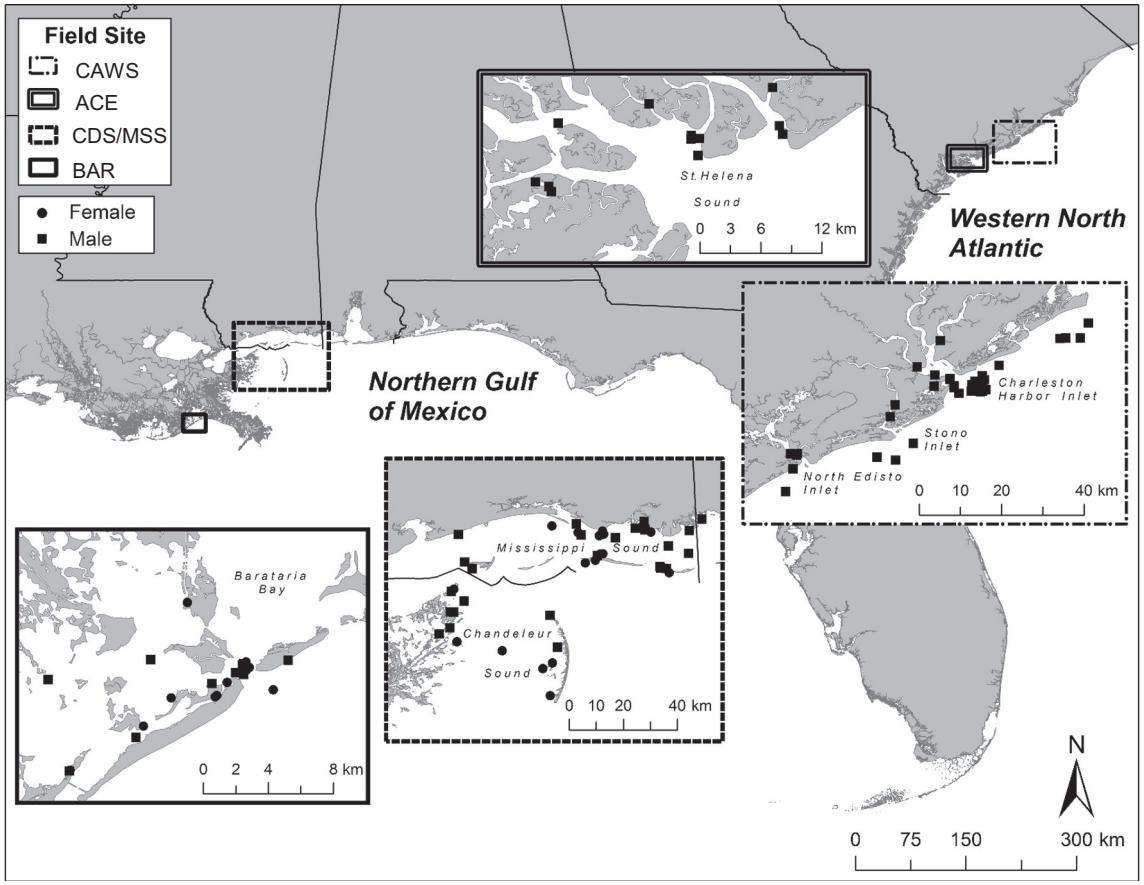

Fig. 1. Location of biopsy samples collected near Charleston, SC: the Cooper, Ashley, Wando, and Stono River Basin (CAWS) and further south in the Ashepoo, Combahee, and Edisto River Basin (ACE) in waters along the southeastern U.S. Atlantic (SE) and in the northern Gulf of Mexico: Barataria Bay (BAR), Chandeleur Sound (CDS), and Mississippi Sound (MSS).

biopsies of skin and blubber have been used extensively to obtain information on contaminant exposure in bottlenose dolphins (Kucklick et al., 2011; Balmer et al., 2015).

Because bottlenose dolphins have a lipid-rich blubber layer, this tissue compartment serves as a primary storage site for lipophilic contaminants. In males, the concentration of blubber contaminants is generally reflective of their environment (Kucklick et al., 2011), however reproductively mature females transfer much of their stored contaminant loads to their offspring through lactation, and thus the concentration and composition of blubber POPs in females may change substantially in relation to reproductive state. For this reason, POP concentrations are typically monitored in males (Yordy et al., 2010). In addition to blubber, skin is often sampled to investigate exposure of bottlenose dolphins to inorganic pollutants, such as mercury (Bryan et al., 2007; Woshner et al., 2008).

Organic or inorganic contaminant concentration in cetacean blubber and skin can help us understand levels of exposure, but these measures do not provide direct insight into the health effects of such exposures. Health assessment studies, which involve temporary capture and restraint to conduct physical and/or ultrasound examination and sampling of blood for diagnostics, have explored health endpoints in bottlenose dolphins in relation to some chemical exposures. For example, hypothyroidism, anemia, and immunosuppression were associated with high, site-specific polychlorinated biphenyl (PCB) contamination in dolphins resident to an embayment adjacent to a Superfund site in Georgia (Wells et al., 2004). Similarly, resident dolphins in Barataria Bay, Louisiana, which was heavily oiled following the explosion of the *Deepwater Horizon* (DWH) drilling platform in the Gulf of Mexico, were found to have a high prevalence of lung disease and adrenal insufficiency, both consistent with exposure to petroleum hydrocarbons (Reif et al., 2006). However, such studies are expensive and labor-intensive to conduct, and are generally limited to dolphin stocks in shallow estuarine waters where the dolphins can be safely captured and temporarily restrained for processing. Thus, health assessment methods that can be conducted remotely (e.g. visual assessment), or via remote sampling of skin or blubber, are highly desirable.

Efforts have been made to interrogate remotely collected samples of blubber and skin for the expression of genes or proteins that demonstrate the activation of detoxification pathways or changes in expression of receptors, which may relate to altered endocrine or other adverse health effect pathways. For example, the expression of the xenobiotic detoxification enzyme cytochrome P450 1A1 (Cyp1A1) correlates with organic contaminant exposure in bottlenose dolphins (Wilson et al., 2007; Montie et al., 2008) and sperm whales (*Physeter macrocephalus*) (Godard-Codding et al., 2011) as assessed by immunohistochemistry. Similarly, qPCR measurements of transcript abundance of selected nuclear receptors involved in xenobiotic detoxification and immune function correlate with persistent organic contaminant levels in harbor seals (*Phoca vitulina*) (Tabuchi et al., 2006) and killer whales (*Orcinus orca*) (Buckman et al., 2011). Therefore, the transcript abundance of this suite of genes has been proposed as a suitable suite of biomarkers for adverse health effects from some contaminant classes in cetaceans.

To broaden the scope of the genes and pathways interrogated in dolphin skin, we recently investigated global gene expression in skin from 94 common bottlenose dolphin remote biopsy samples using a dolphin-specific microarray (Van Dolah et al., 2015). This study using samples from dolphins in the northern Gulf of Mexico (NGoM) identified for the first time a significant seasonal influence on skin gene expression, with one-third of the genes queried being significantly

different in expression level between winter and summer. In parallel, POP concentrations in the blubber of males changed almost two-fold between winter and summer, likely due to seasonal thinning of the blubber (Balmer et al., 2015). Since many genes involved in detoxification pathways are also involved in the biosynthesis and metabolism of endogenous compounds, these results suggest the need to distinguish responses of the skin transcriptome to contaminant exposure versus intrinsic changes associated with temperature, hormonal cycles, and other natural influences. To our knowledge previous studies on gene expression in skin have not taken season into account.

Here we further characterize the bottlenose dolphin skin transcriptome using RNA sequencing (RNA-seq) in a subset of 65 male and female skin samples from NGoM dolphins previously studied by microarray (Van Dolah et al., 2015). In order to interrogate contaminant related gene expression, we next compare samples from 35 NGoM males with 51 samples collected from male dolphins along the southeastern U.S. Atlantic coast (SE), including waters near Charleston, SC: the Cooper, Ashley, Wando, and Stono River Basin (CAWS) and further south in the Ashepoo, Combahee, and Edisto River Basin (ACE Basin, ACE) (Fig. 1). We compare seasonal patterns of gene expression in the two regions, season-independent differences in gene expression between regions, and gene expression associated with different POP concentrations. The RNA-seq method has an advantage over microarrays for investigating both known transcripts and exploring new ones, and has the ability to quantify over a larger dynamic range of expression levels, including transcripts with very low levels of expression, which may include important components of a response pathway. The microarray used in the previous study (Van Dolah et al., 2015), the best tool available at the time (Mancia et al., 2015), contained 24,418 sequence probes; however, only 7281 were annotated, and some of the key xenobiotic pathway members were missing, including aryl hydrocarbon receptor (AhR), as well as cytochrome P450 enzymes, Cyp1A1and Cyp1B1. Since that time, a low coverage (2.59x) common bottlenose dolphin genome has been sequenced and partially annotated (Lindblad-Toh et al., 2011), providing improved ability to identify transcripts expressed. All transcriptomic analyses in this study were performed with the dolphin genome annotation currently available through ENSEMBL as the reference genome.

2. Methods

2.1. Samples

The bottlenose dolphin skin biopsies from the NGoM were collected 2010–2012, prior to, during and after visible oiling in sampling locations following the explosion of the DWH drilling platform on April 20, 2010. The explosion precipitated the release of millions of gallons of Louisiana crude oil that resulted in heavy oiling of coastal embayments of Louisiana and Mississippi and consequent exposure of resident dolphin populations (Lane et al., 2015). Remote biopsy samples included in this study were collected from free ranging bottlenose dolphins at three field-sites (Barataria Bay (n = 22) and Chandeleur Sound (n = 14), Louisiana and Mississippi Sound (n = 29), Mississippi) in the NGoM (n = 65). Samples were collected from Barataria Bay and Mississippi Sound in May 2010, before oil was observed on the coast, and Chandeleur Sound in late May 2010 after initial oiling. Samples were collected again from all three locations, after oiling was visually observed in each location, in August–September 2010, December 2010–February 2011, and the following year in August–September 2010, January–February 2011, and the following year in August and December 2011–February 2012 - February 2012 (Fig. 1).

On the SE coast, Charleston Harbor is the eighth largest port in the United States with contaminant profiles that reflect its urban setting, shipping activity, as well as industrial past (Van Dolah et al., 2006; Van Dolah et al., 2008). In contrast, the ACE Basin is a National Estuarine Research Reserve in South Carolina and was selected for inclusion in this study because it has lower contaminant levels in water and sediments than Charleston Harbor, consistent with its rural location (Scott et al., 1998). Remote biopsy samples included in this study were collected from free ranging male bottlenose at the ACE Basin (n = 13) (June–August 2012); and CAWS (n = 38) (May–July 2012, November–December 2013, February 2014, July–August 2014).

Samples collected in this study were primarily from dolphins that were members of Bay, Sound, and Estuary (BSE) Stocks that have characteristics of long-term residence and localized movements (Waring et al., 2015). In the NGoM, the BSE Stocks sampled were Barataria Bay, Mississippi Sound, Lake Borgne, and Bay Boudreau Stocks. Samples were also collected within Chandeleur Sound, part of the Northern Coastal Stock area. In the southeastern U.S., the BSE Stocks sampled were: the Charleston Estuarine System and Northern Georgia/Southern South Carolina Estuarine System (includes ACE Basin) Stocks. In addition to these BSE Stocks, the Northern Coastal Stock (NGoM) and South Carolina-Georgia Coastal Stock (southeastern U.S.) overlap to some degree with the respective, adjacent BSE Stocks. Individuals from these coastal Stocks may have been sampled and included in subsequent analyses. A detailed list of individuals included in this study is presented in Table 1.

Biopsy samples were collected using previously described protocols (Sinclair et al., 2015), under the authority of Marine Mammal Protection Act Scientific Permits No. 779–1633 and No. 14450. Briefly, biopsy samples were collected using a modified rifle or a Barnett Panzer V crossbow (Barnett Outdoors, LLC, Tarpon Springs, FL, USA) at a distance of 3–10 m, targeting the flank of the animal below the dorsal fin and above the midline. The sample obtained was a cylindrical core measuring approximately 10 mm by 15–20 mm, consisting of skin and a full-thickness section of blubber approximately 0.7–0.8 g in weight. A subsample consisting of one quarter of the skin and blubber was taken for transcriptomics and placed in a 2.5 ml cryovial and snap frozen in a liquid N_2 vapor shipper (NGoM samples) or placed in RNAlater (SE samples) and stored on ice until transfer to the laboratory where they were placed in a refrigerator overnight, and thereafter stored at − 80 °C until processing.

For sex determination, genomic DNA was extracted from a subsample of the epidermis from the biopsy and sex was determined using a multiplex PCR reaction which simultaneously targets the ZFX and SRY genes (Rosel, 2003).

3. Contaminants

Persistent organic pollutant levels in blubber of the NGoM males used in this study were first reported by Balmer et al. (2015). Male dolphins from the NGoM (24), CAWS (13), and ACE (13) were sampled in spring and summer to assess POP concentrations in blubber. POP analyses of the NGoM blubber samples were performed as described in Litz et al. (2007). A reduced set of compounds were used for this study to have a common set of compounds tested from all locations. They included a total of 41 PCB congeners (ΣPCBs) (IUPAC PCB numbers 18, 28, 31, 44, 49, 52, 66, 70, 74, 82, 87, 95, 99, 101, 105, 110, 118, 128, 138, 149, 151, 156, 158, 170, 177, 180, 183, 187, 191, 194, 195, 196, 199, 200, 201, 202, 205,206, 207, 208, 209), 5 polybrominated diphenyl ether congeners (ΣPBDEs) (47, 99, 100, 153,154), 5 dichlorodiphenyl-dichloroethanes (ΣDDTs) (*o,p′*-DDD, *o,p′*-DDE, *o,p′*-DDT, and *p,p′*-DDE, and *p,p′*-DDT), 3 chlordanes (ΣCHLs) (cis-nonachlor, oxychlordane, and trans-nonachlor), ashexachlorobenzene (HCB), dieldrin, and mirex. Total POPs (ΣPOPs) included all 57 analyzed compounds and total organochlorine pesticides (ΣOCPs) included the sum of ΣDDTs, ΣCHLs, HCB, dieldrin, and mirex.

3.1. Statistical analyses

POP concentrations were lipid-normalized to reduce the variations in lipid content due to factors such as nutritional status (Struntz et al.,

Table 1
Samples included in this study, sorted by location.

Field sample #	Collection date	Season	Location[a]	Collection location[b]	Latitude	Longitude	Sex
BW120220-01	2/20/2012	Winter	NGoM	Barataria Bay, Louisiana	29.2172	− 90.0478	Female
BW120221-02	2/21/2012	Winter	NGoM	Barataria Bay, Louisiana	29.2673	− 90.0599	Male
BW120221-05	2/21/2012	Winter	NGoM	Barataria Bay, Louisiana	29.2783	− 90.0032	Male
BW120223-01	2/23/2012	Winter	NGoM	Barataria Bay, Louisiana	29.2168	− 90.0482	Male
BW120223-02	2/23/2012	Winter	NGoM	Barataria Bay, Louisiana	29.2352	− 90.0114	Male
BW120223-05	2/23/2012	Winter	NGoM	Barataria Bay, Louisiana	29.2657	− 89.9609	Female
BW120225-03	2/25/2012	Winter	NGoM	Barataria Bay, Louisiana	29.2577	− 89.9676	Female
R2110815-01	8/15/2011	Summer	NGoM	Mississippi Sound, Mississippi	30.3443	− 88.5962	Male
R2110823-01	8/23/2011	Summer	NGoM	Chandeleur Sound, Louisiana	30.1422	− 89.1988	Female
R2110823-03	8/23/2011	Summer	NGoM	Chandeleur Sound, Louisiana	29.9676	− 89.1887	Female
R2110824-01	8/24/2011	Summer	NGoM	Chandeleur Sound, Louisiana	29.7890	− 88.8787	Female
R2110828-01	8/28/2011	Summer	NGoM	Mississippi Sound, Mississippi	30.3224	− 88.7761	Male
R2110829-02	8/29/2011	Summer	NGoM	Mississippi Sound, Mississippi	30.2598	− 88.7038	Female
R3100508-02	5/8/2010	Spring	NGoM	Mississippi Sound, Mississippi	30.2295	− 88.7621	Female
R3100510-02	5/10/2010	Spring	NGoM	Barataria Bay, Louisiana	29.2701	− 89.9519	Male
R3100510-03	5/10/2010	Spring	NGoM	Barataria Bay, Louisiana	29.2770	− 89.9505	Female
R3100510-04	5/10/2010	Spring	NGoM	Barataria Bay, Louisiana	29.2762	− 89.9519	Male
R3100511-01	5/11/2010	Spring	NGoM	Barataria Bay, Louisiana	29.2710	− 89.9563	Male
R3100511-04	5/11/2010	Spring	NGoM	Barataria Bay, Louisiana	29.2725	− 89.9503	Female
R3100512-02	5/12/2010	Spring	NGoM	Barataria Bay, Louisiana	29.2585	− 89.9667	Female
R3100512-03	5/12/2010	Spring	NGoM	Barataria Bay, Louisiana	29.3099	− 89.9829	Female
R3100512-04	5/12/2010	Spring	NGoM	Barataria Bay, Louisiana	29.2753	− 89.9525	Male
R3100513-02	5/13/2010	Spring	NGoM	Barataria Bay, Louisiana	29.2651	− 89.9694	Male
R3100515-01	5/15/2010	Spring	NGoM	Mississippi Sound, Mississippi	30.2114	− 88.4931	Male
R3100517-02	5/17/2010	Spring	NGoM	Mississippi Sound, Mississippi	30.2379	− 88.7292	Female
R3100517-03	5/17/2010	Spring	NGoM	Mississippi Sound, Mississippi	30.1972	− 88.4843	Female
R3110208-01	2/8/2011	Winter	NGoM	Mississippi Sound, Mississippi	30.2104	− 89.1383	Male
R3110816-03	8/16/2011	Summer	NGoM	Mississippi Sound, Mississippi	30.3249	− 88.7011	Female
R3110817-01	8/17/2011	Summer	NGoM	Mississippi Sound, Mississippi	30.2854	− 88.4865	Male
R3110817-05	8/17/2011	Summer	NGoM	Mississippi Sound, Mississippi	30.2586	− 88.7131	Female
R3110820-03	8/20/2011	Summer	NGoM	Chandeleur Sound, Louisiana	29.8789	− 88.9032	Female
R3110821-02	8/21/2011	Summer	NGoM	Chandeleur Sound, Louisiana	30.1029	− 89.1643	Male
R3110823-02	8/23/2011	Summer	NGoM	Chandeleur Sound, Louisiana	30.05577	− 88.87933	Male
R3110823-03	8/23/2011	Summer	NGoM	Chandeleur Sound, Louisiana	29.9503	− 88.8550	Male
R3110828-01	8/28/2011	Summer	NGoM	Mississippi Sound, Mississippi	30.3316	− 88.5448	Female
R3110828-02	8/28/2011	Summer	NGoM	Mississippi Sound, Mississippi	30.3747	− 88.3757	Male
R3110828-03	8/28/2011	Summer	NGoM	Mississippi Sound, Mississippi	30.3370	− 88.4174	Male
R3110829-01	8/29/2011	Summer	NGoM	Mississippi Sound, Mississippi	30.2173	− 88.5151	Male
R3111202-01	12/2/2011	Winter	NGoM	Mississippi Sound, Mississippi	30.3353	− 88.7051	Female
R3111205-01	12/5/2011	Winter	NGoM	Mississippi Sound, Mississippi	30.3671	− 88.5676	Male
R3111208-01	12/8/2011	Winter	NGoM	Mississippi Sound, Mississippi	30.3392	− 88.5639	Male
R3111208-02	12/8/2011	Winter	NGoM	Mississippi Sound, Mississippi	30.3592	− 88.7920	Male
R3111209-01	12/9/2011	Winter	NGoM	Mississippi Sound, Mississippi	30.2615	− 88.4196	Male
R3111212-02	12/12/2011	Winter	NGoM	Mississippi Sound, Mississippi	30.3198	− 88.7161	Female
R3111216-01	12/16/2011	Winter	NGoM	Mississippi Sound, Mississippi	30.3521	− 88.8733	Female
R3111216-02	12/16/2011	Winter	NGoM	Mississippi Sound, Mississippi	30.3133	− 88.6623	Male
R3111220-01	12/20/2011	Winter	NGoM	Mississippi Sound, Mississippi	30.33044	− 88.7866	Female
TE100909-03	9/9/2010	Summer	NGoM	Mississippi Sound, Mississippi	30.2542	− 88.7217	Male
TE120220-03	2/20/2012	Winter	NGoM	Barataria Bay, Louisiana	29.2571	− 89.9919	Female
TYP052710-01	5/27/2010	Spring	NGoM	Chandeleur Sound, Louisiana	29.8978	− 88.8712	Female
TYP052810-02	5/28/2010	Spring	NGoM	Chandeleur Sound, Louisiana	29.9381	− 89.0385	Female
TYP100814-06	8/14/2010	Summer	NGoM	Barataria Bay, Louisiana	29.2780	− 89.9273	Male
TYP100816-01	8/16/2010	Summer	NGoM	Barataria Bay, Louisiana	29.2740	− 89.9487	Female
TYP100817-02	8/17/2010	Summer	NGoM	Barataria Bay, Louisiana	29.2723	− 89.9528	Female
TYP100818-01	8/18/2010	Summer	NGoM	Barataria Bay, Louisiana	29.2415	− 90.0072	Female
TYP100828-01	8/28/2010	Summer	NGoM	Mississippi Sound, Mississippi	30.3508	− 88.5647	Female
TYP100831-01	8/31/2010	Summer	NGoM	Mississippi Sound, Mississippi	30.2125	− 88.5126	Male
TYP100915-01	9/15/2010	Summer	NGoM	Mississippi Sound, Mississippi	30.2326	− 89.1629	Male
TYP100915-02	9/15/2010	Summer	NGoM	Chandeleur Sound, Louisiana	30.1345	− 89.2074	Male
TYP100915-03	9/15/2010	Summer	NGoM	Chandeleur Sound, Louisiana	30.0665	− 89.1992	Male
TYP100916-02	9/16/2010	Summer	NGoM	Mississippi Sound, Mississippi	30.3239	− 89.1834	Male
TYP100917-02	9/17/2010	Summer	NGoM	Chandeleur Sound, Louisiana	30.0675	− 89.2105	Male
TYP100917-04	9/17/2010	Summer	NGoM	Chandeleur Sound, Louisiana	30.0147	− 89.2121	Male
TYP100918-01	9/18/2010	Summer	NGoM	Chandeleur Sound, Louisiana	29.9940	− 89.2474	Male
TYP110111-02	1/11/2011	Winter	NGoM	Barataria Bay, Louisiana	29.2616	− 89.9354	Female
TYP120502-01	5/2/2012	Spring	SE	CAWS, South Carolina	32.77147	− 79.94424	Male
TYP120502-02	5/2/2012	Spring	SE	CAWS, South Carolina	32.82911	− 79.89382	Male
TYP120502-03	5/2/2012	Spring	SE	CAWS, South Carolina	32.72878	− 79.90706	Male
TYP120517-01	5/17/2012	Spring	SE	CAWS, South Carolina	32.66219	− 80.00282	Male
TYP120517-04	5/17/2012	Spring	SE	CAWS, South Carolina	32.68811	− 79.99229	Male
TYP120619-01	6/19/2012	Summer	SE	ACE, South Carolina	32.50082	− 80.54573	Male
TYP120620-01	6/20/2012	Summer	SE	ACE, South Carolina	32.44863	− 80.56577	Male
TYP120620-02	6/20/2012	Summer	SE	ACE, South Carolina	32.44008	− 80.55183	Male
TYP120620-03	6/20/2012	Summer	SE	ACE, South Carolina	32.43996	− 80.55172	Male

(continued on next page)

Table 1 *(continued)*

Field sample #	Collection date	Season	Location[a]	Collection location[b]	Latitude	Longitude	Sex
TYP120620-04	6/20/2012	Summer	SE	ACE, South Carolina	32.48972	− 80.42776	Male
TYP120628-03	6/28/2012	Summer	SE	CAWS, South Carolina	32.54850	− 80.21537	Male
TYP120628-04	6/28/2012	Summer	SE	CAWS, South Carolina	32.58147	− 80.20526	Male
TYP120629-01	6/29/2012	Summer	SE	CAWS, South Carolina	32.74507	− 79.87231	Male
TYP120718-03	7/18/2012	Summer	SE	CAWS, South Carolina	32.57794	− 80.20734	Male
TYP120718-04	7/18/2012	Summer	SE	CAWS, South Carolina	32.58151	− 80.22098	Male
TYP120724-01	7/24/2012	Summer	SE	ACE, South Carolina	32.47217	− 80.42187	Male
TYP120724-04	7/24/2012	Summer	SE	ACE, South Carolina	32.49846	− 80.34969	Male
TYP120731-01	7/31/2012	Summer	SE	CAWS, South Carolina	32.75371	− 79.90562	Male
TYP120731-03	7/31/2012	Summer	SE	CAWS, South Carolina	32.71359	− 79.85281	Male
TYP120731-04	7/31/2012	Summer	SE	CAWS, South Carolina	32.74626	− 79.87216	Male
TYP120731-05	7/31/2012	Summer	SE	CAWS, South Carolina	32.74382	− 79.87291	Male
TYP120814-03	8/14/2012	Summer	SE	ACE, South Carolina	32.49074	− 80.34679	Male
TYP120814-05	8/14/2012	Summer	SE	ACE, South Carolina	32.53215	− 80.35588	Male
TYP120816-01	8/16/2012	Summer	SE	ACE, South Carolina	32.51784	− 80.46529	Male
TYP120816-02	8/16/2012	Summer	SE	ACE, South Carolina	32.44472	− 80.55397	Male
TYP120823-03	8/23/2012	Summer	SE	CAWS, South Carolina	32.72582	− 79.86312	Male
TYP120824-02	8/24/2012	Summer	SE	ACE, South Carolina	32.48709	− 80.42071	Male
TYP120824-04	8/24/2012	Summer	SE	ACE, South Carolina	32.48656	− 80.42816	Male
TYP131122-01	11/22/2013	Winter	SE	CAWS, South Carolina	32.60400	− 79.95264	Male
TYP131122-02	11/22/2013	Winter	SE	CAWS, South Carolina	32.56745	− 79.99126	Male
TYP131204-02	12/4/2013	Winter	SE	CAWS, South Carolina	32.83449	− 79.58777	Male
TYP131204-04	12/4/2013	Winter	SE	CAWS, South Carolina	32.83353	− 79.63226	Male
TYP131204-05	12/4/2013	Winter	SE	CAWS, South Carolina	32.83473	− 79.62010	Male
TYP131206-01	12/6/2013	Winter	SE	CAWS, South Carolina	32.86685	− 79.56981	Male
TYP140227-02	2/27/2014	Winter	SE	CAWS, South Carolina	32.71804	− 79.80121	Male
TYP140227-03	2/27/2014	Winter	SE	CAWS, South Carolina	32.72046	− 79.80311	Male
TYP140227-04	2/27/2014	Winter	SE	CAWS, South Carolina	32.72742	− 79.80157	Male
TYP140227-05	2/27/2014	Winter	SE	CAWS, South Carolina	32.73713	− 79.80152	Male
TYP140227-06	2/27/2014	Winter	SE	CAWS, South Carolina	32.75239	− 79.80206	Male
TYP140715-01	7/15/2014	Summer	SE	CAWS, South Carolina	32.74315	− 79.7975	Male
TYP140715-04	7/15/2014	Summer	SE	CAWS, South Carolina	32.73018	− 79.8636	Male
TYP140716-01	7/16/2014	Summer	SE	CAWS, South Carolina	32.72718	− 79.80527	Male
TYP140716-05	7/16/2014	Summer	SE	CAWS, South Carolina	32.72194	− 79.79371	Male
TYP140717-03	7/17/2014	Summer	SE	CAWS, South Carolina	32.57394	− 80.03168	Male
TYP140730-05	7/30/2014	Summer	SE	CAWS, South Carolina	32.73994	− 79.81757	Male
TYP140801-04	8/1/2014	Summer	SE	CAWS, South Carolina	32.74166	− 79.80238	Male
TYP140806-01	8/6/2014	Summer	SE	CAWS, South Carolina	32.49926	− 80.23122	Male
TYP140807-01	8/7/2014	Summer	SE	CAWS, South Carolina	32.7746	− 79.76502	Male
TYP140808-01	8/8/2014	Summer	SE	CAWS, South Carolina	32.71722	− 79.80578	Male
TYP140808-05	8/8/2014	Summer	SE	CAWS, South Carolina	32.73223	− 79.82436	Male
TYP140815-02	8/15/2014	Summer	SE	CAWS, South Carolina	32.71884	− 79.82585	Male

[a] Northern Gulf of Mexico (NGoM), southeastern U.S. Atlantic (SE).
[b] Ashepoo, Combahee, Edisto River Basin (ACE), South Carolina.

2004) and were log-transformed to meet the statistical assumptions of equal variance and normality. Concentrations of POPs are reported as μg/g (lipid weight). Prior to statistical analyses, concentration values below the limit of detection (LOD) were replaced with 0.5 of the LOD and analytes with a detection rate of < 75% were removed (Kucklick et al., 2011). The Fit Least Squares method (JMP 12.1.0, SAS Institute, Cary, NC) (JMP, 2015)was used to compare percent lipid, ΣOCPs, and ΣPOPs including concentrations of the various POP classes (ΣPCB, ΣDDT, ΣCHL, ΣPBDE, HCB, dieldrin and mirex) among the NGoM and SE animals and separately the CAWS and the ACE animals. LSMeans Student's t –test was used to identify the statistical differences among the field sites.

4. RNA extraction and sequencing

Samples collected from the NGoM bottlenose dolphins were processed in two tiers, at the end of year 1 (n = 31) (May 2010-Feb 2011) and at the end of the second year (n = 34) (August 2011-Feb 2012). Within a tier, all samples were randomized prior to RNA extraction. The combined ACE and CAWS samples (n = 51) were randomized prior to processing. Approximately 0.05–0.15 g of skin was dissected from the frozen biopsy using a scalpel, on dry ice, just medial to a white line demarcating the fibrous layer of the dermis and further cut into pieces ≤ 0.2 cm thick. For the NGoM samples that were flash frozen and not placed in RNAlater, the frozen dissected sample was transferred to 1.5 ml of RNAlater ICE (Ambion) prechilled to − 80 °C and incubated for at least 16 h at − 20 °C to transition the tissue from − 80 °C to 20 °C.

Samples were removed from the preservative and placed in a 2.0 ml microcentrifuge tube containing 1 ml Qiazol (Qiagen) and a 5 mm stainless steel bead, and homogenized 3 × using a Qiagen Tissuelyser at 20 Hz for 3 min, cooling on ice for 30 s between sets. The homogenate was transferred to a new 2.0 ml microcentrifuge tube and incubated on the benchtop at room temperature for 5 min before adding 200 μl of chloroform. The tube was then shaken vigorously for 15 s then incubated on the benchtop at room temperature for 3 min. After 15 min of centrifugation at 12,000 × g at 4 °C, 500 μl of the upper aqueous phase was transferred to a new 1.5 ml microcentrifuge tube containing 500 μl of 70% ethanol, mixed by pipetting, and then immediately transferred to a Qiagen RNeasy spin column. The protocol for Purification of Total RNA using the RNeasy Lipid Tissue Mini Kit with on-column DNase digestion (Qiagen RNeasy Lipid Tissue Handbook 02/2009) was followed exactly from this point on with a 60 μl final elution in RNase-free water. RNA quantity was determined using a Nanodrop ND-1000 spectrophotometer and quality was assessed using an Agilent 2100 Bioanalyzer. Only samples with an RNA integrity number (RIN) of ≥ 7.0 were used in the study. Samples were sent to North Carolina State University Genomics Service Laboratory for library preparation and sequencing using a NEBNext Ultra Directional RNA Library Prep Kit for

Illumina, indexed with the NEBNextMulitplex Oligos for Illumina. Sequencing was performed on an Illumina HiSeq 2500 sequencer, at a targeted depth of 28 million, 100 nucleotide single end reads.

5. Sequence processing and analysis using the ensembl bottlenose dolphin genome

Illumina BCL output files were converted to FASTQ-sanger file format in Galaxy (Goecks et al., 2010; Blankenberg et al., 2010a; Giardine et al., 2005) with FASTQ Groomer (Blankenberg et al., 2010b). Sequence quality trimming was performed using Trimmomatic (Bolger et al., 2014) with a minimum quality score of phred > 20 over the length of the reads. The trimmed reads were then quality checked using the FASTQC tool. Reads were mapped to the Ensembl *T. truncatus* genome, version turTru1 v76.1, with Bowtie2 v 2.2.4 (Langmead and Salzberg, 2012) as the alignment engine within the package RNA-Seq by Expectation Maximization (RSEM; (Li and Dewey, 2011)), generating mapped read counts as FPKM (fragments per kilobase of transcript per million mapped reads). The raw reads and summarized FPKMs for all samples are available on GEO (Series GSE90941). Differential expression was performed using EBseq (Leng et al., 2013) with quartile normalization, a minimum alignment count of 10, and using a false discovery rate of 0.05. Gene enrichment analysis (Fishers Exact test) was performed in Blast2GO (Conesa et al., 2005; Conesa and Götz, 2008; Götz et al., 2008; Götz et al., 2011). Pathway mapping was performed in Blast2GO and WebGestalt (Zhang et al., 2005; Wang et al., 2013).

6. Principal components analysis

Principal components analysis (PCA) was performed on $\log_2$ transformed FPKM values to identify subgroups within the samples based on the variance of the first and second principle components. To avoid correlation artifacts due to zero read counts, we excluded genes with zero read counts in more than the sample size for the condition with the most replicates in each comparison, and then further removed genes with an average expression < 1 FPKM across all samples, which can have artificially high variance due to sampling depth. PCA was performed using the prcomp function from the stats package in R Studio (v 0.99.878) (RStudio_Team, 2015). The plots were visualized using ggplot2 (Wickham, 2009).

7. Cluster analysis

Unsupervised two-dimensional hierarchical clustering was carried out in R Studio (v 0.99.878) on $\log_2$ transformed FPKM values filtered as for PCA. This was used to identify samples clustering together based on variation in gene expression. The DESeq2 package (v 1.10.1) and pheatmap package (v 1.0.8) utilized a Euclidean distance metric and Ward's linkage rule.

8. Co-expression network analysis

A gene co-expression network was generated using WGCNA (Langfelder and Horvath, 2008) using $\log_2$ transformed FPKM values, filtered as for PCA. An unsigned co-expression network was constructed on all pairwise Spearman correlations of gene expression. To weight highly correlated genes, correlation coefficients were then raised to a soft thresholding power (β) of 10, as determined by scale-free topology (Zhang and Horvath, 2005). For network construction, a minimum module size of 30 was used with a detect cut height of 0.95 and a merge cut height of 0.25. The resulting modules were then tested for their association with sample traits by correlating module eigengenes with physical measurements (e.g., season, location, contaminants). Gene ontology enrichment analysis was performed on individual gene co-expression modules compared to a background of all genes expressed in skin (ave FPKM ≥ 1 and FPKM > 0 in at least 65 samples) using Fishers exact test in Blast2GO (Conesa et al., 2005; Conesa and Götz, 2008; Götz et al., 2008; Götz et al., 2011). Pathways enrichment analysis was conducted in Webgestalt (Zhang et al., 2005; Wang et al., 2013) using the hypergeometric test.

9. Results and discussion

9.1. The common bottlenose dolphin skin transcriptome

A genome-guided transcriptome assembly was conducted using the Ensembl *T. truncatus* genome, version turTru1 v76.1 which includes 21,265 genes. To limit analyses to only the expressed transcripts, a set of 17,475 sequences, comprised of coding sequences and pseudogenes (included after identifying mapped reads in skin), were selected from the genome for further analyses. Next, to avoid correlation artifacts due to zero read counts, we excluded genes with zero read counts in more than the sample size for the condition with the most replicates (NGoM, $n = 65$), then further removed genes with an average expression < 1 FPKM across all samples. Using these criteria, skin was found to express 11,254 genes (64.4%), representing a wide range of molecular functions and biological processes. This set of 11,254 genes was used as the background skin expression for all other analyses. GO terms significantly enriched in the skin transcriptome, relative to the entire genome, included those associated with translation, peptide biosynthesis, keratin, the cornified envelope, and mitochondrial electron transport (Fisher's Exact test, FDR < 0.05). Only signal transduction was significantly under-enriched among skin transcripts (Fisher's Exact test, FDR = 0.038). The GO terms assigned to the 100 most highly expressed transcripts in skin represent molecular functions, biological processes, and cellular components involved in macromolecule and nitrogen compound biosynthesis, protein metabolism, gene expression, oxioreductase functions and cytoskeletal structure (Fig. 2), with significant enrichment of functions and processes associated with translation, keratin, the cornified envelope, and mitochondrial electron transport (Fisher's Exact test, FDR < 0.05).

A previous microarray study of gene expression in dolphin skin from the NGoM identified a strong seasonal component to gene expression, wherein approximately 25% of array features were differentially expressed between winter and summer/spring, and season accounted for 32.6% of variability by PCA (Van Dolah et al., 2015). A subset of samples from this study was analyzed by RNA-seq here, which resulted in nearly double the number of annotated sequences available for analysis. Similar to the previous microarray study, PCA on the 35 male and 30 female dolphins from the NGoM revealed a strong seasonal effect on global gene expression. A plot of principle components 1 (26% of variance) and 2 (15% of variance) clearly shows the samples clustering into two groups with winter as one group and spring and summer together as a group along the PC1-axis (Fig. 3A). No grouping was apparent according to sex, as previously observed in the microarray study. In fact, only eight genes were differentially expressed between males and females (EBseq, FDR < 0.05). Of the five that were annotated, KRT36 is an autosomal gene in humans while CLCN4, KDM5C, WWC3, and SYAP1 are all located on the X chromosome in humans. CLCN4 is expressed more highly in males, while all other x-linked genes are more highly expressed in females. Loose groupings by location were somewhat apparent on the PC2-axis, but not as distinctly as season in PC1. Gene set enrichment analysis of the top 100 and 200 genes for PC1 or PC2 based on loadings did not reveal significant enrichment for specific Gene Ontologies or KEGG pathways.

As in the NGoM animals, we also saw a strong seasonal effect on global skin gene expression in CAWS dolphins collected during the winter and summer months, all of which were males (Fig. 3B). PCA clearly identified two groups along PC1 (x-axis) corresponding to season, which explains 47% of the variance. Three groups are apparent within PC2, but PC2 only explains an additional 8% of the total

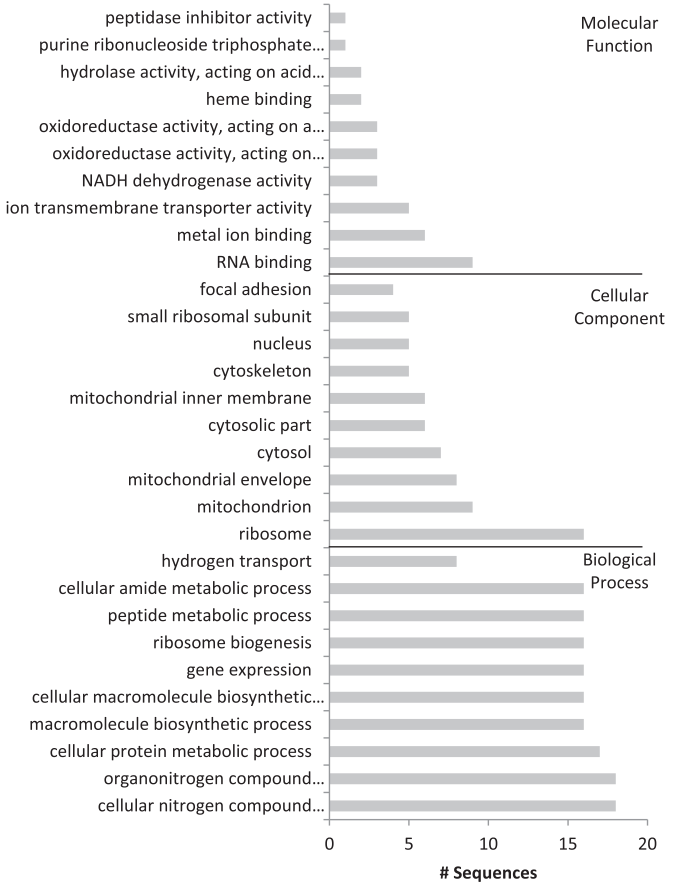

Fig. 2. Top 10 Gene Ontology (GO) annotations (level 5) from the 100 most highly expressed transcripts in dolphin skin. Forty-seven of the top 100 expressed genes mapping to the dolphin genome were annotated in Blast2GO.

variance. The attributes contributing to the variance shown with PC2 cannot be explained with the physical and physiological data available for these animals.

PCA of the 86 males from both the NGoM and CAWS was next performed, since subsequent analyses assessing the ability of skin gene expression to reveal information on contaminant exposure were limited to males. This analysis similarly showed two tight seasonal groups, with separate regional groups apparent within each season (Fig. 3C). In this plot, PC1 explains 33% of the variance while PC2 explains 14%. Three NGoM animals cluster separately from the rest of the animals on the PC2-axis, but their variance along the PC2-axis cannot be explained by any physical or measured attributes available for these animals. Two-dimensional hierarchical cluster analysis of the 86 males further illustrates that season (winter vs. spring/summer) is the prevailing source of variation in gene expression (Fig. 4). Within season, samples cluster by region, but neither specific locations within a region nor spring vs summer collection appeared to greatly impact gene expression.

9.2. Seasonal patterns of skin gene expression

Among NGoM animals, 60% of genes in the dolphin genome were differentially expressed between winter and spring/summer months (EBseq, FDR < 0.05, Supplemental Table S1), higher than that observed by microarray (Van Dolah et al., 2015). As in the earlier microarray study, approximately half were more highly expressed in winter (0.12 to 5.43 $\log_2$ fold change, ave. = 0.75) while the other half were more highly expressed in warmer months (−0.15 to −5.42 $\log_2$ fold change, ave. = −0.95). When all differentially expressed genes were considered together, there was no significant enrichment of KEGG

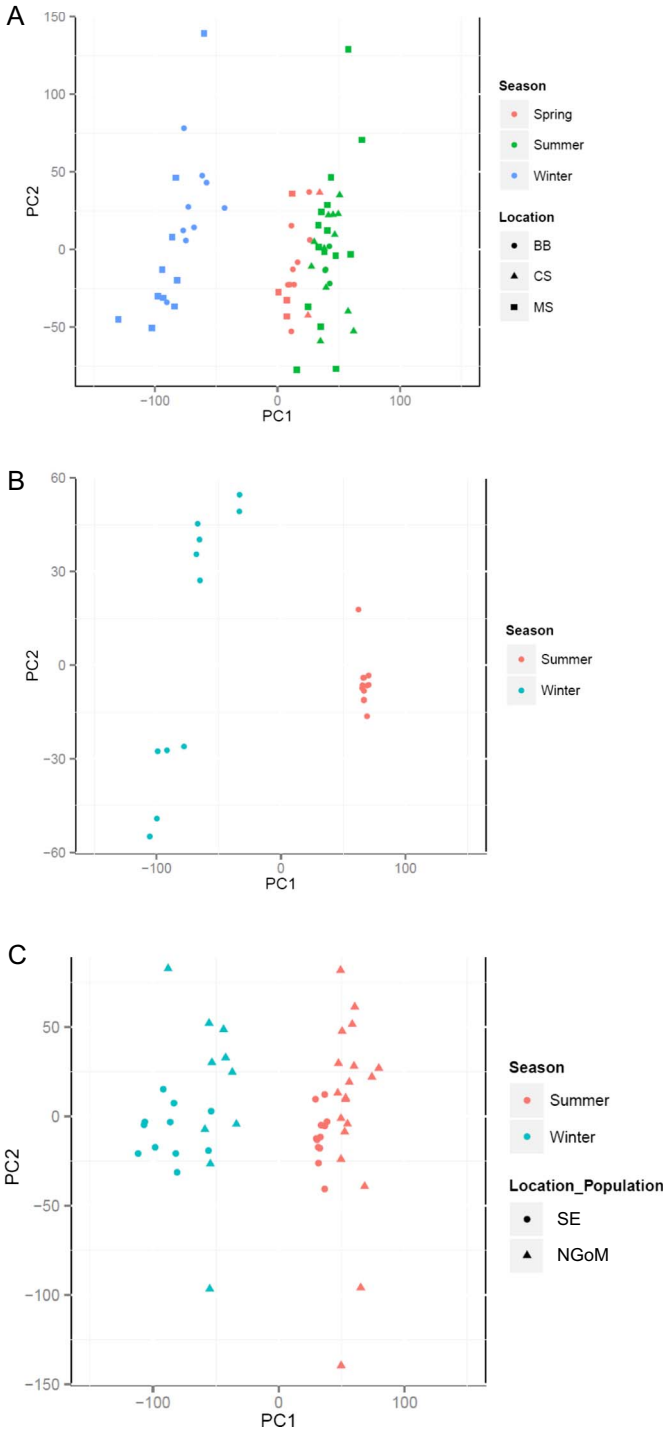

Fig. 3. A. Principle components analysis (PCA) of 65 male and female dolphins collected in the NGoM with samples collected in the winter months clustering as one group and spring and summer as another group along the PC1 axis. B. PCA of CAWS male dolphin samples collected in summer and winter months shows two distinct groups clustering along the PC1 axis corresponding to season. C. PCA of 86 male dolphins from both the NGoM and CAWS shows two tight seasonal groups along the PC1 axis with separate regional groups apparent within each season.

or Wiki pathways. However, enrichment was evident when examining gene sets expressed more highly in spring/summer or winter separately.

Genes expressed more highly in spring/summer map with significant enrichment to the following KEGG pathways: metabolic pathways ($p_{adj.} = 2.69e^{-7}$) and peroxisome ($p_{adj.} = 0.0142$). In contrast, genes expressed more highly in winter were significantly enriched for

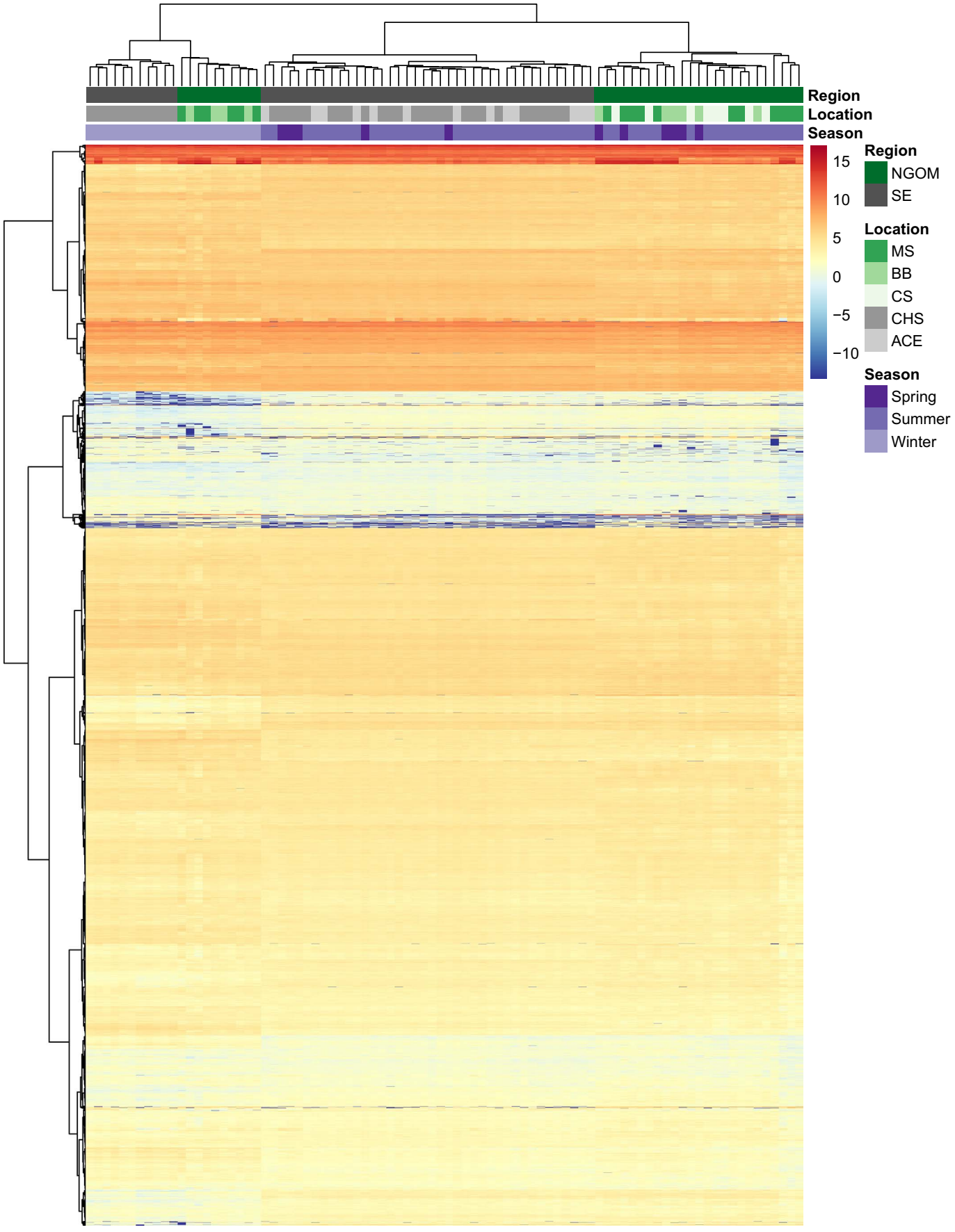

Fig. 4. Two-dimensional hierarchical cluster analysis of 86 male dolphins collected in the NGoM or CAWS using gene expression data from the 11, 550 genes defined as expressed in dolphin skin from this study. Season (winter v spring/summer) is the primary variable driving clustering, followed by region. Clustering was performed using a Euclidean distance metric and wards linkage rule.

the Wnt signaling pathway ($p_{adj.}$ = 0.0155, KEGG), Hippo signaling pathway (P_{adj} = 0.0155, KEGG) diurnally regulated genes with circadian orthologs ($p_{adj.}$ = 0.00929, Wiki pathways), FSH signaling ($p_{adj.}$ = 0.00929; Wiki pathways), BDNF-TrkB signaling ($p_{adj.}$ = 0.00929; Wiki pathways), EGF/EGFR signaling pathway ($p_{adj.}$ = 0.00929; Wiki pathways), serotonin receptor 2 and ELK-SRF/GATA4 signaling pathway ($p_{adj.}$ = 0.0211; Wiki pathways), and TNF alpha signaling pathway ($p_{adj.}$ = 0.0303; Wiki pathways). The canonical Wnt signaling pathway functions by modulating the levels of β-catenin, a transcriptional co-activator that controls key developmental gene expression programs (MacDonald et al., 2009). Wnt signaling is involved in early stages of skin development, hair follicle morphogenesis, and melanocyte development and homeostasis (Lim and Nusse, 2013). A critical component of the signaling pathway, β-catenin, is involved in FSH-mediated regulation of estrogen production, but is not identified in the dolphin genome. Nonetheless, the interaction between the Wnt and FSH signaling pathways is well documented and may be involved in steroidogenesis as well as skin cell differentiation (Lim and Nusse, 2013; Stapp et al., 2014). Noncanonical or alternative Wnt signaling mediates biological processes including gene expression, osteogenic differentiation, cell migration, tissue regeneration and tumorigenesis that do not involve β-catenin activity (Park Hyun et al., 2015) and may be, in the absence of an identified β-catenin, the primary pathways present in dolphin. This is further evidenced by enrichment of the Hippo signaling pathway, which has a negative role in cell proliferation and is involved in cell adhesion and cell polarity. There is significant cross talk between regulators of the Wnt (canonical and non-canonical) and Hippo signaling pathways. YAP/TAZ, both identified in the dolphin skin transcriptome and more highly expressed in winter months, are transcriptional co-activators in the Hippo signaling pathway and function as downstream effectors of the non-canonical Wnt signaling pathway (Park Hyun et al., 2015; Kim and Jho, 2014). In fact, there is known crosstalk between all pathways up-regulated in winter months and these pathways may be considered a complex network with roles in cell proliferation, differentiation, and adhesion.

When the geographic range of the study is expanded to include all male samples from the NGoM and the SE, just over 66% of genes are differentially expressed between spring/summer and winter (Supplemental Table S2). As in the NGoM-only analyses, among the genes differentially expressed by season in the full set of 86 male animals, there was no significant enrichment of any KEGG or Wikipathways, likely due to the breadth of gene functions present in the large gene set. However, when queried based on season of peak expression, differential enrichment was observed, and consistent with that seen in analysis of animals from the NGoM. The 3824 genes more highly expressed in summer (0.12 to 6.3 $\log_2$ fold change, ave. = 0.97) are enriched in the KEGG metabolic pathways ($p_{adj.} = 9.46e^{-8}$) and carbon metabolism ($p_{adj.}$ = 0.0.0357). The 3656 genes more highly expressed in winter (0.15 to 6.52 $\log_2$ fold change, ave. = 0.78) are significantly enriched in genes mapping to the Hippo ($p_{adj.}$ = 0.00467) and Wnt (padj. = 0.00467) signaling KEGG pathways, the mRNA surveillance KEGG pathway ($p_{adj.}$ = 0.0341) and the TNF alpha signaling Wikipathway ($p_{adj.}$ = 0.00666). Thus, it appears that the seasonal impacts on gene expression are conserved between distinct geographic regions.

A recent study of the dolphin blood transcriptome identified only a small seasonal response (< 2% of genes differentially expressed between seasons) in managed dolphins from Kona, Hawaii (Morey et al., 2016). Interestingly, 32% (70 genes) of the seasonally expressed genes in blood are also seasonally expressed in skin, and these genes are significantly enriched for several KEGG pathways: Jak-STAT signaling pathway ($p_{adj.}$ = 0.0072), glutathione metabolism ($p_{adj.}$ = 0.0382), and antigen processing and presentation ($p_{adj.}$ = 0.0382). The Antigen Processing and Presentation KEGG map includes components of both the MHC I and MHC II pathways. Additional immune related functions were found in enriched WikiPathways for Type III and Type II interferon signaling ($p_{adj.}$ = 0.0007 and 0.0256, respectively), IL-5, and IL-7 signaling pathways ($p_{adj.}$ = 0.012 and 0.0256, respectively). These pathway enrichments are driven by the presence of STAT1, STAT2, and STAT5A in the gene set, possibly indicative of gene expression involved with response to cell stimuli and pathogens on a seasonal basis.

9.3. Regional patterns of skin gene expression

When the 35 NGoM male dolphins were compared to the 51 SE male dolphins there were 2855 genes with significantly different expression by region (23.5%, EBSeq, FDR < 0.05, Supplemental Table S3). There was significant overlap with the seasonal gene set, with 53% of these genes present in both sets. Overall, these genes exhibited greater change in response to season than to region; however, this was not a significant or highly conserved trend. When the genes that exhibited significantly different expression only in response to region (i.e., not also significantly changing between season) were queried, enrichment of the oxidative phosphorylation ($p_{adj.} = 4.3e^{-3}$), *Vibrio cholerae* infection ($p_{adj.} = 4.3e^{-3}$), Parkinson's disease ($p_{adj.}$ = 0.0386), Pathogenic *Escherichia coli* infection ($p_{adj.}$ = 0.0412), and spliceosome ($p_{adj.}$ = 0.0412), KEGG pathways and electron transport chain Wikipathway ($p_{adj.}$ = 0.0315), were observed. The enrichment of the *V. cholerae* or *E. coli* infection pathways or Parkinson's disease pathway does not likely indicate the presence of infection or disease in these animals, however, as the genes identified in the pathway were not those activated via the *V. cholerae* pathogenic cycle, rather they are genes involved in downstream responses involved in oxidative phosphorylation, membrane transport, actin polymerization, and tight junctions.

Pathway enrichment analysis of all 2855 genes significantly different between regions yielded additional evidence of regional variations in tissue and cytoskeletal structure mechanisms with significant enrichment of the adherens junction ($p_{adj.} = 9.65^{e-3}$), spliceosome ($p_{adj.} = 9.65^{e-3}$), cell adhesion molecules (CAMs) ($p_{adj.}$ = 0.0133) and tight junction ($p_{adj.}$ = 0.0144) KEGG pathways. The adherens junction, CAMs, and tight junction pathways were extremely well represented by transcripts present in dolphin skin, with only two genes in the CAMs pathway (TCR and GlyCAM1) not identified in the dolphin genome. All 28 genes differentially expressed in the adherens junction pathway are more highly expressed in animals from the SE, as are all 23 or 24 genes differentially expressed in the CAMs or tight junction pathways, respectively. Even though more than half of the genes mapping to this pathway are also differentially expressed by season, their seasonal responses are varied (13 higher in winter, 16 higher in summer) and there is no significant enrichment of the adherens junction CAMs, or tight junction pathway among the seasonally expressed genes, indicating that location has the greater impact on expression. Nonetheless, it is interesting to note that there is significant crosstalk between the mechanisms that control cellular architecture and Wnt pathways with diverse impacts on tissue and organ formation and tumorigenesis (Amin and Vincan, 2012). For example, decreased cadherin levels coupled with increased Wnt signaling increases the epithelial-mesenchymal transition in carcinomas (Heuberger and Birchmeier, 2010). Seasonal gene expression from this study exhibits this pattern with increased Wnt signaling and decreased expression of cadherin during cooler months.

Given that adherens junctions are important in tissue architecture and cell polarity, and can limit cell movement and proliferation, that CAMs play a critical role in diverse biological processes including cell proliferation, differentiation and survival, regulation of gene expression, hemostasis, immune response, and inflammation, and that tight junctions are fundamental to establishing a selectively permeable barrier between cells, we queried the genes more highly expressed in each region separately in an attempt to elucidate more specific regional differences. Among the genes more highly expressed in the SE, there is significant enrichment of several additional pathways (Table 2), indicating a strong cytoskeletal reaction. Likewise, enrichment of the KEGG pathway for arrhythmogenic right ventricular cardiomyopathy

Table 2
KEGG pathways significantly enriched among the genes more highly expressed in the south eastern U.S.

KEGG pathway	*p*-value	# Genes[a]
Adherens junction	5.28E-07	26/51
Tight junction	6.26E-07	35/85
Cell adhesion molecules	2.98E-06	22/43
Focal adhesion	7.15E-05	42–133
Leukocyte transendothelial migration	1.42E-04	26/68
Proteoglycans in cancer	5.70E-04	42/145
Pathogenic *Escheria coli* infection	1.56E-03	16/37
Amoebiasis	2.25E-03	20/54
Hippo signaling pathway	3.21E-03	32/109
Regulation of actin cytoskeleton	3.44E-03	36/129
PI3K-Akt signaling pathway	7.29E-03	48/195
Bacterial invasion of epithelial cells	8.65E-03	19/56
AGE-RAGE signaling pathway in diabetic complications	1.06E-02	23/75
Endocytosis	1.33E-02	45/186
Gap junction	1.92E-02	16/47
Malaria	1.94E-02	23-Oct
EGFR tyrosine kinase inhibitor resistance	2.03E-02	19/61
Pentose phosphate pathway	2.27E-02	9/20
Shigellosis	2.27E-02	20/53
ErbB signaling pathway	2.30E-02	20/67
Fc gamma R-mediated phagocytosis	2.30E-02	18/58
Vibrio cholerae infection	2.80E-02	13/37
Salmonella infection	3.59E-02	17/56
HIF-1 signaling pathway	3.59E-02	21/75
Complement and coagulation cascades	3.66E-02	9/22
Pertussis	3.66E-02	14/43
Platelet activation	3.79E-02	21/76
Long-term depression	4.09E-02	12/35
endocrine and other factor-regulated calcium reabsorption	4.46E-02	10/27
Arrhythmogenic right ventricular cardiomyopathy	4.95E-02	12/36

[a] The number of genes shown is the number of genes mapping to the given pathway among the genes more highly expressed in the SE over the total number of genes mapping to the pathway from the dolphin skin transcriptome.

was driven by the presence of all but two genes involved in the adherens junction, desmosome, or gap junction portion of the pathway among the genes most highly expressed in the SE. In addition to the numerous pathways associated with CAMs, genes more highly expressed in the SE are also enriched for endocytosis, complement and coagulation cascades and Fc gamma R-mediated phagocytosis KEGG pathways, which may be involved in host-defense mechanisms. Diverse signaling pathways (ErbB, EGFR, Hippo, proteoglycans) are involved in cell proliferation, differentiation, motility, and survival and are known to be involved in progression of cancer in humans (Holbro and Hynes, 2004). The enrichment of pathways including bacterial invasion of epithelial cells, pathogenic *Escherichia coli* infection, amoebiasis, shigellosis, salmonella infection, malaria, *Vibrio cholera* infection, prion diseases, and leukocyte transendothelial migration largely involve genes associated with cytoskeletal structure and may indicate that animals in the SE may be mounting a response to a pathogenic attack. Conversely, it could be inferred that dolphins in the NGoM are unable to mount these defenses, perhaps due to suppressed immune responses associated with oil exposure. A repression of immune responses is supported by the conclusions of a recent review which found that contaminant exposure results in suppressed immune function in marine mammals (Desforges et al., 2016). Similarly, the decreased expression of genes in the Pertussis pathway may be indicative of the increased lung disease observed in dolphins following the DWH spill (Schwacke et al., 2014).

In contrast, among the genes more highly expressed in animals from the NGoM, the KEGG pathways for spliceosome ($p_{adj.} = 2.34e^{-8}$), oxidative phosphorylation ($p_{adj.} = 2.34e^{-8}$), ribosome ($p_{adj.} = 1.2e^{-7}$), Parkinson's disease ($p_{adj.} = 1.21e^{-4}$), Huntington's disease ($p_{adj.} = 8.29e^{-4}$), ribosome biogenesis in eukaryotes ($p_{adj.} = 1.23e^{-3}$), Alzheimer's disease ($p_{adj.} = 2.2e^{-3}$), and non-alcoholic fatty liver disease (NFALD) ($p_{adj.} = 9.39e^{-3}$) were significantly enriched. This enrichment is similar to that of the regional gene set unimpacted by season. The KEGG pathways for Parkinson's disease, Huntington's, Alzheimer's, and NFALD disease pathways were enriched largely due to the impact of genes involved with oxidative phosphorylation and mitochondrial dysfunction. It follows that the increased expression of many of these genes may be in response to increased oxidative stress. While the exact source of this stress is uncertain, most of the animals sampled in the NGoM were potentially exposed to crude oil following the DWH oil spill and the production of free radicals and reactive oxygen species during the metabolism of polycyclic aromatic hydrocarbons (PAHs) is known to increase oxidative stress burdens (Halliwell and Gutteridge, 2007).

9.4. Differential contaminant burdens in dolphins between and among NGoM and SE field sites

For the analysis of contaminant burdens, only samples from males collected during spring and summer from the NGoM and SE were included, due to seasonal differences previously reported in POP concentrations and blubber percent lipid content in the NGoM animals (Balmer et al., 2015). Blubber percent lipid content was significantly higher for NGoM males (30%) than SE males (24%) (F-test, $p = 0.0105$), with no significant difference observed in these SE animals sampled from ACE (24%) and CAWS (25%) (F-test, $p = 0.5977$). ΣPOPs were significantly higher in the SE males than NGoM males (F-test, $p = 0.0034$, Fig. 5A), with no significant difference observed in animals sampled from ACE and CAWS (F-test, $p = 0.9363$, Fig. 5B). Within the individual POP classes, ΣPBDEs (F-test, $p = 0.577$) and HCB (F-test, $p = 0.4991$) concentrations were not significantly different between NGoM and the SE (Fig. 5A), however within the latter animals, ΣPBDEs (F-test, $p = 0.0430$), HCB (F-test, $p = 0.0145$), and dieldrin (F-test, $p = 0.0003$) were significantly higher in CAWS than ACE (Fig. 5B). There was significantly lower ΣOCPs in NGoM males than SE males (F-test, $p = 0.0042$, Fig. 5A), with no significant difference in ΣOCPs between the CAWS and the ACE males (F-test, $p = 0.9682$, Fig. 5B).

While differences between NGoM and SE males in ΣPOP concentrations were expected, we were surprised to see no significant difference between the total POP concentrations, specifically ΣPCBs, ΣDDTs, ΣCHLs, and mirex, between the CAWS and ACE Basin. The finding of significantly higher concentrations of ΣPBDEs, dieldrin, and HCB in blubber of CAWS dolphins compared to ACE Basin dolphins is consistent with previous reports of high concentrations of these POP classes in Charleston (Kucklick et al., 2011).

9.5. Gene co-expression modules show correlation with contaminant burdens

To investigate the co-regulation of genes that may relate to environmental factors or contaminant exposure, a gene co-expression network was constructed in WGCNA using male samples from the NGoM ($n = 24$) and the SE ($n = 26$) for which we had contaminants measurements from blubber. All samples included in the analysis were collected in spring (May) or summer (June, July, August) to minimize seasonal effects on gene expression profiles. Eight co-expressed gene modules were identified (Fig. 6). The vast majority of genes were not significantly co-expressed (7507 genes), and thus were assigned to the default grey module representing genes not co-regulated. Pairwise correlations between each module eigengene (representative gene) and each of the measured parameters revealed several modules with significant associations (Fig. 6). One module of co-expressed genes showed correlation with season (brown module, 399 genes), and no other parameters measured. This module was enriched in GO terms “Rho signaling” ($p_{adj.} = 8.9e^{-06}$), histone modification ($p_{adj.} = 8.9e^{-06}$), neuron projection morphogenesis ($p_{adj.} = 1e^{-04}$), KEGG pathways focal adhesion ($p_{adj.} = 4e^{-03}$) and Notch signaling ($p_{adj.} = 1e^{-02}$).

Modules with the strongest positive correlation to any condition

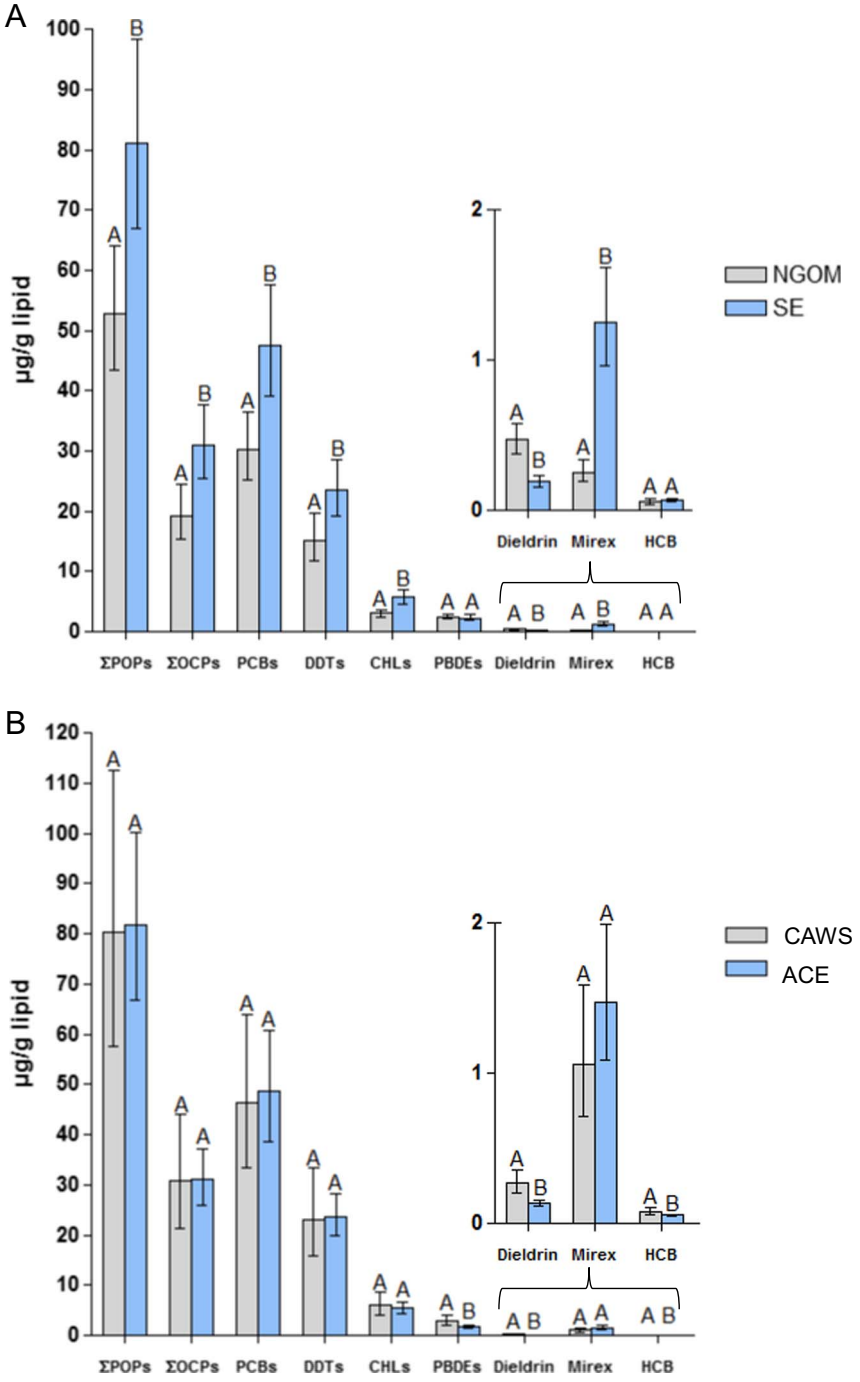

Fig. 5. A. POP concentrations (µg/g lipid; geometric mean, 95% CI) measured in remote biopsy blubber samples of male bottlenose dolphins collected in the NGoM and SE during spring and summer months. Statistically homogeneous groups are indicated by the same letter subscripts. B. POP concentrations (µg/g lipid; geometric mean, 95% CI) measured in remote biopsy blubber samples of male bottlenose dolphins collected in the ACE and CAWS basins during spring and summer months. Statistically homogeneous groups are indicated by the same letter subscripts.

were the pink (49 genes, $r = 0.84$; $p_{adj.} = 2e^{-14}$) and blue (1361 genes, $r = 0.82$; $p_{adj.} = 2e^{-13}$) modules and the region the sample was collected from (i.e., positively correlated with the SE). These modules were also more modestly correlated to mirex ($r = 0.39$–0.45), chlordane ($r = 0.34$), ΣPCBs ($r = 0.32$–0.36) and ΣPOPs ($r = 0.28$–0.32), all of which were higher in the SE animals than NGoM, and were inversely correlated with dieldrin ($r = 0.46$–0.48), which was lower in the SE animals compared to the concentrations in the NGoM animals. The blue module was most significantly enriched in GO process terms antigen processing ($p_{adj.} = 1.9e^{-03}$), epidermis development ($p_{adj.} = 2.4e^{-03}$) and nerve growth factor receptor signaling ($p_{adj.} = 5.3e^{-03}$) and KEGG pathways protein processing in the ER ($p_{adj.} = 2.0e^{-04}$), pentose phosphate pathway ($p_{adj.} = 7e^{-04}$), and neurotrophin signaling ($p_{adj.} = 7e^{-03}$). The pink module contained only six annotated sequences of the 49 present in the module and thus could not be tested for enrichment. When both modules were combined, the enrichment did not differ from that of the blue module alone. The GO term enrichment of epidermis development observed in these modules correlated with increased gene expression in animals from the SE is indicates similar responses as described by KEGG pathway enrichment (CAM and adherens junction) in the differential expression analysis above.

Two modules were inversely correlated with region (i.e., positively correlated with NGoM origin): the yellow (168 genes, $r = -0.66$; $p_{adj.} = 2e^{-07}$) and green modules (130 genes, $r = 0.52$; $p_{adj.} = 1e^{-04}$). The green module was positively correlated with dieldrin ($r = 0.54$), which was higher in NGoM animals. The enriched GO terms in this module included cilium ($p_{adj.} = 3e^{-04}$), chromatin remodeling

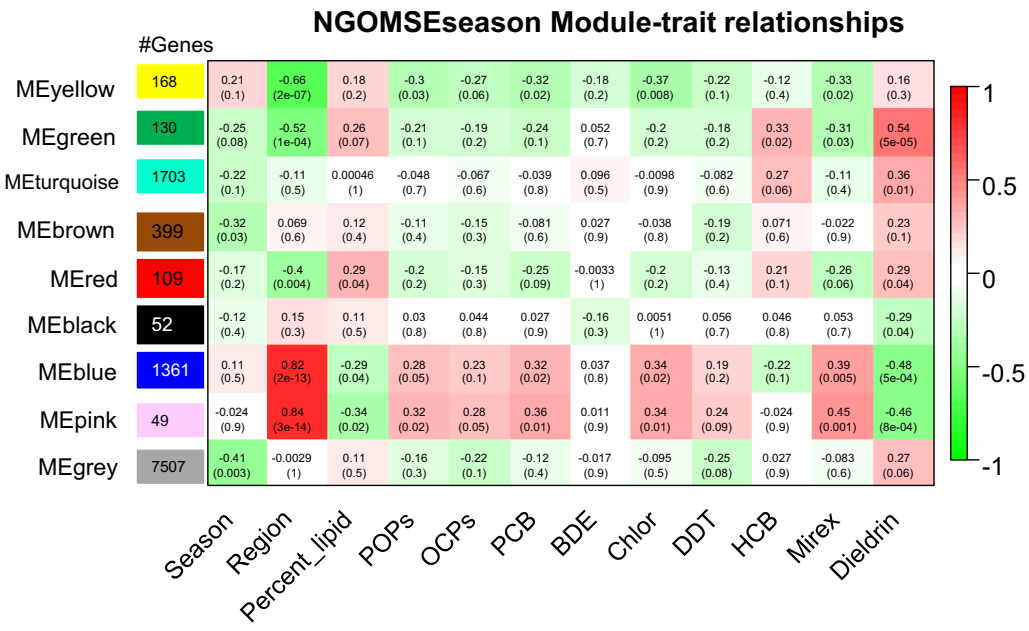

Fig. 6. Weighted gene expression co-variance network analysis (WGCNA) of male samples from the northern Gulf of Mexico (NGoM) (n = 24) and the southeastern U.S. Atlantic coast (SE) (n = 26) identified eight co-expressed gene modules. Correlation matrix of modules with sample traits and contaminants: red is positively correlated, green is negatively correlated. The correlation coefficient between the module eigengene and the measured trait is listed for each pairwise correlation, with significance in parentheses (adjusted P value). Number of genes in each module is listed at left. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

($p_{adj.} = 3.7e^{-02}$), and transcription ($p_{adj.} = 2.6e^{-02}$). No KEGG pathways were enriched. The yellow module was modestly inversely correlated with most other contaminants, consistent with their lower concentration in NGoM animals. The yellow module was strongly enriched in ribosome ($p_{adj.} = 1.3e^{-47}$) and oxidative phosphorylation ($p_{adj.} = 4.9e^{-15}$), which were also found more highly expressed in NGoM animals in the differential expression analysis above.

To further explore the association of gene expression profiles with contaminant burdens, we ran the gene co-expression network analysis on the 26 SE animals only, with animals assigned as CAWS or ACE Basin (Fig. 7). This network identified four modules, with the vast majority of genes not displaying significant co-expression (default grey module). The blue module was positively correlated with HCB (r = 0.41; $p_{adj.} = 0.04$). This module was enriched for the GO processes epidermal differentiation ($p_{adj.} = 6.4e^{-03}$) and keratinocyte differentiation ($p_{adj.} = 7.8e^{-03}$). No KEGG pathways were enriched. The turquoise module was mildly inversely correlated with most contaminants ($r = -0.35$–0.39; $p < 0.05$). This module was strongly enriched for the GO process cell cycle ($p_{adj.} = 7e^{-26}$), more specifically mitosis ($p_{adj.} = 1.6e^{-25}$) and the KEGG pathway cell cycle ($p_{adj.} = 1.4e^{-09}$).

9.6. Expression of genes sets with known responses to PAHs

To further investigate if the a signature of oil exposure could be seen in these samples, genes known to respond to crude oil were obtained from the Comparative Toxicogenomics Database (CTD) (Davis et al., 2015). Using this gene set, 2D hierarchical clustering was conducted on gene expression in the 50 male dolphins for which we have contaminants data. A conserved response to crude oil was not observed in the dolphin skin transcriptome among the 196 genes known to respond to crude oil in other organisms (Supplemental Fig. S1). Overall, the differences in the expression of this gene set were minimal across all

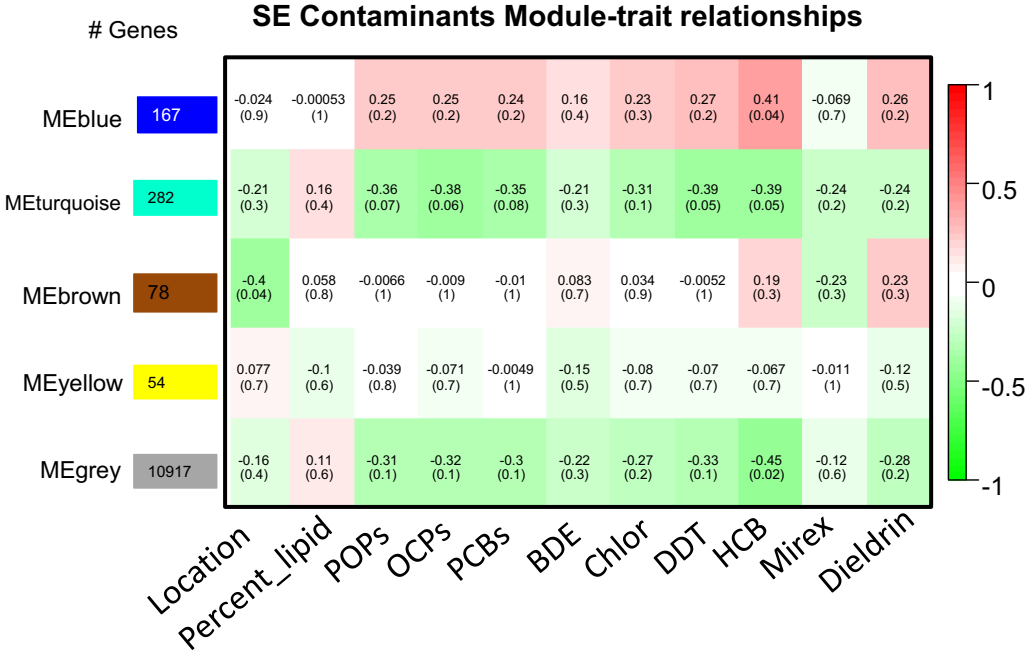

Fig. 7. Weighted gene expression co-variance network analysis (WGCNA) of male samples from the southeastern U.S. Atlantic coast (SE) (n = 26) identified four co-expressed gene modules. Correlation matrix of modules with sample traits and contaminants: red is positively correlated, green is negatively correlated. The correlation coefficient between the module eigengene and the measured trait is listed for each pairwise correlation, with significance in parentheses (adjusted P value). Number of genes in each module is listed at left. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

animals. Although there was some clustering associated with region, it did not completely separate animals. It is interesting to note that most animals sampled during spring in the NGoM clustered somewhat independently from the remaining NGoM animals. The spring animals were sampled prior to known oiling, whereas animals sampled during the summer in the NGoM were suspected to have been exposed to oil; however, this clustering may alternatively reflect subtle seasonal differences in gene expression. Although this analysis was based on a set of genes listed as responsive to oil in the CTD, these genes are based on many different species and tissues, none of which include dolphins, and many of which are not expressed in skin. Thus, the lack of a clear oil-response signature from the CTD gene set does not preclude the use of dolphin skin gene expression as a marker of exposure. Downstream pathways, including those involved in cellular architecture, immune response, and oxidative stress, observed in our differential expression analysis above, may be informative markers in bottlenose dolphin skin. To confirm this possibility, controlled studies to map the genes responsive to various contaminant classes in dolphin skin are needed.

10. Conclusions

The current study using RNA-Seq technology and the 2.59x dolphin genome enhanced our insight into of the bottlenose dolphin skin transcriptome, nearly doubling the number of annotated transcripts detected for analysis as compared with our previous microarray study (Van Dolah et al., 2015). Season is a major influence on gene expression, overwhelming and intermingling with variations in gene expression based on other factors such as location and contaminant burdens. Thus, this seasonal variation must be taken into account; future studies should limit comparisons to within a season and baseline data for every season must be archived for future event responses. The current study identified co-expressed gene modules that correlate with contaminant burdens of various POPs classes. We did not observe significant differences in the expression of a known oil-responsive gene set between NGoM animals and non-oil exposed animals from SE coastal waters. However, global differential expression analyses revealed differences in possible downstream pathways, including those involved in tissue architecture, immune function, and oxidative stress, that may prove responsive to oil exposure in dolphin skin. Future studies involving animals with significantly different contaminant profiles and/or controlled laboratory exposures of cells/tissues are needed to further define the transcriptomic response to contaminant exposures in cetaceans.

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