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GENE EXPRESSION IN AMERICAN LOBSTER (*HOMARUS AMERICANUS*) WITH EPIZOOTIC SHELL DISEASE

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ABSTRACT Epizootic shell disease (ESD) has been reported widely in American lobster (*Homarus americanus,* Milne Edwards) in southern New England. The appearance of irregular, deep lesions—characteristic of ESD—has been associated previously with elevated levels of ecdysteroids and premature molting, but the underlying molecular and physiological changes associated with ESD remain poorly understood. Previously, we identified several genes, including arginine kinase and hemocyanin, that were expressed differentially in lobsters exhibiting signs of ESD (diseased) versus those lobsters exhibiting no signs of ESD (assumed healthy), and quantified their expression. In this study, we extend these findings and measure expression of a suite of 12 genes in tissues from 36 female lobsters of varying disease condition. In addition, molt stage is evaluated as a possible confounding factor in the expression of the selected genes. The expression of several genes changed significantly with disease stage. Arginine kinase expression decreased significantly in thoracic muscle of lobsters with signs of ESD. Ecdysteroid receptor expression was elevated significantly in both muscle and hepatopancreas of lobsters with signs of ESD. CYP45, a cytochrome P450 form that was shown previously to covary with ecdysteroid levels and to be inducible by some xenobiotics, showed significantly increased expression in hepatopancreas of lobsters with signs of ESD. Together, these results demonstrate that the expression of several genes is altered in lobsters showing signs of ESD, even when accounting for variation in molt stage. Given the observed changes in ecdysteroid receptor, arginine kinase, and CYP45 expression, further investigations of the association, if any, between molting, muscular function and xenobiotic metabolism and ESD are warranted.

KEY WORDS: arginine kinase, 100 lobsters, cytochrome P450, ecdysteroid, endocrine, hepatopancreas, heat shock protein, epizootic shell disease, American lobster, *shade*

INTRODUCTION

The American lobster, *Homarus americanus,* supports an iconic and economically valuable fishery along the Atlantic coast of the United States and Canada. Epizootic shell disease (ESD) is a syndrome associated with the progressive development of irregular, deep lesions on the carapace (Smolowitz et al. 2002, Smolowitz et al. 2005). Lobsters with signs of ESD exhibit increased mortality in captivity and sublethal effects on growth (Castro et al. 2006, Stevens 2009). The high incidence of ESD in southern New England (SNE) has been suggested as a possible causative factor in the observed decline in the SNE lobster stock since 2001 (Howell 2012). The disease etiology is likely multifactorial and complex, possibly resulting from interactions among the environment, infectious agents, and the underlying host physiology (Castro et al. 2006, Tlusty et al. 2007). The microbial community associated with lesions has been described previously and is characterized further in other articles in this special issue (Chistoserdov et al. 2005, Bell et al. 2012, Chistoserdov et al. 2012, Meres et al. 2012). Progress has also been made toward identifying the infective agents and toward replicating ESD under controlled laboratory conditions (Quinn et al. 2012, Tlusty & Metzler 2012). The idea that a permissive physiological state in the lobster, which may occur in response to environmental conditions, enables initiation and progression of infection has been termed the host susceptibility hypothesis (Tlusty et al. 2007). A variety of conditions have been suggested as relevant to lobster susceptibility to ESD, including diet, temperature, contaminant exposure, and behavior (Castro & Angell 2000, Glenn & Pugh 2006, Tlusty et al. 2008, Laufer et al. 2012, Tlusty & Metzler 2012); however, the underlying molecular and physiological changes associated with ESD remain poorly understood.

Our approach is to use molecular techniques to identify genes that are associated with ESD. Our working hypothesis is that the identification of such genes and quantification of their expression with disease state could lead to a mechanistic understanding of ESD. We previously conducted a suppressive subtractive hybridization as a conceptually unbiased way to identify genes differentially expressed in lobsters exhibiting signs of ESD (Tarrant et al. 2010). Next, we evaluated the expression of the differentially expressed genes in a small number of lobsters and identified arginine kinase (AK), hemocyanin, and α2-macroglobulin as genes of interest for further investigation. In the current study, we extend these results by quantifying the expression of an additional suite of genes, chosen *a priori* for their known or suspected role in cellular stress responses, endocrine regulation, or xenobiotic metabolism. We measured gene expression in tissues from female lobsters that showed no signs, mild signs, or severe signs of ESD. The lobsters analyzed were a subset of 120 animals of varying disease states that were collected as a part of the Lobster Shell Disease Research Initiative and used to support a collaborative research effort called the 100 Lobsters Project (Shields et al. 2012a). The results of the other research groups that make up the Lobster Shell Disease Research Initiative and that worked on the 100 Lobsters Project are reported elsewhere in this special issue (LeBlanc & Prince 2012, Shields et al. 2012a, Shields et al. 2012b). Here we report the altered expression of several genes in lobsters showing signs of ESD. Gene expression patterns were consistent with disruption of molting, muscular function and xenobiotic metabolism in lobsters with signs of ESD.

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MATERIALS AND METHODS

Animals

Lobsters were collected from Rhode Island Sound, RI, as a part of the 100 Lobsters Project ((Shields et al. 2012b) although this collaborative project also included specimens from Maine, only samples from the Rhode Island population were included in our study). The collection site coordinates were 41.5073° N and 71.3463° W, and depth was 35 m. Details of sample collection and dissection are provided by Shields et al. (2012b). The specimens analyzed in this study were collected in June and July 2008. During these months, the temperature at the site ranged from 12.9–19.1°C. Shortly after collection using baited ventless traps, live lobsters were shipped to the Virginia Institute of Marine Science (VIMS) via overnight delivery in coolers packed with cold packs and moist newspaper. At VIMS, thoracic muscle, hepatopancreas, and ovary were dissected, stored in RNAlater at ≤ –20°C (Ambion, Austin TX), and subsequently shipped to Woods Hole Oceanographic Institution (WHOI) on dry ice. On arrival at WHOI, all samples were stored at –80°C until analysis. Molt stage was assessed from histological sections (Shields et al. 2012a). A subset of 36 female lobsters was selected for analysis of gene expression (Table 1), with the distribution divided among lobsters with no signs (stage 0, $n = 15$), mild signs (stage 1, $n = 9$), and moderate to severe signs (stages 2 and 3; $n = 3$ and $n = 9$, respectively) of ESD. Carapace length ranged from 63.2–86.2 mm (79 ± 6.62 SD). Female lobsters were used in this study to reduce the variability that would have been introduced by analyzing lobsters of both genders, because severe cases of ESD are relatively common in ovigerous females, and because abnormal molting and ecdysone levels have been reported in females with signs of ESD (Laufer et al. 2005, Cobb & Castro 2006). Additional details regarding these animals are available via an online database (http://www.vims.edu/research/departments/eaah/programs/crustacean/research/lobster_shell_disease/index.php (Shields et al. 2012b)).

TABLE 1.

Lobsters used in gene expression studies.

	Late Postmolt (C3)/Intermolt (C4) $n = 22$	**Premolt (D0–D3)/ Molt (E) $n = 14$**
No signs (stage 0) $n = 15$	$n = 10$ *C3: 9, 10, 13, 15, 22, 30* *C3/C4: 48* *C4: 47, 57, 58*	$n = 5$ *C4/DO: 60* *D0: 49* *D0/D1: 43* *D1: 50* *D3: 44*
Mild signs (stage 1) $n =9$	$n = 8$ *C3: 7, 18, 42* *C3/C4: 14, 20* *C4: 29, 36, 38*	$n = 1$ *D3/E: 51*
Moderate to severe signs (stage 2–3) $n = 12$	$n = 4$ Stage 3 *C4:12, 19, 33, 67*	$n = 8$ Stage 2 *C4/D0: 21, 46* *D0: 52* Stage 3 *C4/D0: 40, 45* *D0/D1: 27, 37, 28*

All lobsters were females collected from Rhode Island Sound as a part of the "100 Lobster Project." Numbers at the top of each cell indicate the sample size for each category. Italicized text indicates the individual lobsters within each molt stage. Stages as described by Smolowitz et al. (1992) and references therein (C3, late postmolt; C4, intermolt; D0–D3, premolt; E, ecdysis). Sample numbers refer to the individuals within the "100 Lobster Database" (e.g., *"9"* refers to "RI009"). See methods and Shields et al. (2012a) for additional details. Additional idiopathic conditions were diagnosed in several individuals, the most severe or extensive include ((Shields et al. 2012b) sample numbers underlined in table): necrotizing hepatopancreatis (*44, 48, 67*), moderate to extensive granulomas (relative level 3 or greater, *18, 19, 20, 42, 45, 58, 67*), and moderate to severe ommatidial lesions (covering ≥ 66% of area in cross-section, *7, 14, 21*).

RNA Extraction and cDNA Synthesis

Approximately 100 mg of each tissue sample was homogenized in 1 mL PureZOL (a reagent containing phenol and guanidine isothiocyanate; Bio-Rad, Hercules, CA). Total RNA was extracted using the Aurum Fatty and Fibrous Tissue kit (Bio-Rad), according to the manufacturer's directions, including on-column DNase treatment. RNA quality was visualized for selected samples on a denaturing agarose gel. RNA concentration and purity were assessed using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). Complementary DNA (cDNA) was synthesized from total RNA using the IScript Reverse Transcription Kit (Bio-Rad), using 1 µg total RNA per 20-µL reaction.

Identification, Cloning, and Sequencing of Candidate Genes

Candidate genes were selected based on their known or suspected role in crustacean immune or stress responses, endocrine signaling, or xenobiotic metabolism. Sequences for AK, α2-macroglobulin, hemocyanin (Tarrant et al. 2010), ecdysteroid receptor, and retinoid X receptor (Tarrant et al. 2011) were described previously. The remaining sequences were identified through searches of the NCBI database, including the EST database, and annotated provisionally based on sequence identity with known genes (Table 2). To create standards for qPCR quantification, portions of each gene were amplified from cDNA using specific primers (Table 2; in some cases, the primers were the same as those used for qPCR and, in other cases, a separate set of primers was used to amplify a larger portion of the gene), cloned into pGEMT-Easy (Promega, Madison, WI) and sequenced by Eurofins MWG Operon (Huntsville, AL). The DNA concentration (measured in nanograms per microliter) of each plasmid was determined using the Nanodrop spectrophotometer and used to calculate the titer of the standard (measured in molecules per microliter). Plasmids were diluted serially with molecular biology-grade water to produce standards ranging in concentration from 10^3–10^9 molecules DNA/µL solution.

Quantification of Gene Expression

Primers were designed to amplify 75–150-bp fragments of genes of interest (Table 2). Expression of each gene was measured in thoracic muscle, ovary, and/or hepatopancreas, with tissues selected on the basis of previous studies (e.g., Snyder 1998, Chang 2005, Tarrant et al. 2010) and the known or hypothesized gene function. That is, genes were amplified

TABLE 2.

Accession numbers, provisional annotations, and primers used for amplification of lobster genes quantified in this study.

Provisional Identification	Accession No. (Citation)	Reference Species (Accession No.)	E Value	Sequences	Amplicon Size (bp)
Hsp70	DQ173923	*Marsupenaeus japonicus* ABK76338	0.0	F: 5′-TCGTCTCCGTACTGCCTGTGAG-3′ R: 5′-TCCTTCTCGCCTAGCTGGTTTG-3′ F: 5′-CCTCAGCAAAGAGGAAATCG-3′ R: 5′-CGATCCCTTTGCTTCTCATC-3′	986 (cloning) 78 (qPCR)
Hsp90	AY851302	*Eriocheir sinensis*	4e-159	F: 5′-TCCCATCAAGCTGTTGGTTGAG-3′ R: 5′-TTCAGGGAGCAGATGTCGTCAC-3′ F: 5′-AGCTCCATTTGACCTGTTCG-3′ R: 5′-CTCCGTTGAGGAAGTTCAGG-3′	1,008 (cloning) 125 (qPCR)
CYP45	AF065892 (Snyder 1998)	N/A	N/A	F: 5′-ATGACCTGATCCGTCTCCTC-3′ R: 5′-ACGACCTTGGTCAGATGCTC-3′	79
CYP314A1-like (*shade*-like)	CN952331	*Drosophila melanogaster* AAQ05972.1	5e-9	F: 5′-CGAGCGTGCTAAGGTGTAGG-3′ R: 5′-TCGCAGTCATCAGGGTTCAC-3′ (forward primer same as cloning) R: 5′-GGTCTTGCTGCGACTTTCTG-3′	340 (cloning) 148 (qPCR)
CYP2L1-like	CN951298 (Towle & Smith 2006)	*Panulirius argus* Q27712 (James et al. 1996)	2e-24	F: 5′-GTCGTGGCTCACACTCAGAC-3′ R: 5′-TCGACCAGTTCCTGAGCTTC-3′	108
CYP6H-like	CN854020 (Towle & Smith 2006)	*Locusta migratoria* AAD39748.1 (Winter et al. 1999)	3e-24	F: 5′-CGAACGACATGCTAACGAAC-3′ R: 5′-GACCAGCCAGTTGAAGAAGG-3′	144
EcR	HQ335007 (Tarrant et al. 2011)	N/A	N/A	F: 5′-AATTGCGAACCCTTGGAAACC-3′ R:5′-CCAAATCTCGGCCAGGAAAG-3′	92
RXR	HQ335007 (Tarrant et al. 2011)	N/A	N/A	F: 5′-TTACTCAAGGCTGGGTGGAACG-3′ R:5′-CTGGTGAGCGCTACTTCTGTGC-3′	120

Sequences of arginine kinase, hemocyanin, and α2-macroglobulin were described in our previous studies (Tarrant et al. 2010) and are not shown here. EcR and RXR cloning was described previously (Tarrant et al. 2011). qPCR primer sequences are given in the table.

from the tissues in which they were previously shown to be differentially expressed (e.g., AK in muscle (Tarrant et al. 2010)) or from tissues in which they were thought to be active (e.g., xenobiotic metabolism by several cytochrome P450 (CYP) enzymes in the mammalian liver, which corresponds to the lobster hepatopancreas). Ecdysteroid receptor (EcR) and retinoid X receptor (RXR) primers were designed to amplify both known isoforms of each gene (Tarrant et al. 2011). All qPCR assays were performed using a MyCycler iQ Real-Time PCR detection system (Bio-Rad). Samples were run in duplicate wells using IQ Supermix (Bio-Rad) in 25-μL reactions. Cycling conditions were 95°C for 3 min, 40 cycles of 95°C for 15 s and 62–64°C for 45 s; the annealing temperature was 62°C for RXR and 64°C for all other genes. After 40 cycles, the PCR products were subjected to melt-curve analysis to ensure that a single product had been amplified. Expression was quantified by comparing the threshold cycle against a serially diluted plasmid standard, normalized to 16S ribosomal RNA expression, as described previously (Tarrant et al. 2008).

Statistical Analysis

16S-normalized expression values were log transformed to satisfy assumptions of normality. Although log-transformed data were analyzed statistically and presented graphically (Figs. 1–3), data were back transformed to report the magnitude of change in expression ("Fold change" reported in results). Expression was compared among animals with no signs (stage 0), mild signs (stage 1), and moderate to severe signs (stages 2–3) of ESD. One sample (specimen 14) was discarded from analysis of gene expression in muscle (all genes) and hepatopancreas (EcR, hemocyanin, CYP314-like, CYP45, CYP6H-like, CYP330-like) because of extremely low amplification and/or poor melt curves. To explore the potentially confounding effects of molt stage on disease-associated gene expression, lobsters were divided into 2 groups: late postmolt/intermolt (C3–C4) and premolt/molt (C4/D0–E). These data were analyzed as a 2-way ANOVA within Prism (GraphPad, La Jolla, CA). Post hoc comparisons (Bonferroni test) were conducted for statistically significant main effects provided the interaction term (interaction between molt stage and disease condition) was not significant. Significance was assessed at $P < 0.05$.

RESULTS

Expression of AK, EcR, heat shock protein 70 (Hsp70), and heat shock protein 90 (Hsp90) was measured in thoracic muscle (Fig. 1). AK expression varied significantly as a function of both molt stage ($P = 0.036$) and disease condition ($P = 0.0013$). AK expression was higher during late postmolt (C3) and intermolt (C4) than during premolt. Consistent with our previous results (Tarrant et al. 2010), AK expression was higher in lobsters with no signs of ESD than in those exhibiting signs of disease. EcR expression showed a significant interaction ($P = 0.015$) between molt stage and disease condition. Within C3–C4 lobsters, expression was significantly higher in animals exhibiting moderate to severe (stages 2–3) signs of disease (5.4-fold increase relative to stage 0). EcR expression in these individuals was

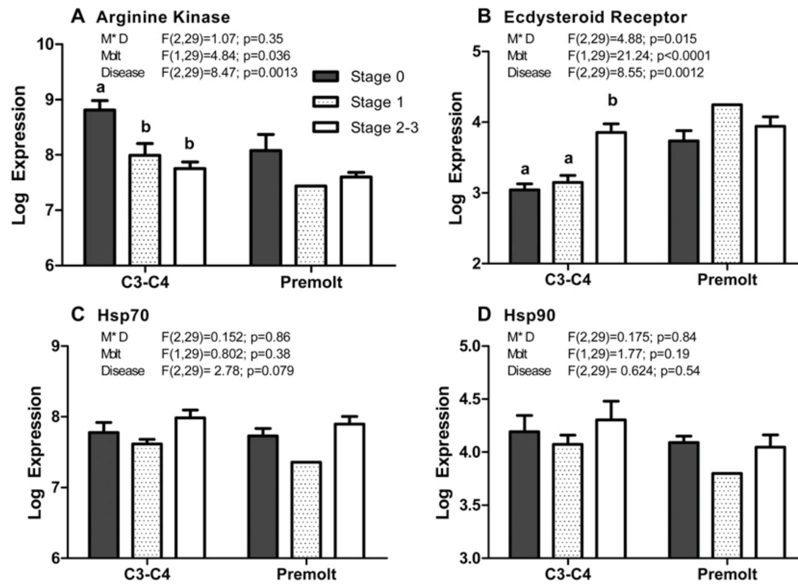

Figure 1. (A–D) Log(10)-transformed expression of arginine kinase (A), ecdysteroid receptor (B), Hsp70 (C), and Hsp90 (D) in thoracic muscle of lobsters of varying molt stage and disease condition. Note that the y-axis varies among genes. Bars indicate mean ± SEM for lobsters exhibiting no signs (stage 0, gray bars), mild signs (stage 1, dotted bars), and moderate to severe signs (stages 2–3, open bars) of ESD. Sample sizes are indicated in Table 1 (only one stage 1 premolt individual). Results of 2-way ANOVA (*P* values) are indicated; letters indicate significant differences (Bonferroni posttest, $P < 0.05$) between disease conditions within a molt stage.

comparable with that observed during lobsters with no signs of disease in premolt. Expression of Hsp70 and Hsp90 did not vary significantly with molt stage or disease condition.

Expression of CYP45, EcR, and α2-macroglobulin was measured in ovary (Fig. 2). None of these genes varied significantly with molt stage or disease condition. α2-Macroglobulin showed a slight and nonsignificant trend toward increased expression in individuals showing signs of disease (e.g., in C3–C4, mean expression was 1.2-fold higher in stage 1 animals relative to stage 0).

In hepatopancreas, expression of EcR, RXR, hemocyanin, and 5 CYP genes was measured (Fig. 3). EcR expression in hepatopancreas showed a significant interaction between molt stage and disease condition ($P = 0.037$). Overall, expression was

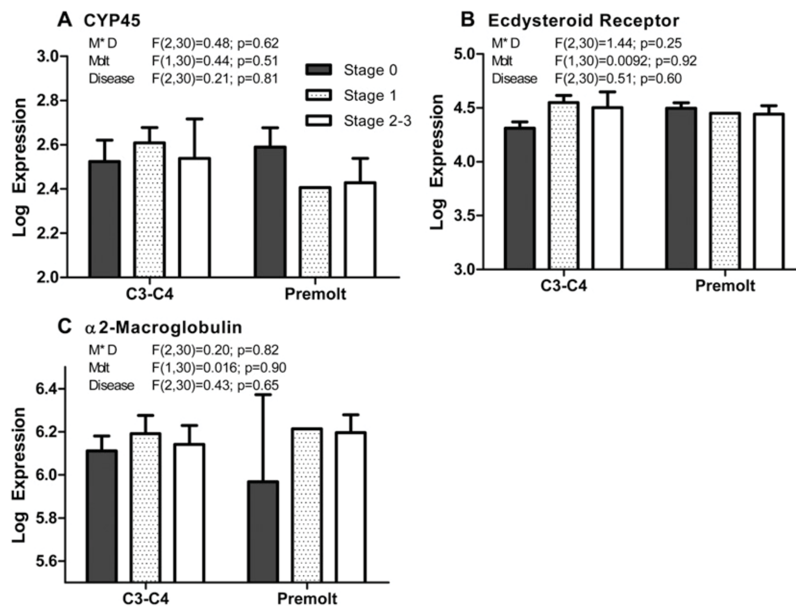

Figure 2. (A–C) Log(10)-transformed expression of CYP45 (A), ecdysteroid receptor (B), and α2-macroglobulin (C) in ovary of lobsters of varying molt stage and disease condition. Note that the y-axis varies among genes. Bars indicate mean ± SEM for lobsters exhibiting no signs (stage 0, gray bars), mild signs (stage 1, dotted bars), and moderate to severe signs (stages 2–3, open bars) of ESD. Sample sizes are indicated in Table 1 (only one stage 1 premolt individual). Results of 2-way ANOVA (*P* values) are indicated; letters indicate significant differences (Bonferroni posttest, $P < 0.05$) between disease conditions within a molt stage.

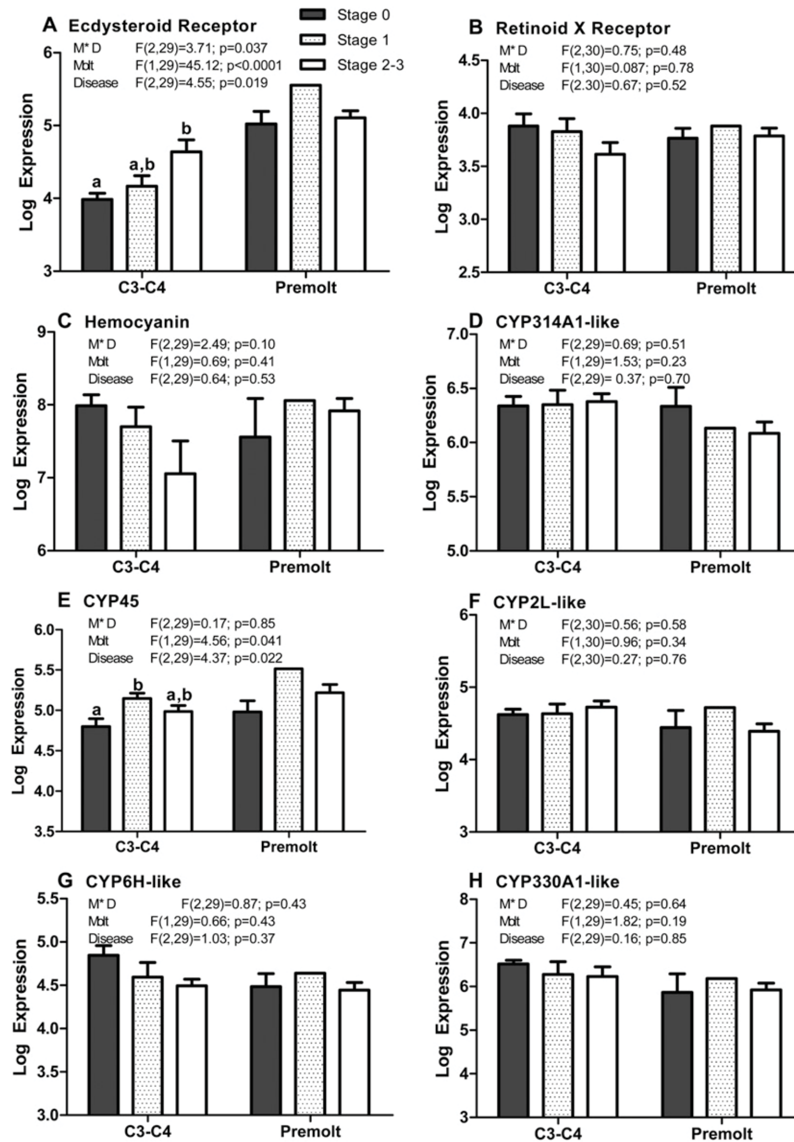

Figure 3. (A–H) Log(10)-transformed expression of ecdysteroid receptor (A), retinoid X receptor (B), hemocyanin (C), "CYP314A1-like" (D; putative ecdysteroid hydroxylase), CYP45 (E), CYP2L-like (F), CYP6H-like (G), and CYP330A1-like (H) in hepatopancreas of lobsters of varying molt stage and disease condition. Note that the y-axis varies among genes. Bars indicate mean ± SEM for lobsters exhibiting no signs (stage 0, gray bars), mild signs (stage 1, dotted bars), and moderate to severe signs (stages 2–3, open bars) of ESD. Sample sizes are indicated in Table 1 (only one stage 1 premolt individual). Results of 2-way ANOVA (*P* values) are indicated; letters indicate significant differences (Bonferroni posttest, $P < 0.05$) between disease conditions within a molt stage.

higher during premolt. Similar to the pattern observed in thoracic muscle (Fig. 1B), during late postmolt and intermolt, expression was elevated 4.5-fold in individuals with moderate to severe signs of disease, relative to lobsters with no signs of disease. CYP45 (Fig. 3E) expression varied significantly as a function of both molt stage ($P = 0.041$) and disease condition ($P = 0.022$). CYP45 expression was elevated during premolt and in lobsters showing signs of disease, with a significant increase (1.5-fold) in lobsters with signs of mild disease during C3–C4. Expression of the other genes (RXR, hemocyanin, CYP314A1-like, CYP2L-like, CYP6H-like, CYP330A1-like) did not vary significantly with either molt stage or disease condition. Hemocyanin showed a trend toward decreased expression in C3–C4 individuals that showed signs of disease.

Histological analysis revealed additional idiopathic conditions (conditions with no specific or known causality) in 12 of the lobsters analyzed in this study (Shields et al. 2012a). Three individuals exhibited necrotizing hepatopancreatitis (44, 48, and 67; numbers reflect specimen numbers as shown in Table 1), 3 had extensive (affecting ≥ 66% of the surface) lesions of the ommatidia (7, 14, 21), and 7 had moderate to extensive granulomas (18, 19, 20, 42, 45, 58, 67; animal 67 also had necrotizing hepatopancreatitis, as noted earlier). Given the relatively small sample size and potentially confounding effects of molt stage and disease condition, it was not possible to evaluate formally associations between gene expression and these conditions. Exploratory inspection of gene expression profiles of these individuals did not reveal any consistent or extreme (i.e., outliers)

patterns for any of the targeted genes. Reanalysis of the data set after removal of these 12 animals generally resulted in relatively small changes, many of which may be, at least in part, attributed to the associated decrease in sample size (i.e., results that were statistically significant on the basis of the larger data set became statistically nonsignificant, although trends generally remained unchanged). In muscle, AK expression remained a significant function of disease condition, but effects of molt stage and the interaction between disease condition and molt stage were no longer significant (interaction $F(2,18) = 0.3087$, $P = 0.7389$; disease condition $F(2,18) = 5.912$, $P = 0.0106$; molt stage $F(1,18) = 2.68$, $P = 0.119$). In ovary, no changes in significance were observed. In hepatopancreas, effects of disease stage and molt–disease interaction on expression of EcR and CYP45 became marginally nonsignificant (EcR: $F(2,18) = 3.058$, $P = 0.0719$; disease condition $F(2,18) = 2.213$, $P = 0.1383$; molt stage $F(1,18) = 48.98$, $P < 0.0001$; CYP45: $F(2,18) = 0.5188$, $P = 0.6039$; disease condition $F(2,18) = 3.143$, $P = 0.0675$; molt stage $F(1,18) = 5.919$, $P = 0.0256$). In this new analysis, significant effects of disease condition were detected for hemocyanin and CYP6H-like (hemocyanin: interaction $F(2,18) = 2.829$, $P = 0.0854$; disease condition $F(2,18) = 4.195$, $P = 0.0319$; molt stage $F(1,18) = 3.789$, $P = 0.0674$; CYP6H-like: interaction $F(2,18) = 1.515$, $P = 0.2465$; disease condition $F(2,18) = 4.754$, $P = 0.022$; molt stage $F(1,18) = 0.6263$, $P = 0.430$). Expression of hemocyanin and CYP6H-like were both lower in animals that exhibited signs of disease.

DISCUSSION

In this study, we identified characteristic gene expression patterns in lobsters exhibiting signs of ESD. The etiology of ESD has been described as strictly dermal, largely because pathogenic changes are restricted to the carapace, and the appearance of disease signs is not correlated with microbial infection of the hemolymph (Chistoserdov et al. 2005, Smolowitz et al. 2005). Our data show significant changes in gene expression in both hepatopancreas and muscle, and implicate tissues beyond the carapace, consistent with the host susceptibility hypothesis previously described by Tlusty et al. (2007), including a potentially systemic disruption of endocrine signaling and energetic metabolism.

AK helps to regulate cellular levels of adenosine triphosphate (ATP) and thus plays a key role in energetic homeostasis (Ellington 2001). Previously, we reported decreased AK expression in muscle (a mixture of claw and thoracic muscle) of male and female lobsters exhibiting signs of ESD (Tarrant et al. 2010); the current study corroborates and extends these results. We observed strongly decreased expression of AK in thoracic muscle of lobsters with disease signs and during premolt (Fig. 1A). Expression was decreased significantly even during early stages of infection (stage 1), and we observed a trend toward decreased expression as the severity of disease signs increased. Biochemical studies have also indicated that lobsters exhibiting signs of impoundment shell disease have altered metabolic profiles, including reduced carbohydrate and arginine in the muscle, and reduced lipids in the hepatopancreas (Floreto et al. 2000). Both ESD and impoundment shell disease are characterized by lesions on the carapace (albeit different in appearance), and our results extend the findings of previous studies that indicate that these external infections are accompanied by systemic changes in energetic metabolism.

To our knowledge, AK expression has not been documented previously in muscle during the crustacean molt cycle. However, Chung & Webster (2003) noted a 2-fold decrease in AK expression during premolt in neural tissue of the eyestalk of the green crab, *Carcinus maenas*. Molting in decapods is accompanied by extensive protein remodeling, which is most pronounced in claw muscle (Mykles & Skinner 1990, Haj et al. 1996, MacLea et al. 2010). Thus, altered expression of AK across molt stages is not, in itself, surprising; however, AK expression was altered significantly in lobsters with signs of ESD, even when accounting for variation resulting from molt stage. Intermolt duration in lobster has been shown to be shortened by ESD, and molting appears to be a defense mechanism that allows lobsters to shed external signs of infection (Castro et al. 2006). Decreased AK expression in lobsters with signs of ESD may therefore be related to induction of molting processes; however, if this is the case, decreased AK expression (in C3–C4 individuals with signs of ESD) precedes morphological changes in the cuticle associated with premolt.

In crustaceans, similar to other arthropods, ecdysteroid concentrations in the hemolymph increase during premolt (Styrishave et al. 2004) and activate heterodimers formed by EcR and RXR (Wu et al. 2004, Wang & LeBlanc 2009, Tarrant et al. 2011). This regulates expression of target genes to coordinate muscular atrophy, metabolic shifts, cuticle separation, and ultimately molting (Kim et al. 2005, Shechter et al. 2007). In our study, EcR expression increased during premolt in both muscle and hepatopancreas, and also increased in both tissues in lobsters with signs of ESD (Figs. 1B and 3A). Expression of EcR in ovary did not vary significantly with molt stage or disease condition. Laufer et al. (2005) previously reported elevated concentrations of ecdysone in hemolymph of lobsters with signs of ESD, particularly in ovigerous females, which typically have reduced ecdysteroid levels. EcR, similar to the pattern observed for AK, showed elevated expression in lobsters showing signs of ESD. Expression in C3–C4 lobsters with signs of ESD was comparable with that observed in lobsters with no signs of ESD during premolt. Molt-associated EcR and RXR expression patterns have not been described previously in lobsters, but are known to vary among crustaceans. For example, in kuruma prawn, like American lobster, EcR expression is elevated in thoracic muscle and hepatopancreas during premolt (Asazuma et al. 2007). In contrast, in land crab, increases in ecdysteroid concentration induced by eyestalk ablation resulted in upregulation of EcR in claw muscle but not thoracic muscle (Kim et al. 2005). In our study, RXR was measured only in hepatopancreas, and did not vary significantly with molt stage or disease condition. Similarly, in kuruma prawn, RXR expression did not vary across molt stages in hepatopancreas (Asazuma et al. 2007). Although RXR is the dimerization partner of EcR in lobsters and other crustaceans, it also forms heterodimers with several other nuclear receptors (Chawla et al. 2001, Zhu et al. 2003), so more stable RXR expression levels may reflect its dimerization behavior with multiple partners.

We measured expression of 5 CYPs in hepatopancreas (CYP314A1-like, CYP45, CYP2L-like, CYP6H-like, and CYP330A1-like) and 1 in ovary (CYP45). These 5 CYPs were selected based on previously described expression patterns in lobsters, expression patterns of similar genes in other crustaceans, and/or similarity to genes with known function in other arthropods (James et al. 1996, Snyder 1998, Winter et al. 1999,

Rewitz et al. 2003, Rewitz et al. 2007). Among them, only CYP45 expression varied significantly, with an increase in expression in animals with signs of disease and during premolt in hepatopancreas (Fig. 3E), similar to the pattern described for EcR expression. Expression of CYP45 has been shown to increase during premolt in lobsters and in response to exposure to 20-hydroxyecdysone, phenobarbital, or heptachlor (Snyder 1998). Although the physiological function of CYP45 is unknown, it is thought to be involved in ecdysteroid catabolism (Snyder 1998). Altered expression of CYP45 in the hepatopancreas of lobsters with signs of disease is consistent with more significant changes in energetic metabolism (Shechter et al. 2007) and early progression toward molting. Among the CYPs that did not vary significantly with molt stage or disease condition, the CYP314A1-like gene is of particular interest. CYP314A1, designated *shade* in *Drosophila*, is a 20-hydroxylase responsible for the conversion of ecdysone to active molting hormone, 20-hydroxyecdysone (Rewitz et al. 2007). Given the observed association between the expression of the molting-related genes EcR and CYP45 (this study), previously described changes in ecdysteroid concentration (Laufer et al. 2005), and ESD, it is somewhat surprising that we did not observe variable expression of a putative 20-hydroxylase. However, the similarity of the lobster CYP314A1-like gene to insect 20-hydroxylases is only moderate (31% amino acid identity over 118 residues, E value 4e-9), so the lobster CYP may serve a different function.

We also measured the expression of 2 heat shock proteins: Hsp70 and Hsp90. In lobster, these genes can be induced in abdominal muscle in response to elevated temperatures or osmotic stress (Chang 2005). Bacterial or viral challenge has also been shown to induce Hsp70 expression in tissues of other decapods (Cui et al. 2010, Zhou et al. 2010); however, in the current study we did not observe significant changes in Hsp70 or Hsp90 expression in muscle associated with disease condition or molt stage. Because these genes are part of a large superfamily of stress-responsive chaperone proteins, it is possible that other forms are involved in the lobster immune response associated with ESD. In addition, the cited studies measured short-term (0–3- h) responses to pathogen challenge, whereas ESD may represent a more chronic stressor that induces different genes. Although all reasonable efforts were made to minimize potential stress associated with transport and handling of lobster specimens, it cannot be ruled out that transport and handling stress resulted in changes in gene expression, which may have obscured differences among groups. That said, observed differences in the expression of marker genes used in our study appeared to be relatively robust in that they could be detected against the background of the potential handling stress experienced by the lobsters in this study.

Finally, in addition to the genes described earlier, we quantified expression of 2 genes shown previously to be associated with ESD: α2-macroglobulin and hemocyanin (Tarrant et al. 2010). Based on a much smaller number of lobsters, previously we found increased expression (not statistically significant) of α2-macroglobulin in ovary and decreased expression (not statistically significant) of a form of hemocyanin in the hepatopancreas of lobsters with signs of disease. Similar trends, which were not statistically significant, were observed in the current study in C3–C4 lobsters (Figs. 2C and 3C). Because many forms of hemocyanin have been described in crustaceans, and some are upregulated after microbial challenge (Lei et al. 2008, Tarrant et al. 2010), it would be of interest to profile the expression patterns of additional forms of hemocyanin in future studies of lobsters with signs of disease. In the case of α2-macroglobulin, we have detected expression in ovary, and α2-macroglobulin does play a role in mammalian ovulation (Zhu & Woessner 1991); however, α2-macroglobulin is expressed primarily in hemocytes of other crustaceans and can be induced by microbial challenge (Rattanachai et al. 2004, Lu et al. 2008, Ho et al. 2009). Thus, future studies should characterize expression of α2-macroglobulin and other components of the innate immune system in hemocytes in association with ESD.

The observed significant associations between ESD condition and AK and EcR expression in muscle were not affected by reanalysis of the data set after removal of animals exhibiting other idiopathic conditions. However, significant associations between ESD condition and EcR and CYP45 expression in hepatopancreas were no longer significant after removing these samples. The loss of significance may be, at least in part, attributed to sample size reduction (35–36 specimens in the initial analysis; 24 in the subsequent analysis). Conversely, significant associations with disease condition were detected for hemocyanin and CYP6H-like after removal of these samples. These results indicate that analysis of a larger data set would be desirable, particularly of animals exhibiting signs of only ESD. In addition, a systematic investigation of gene expression patterns in animals exhibiting signs of necrotizing hepatopancreatitis, extensive granulomas, and ommatidial lesions may provide insight into the physiological and immunological responses to those conditions.

Together, these studies provide convincing evidence of an association between disruption of endocrine signaling and ESD. Our results also provide further evidence for the host susceptibility hypothesis in the development of ESD; however, it is not yet known whether disruption of endocrine signaling precedes the establishment of ESD (i.e., is part of the "cause") or whether it is an additional sign of ESD. Our results also point to significant gaps in our fundamental understanding of lobster physiology. For example, although changes in ecdysteroid levels have been associated with ESD (Laufer et al. 2005), it is unknown whether this is the result of altered ecdysteroid synthesis and/or clearance rates. Direct measurements of these processes are needed, and additional study is needed to identify conclusively the responsible gene and enzymes. Future study of disruption of ecdysteroid signaling in association with ESD should also include analysis of glandular tissues, such as the Y-organ, which is responsible for the initial steps of ecdysteroid synthesis (Mykles 2010). The current study was restricted to analysis of gene expression in female lobsters collected from a single site in Rhode Island. In the future, additional samples should be analyzed from both male and female lobsters from a variety of populations, including populations that are not widely infected by ESD (e.g., Maine populations). Most important, future studies should take advantage of a laboratory model system (Tlusty et al. 2008, Quinn et al. 2012, Tlusty & Metzler 2012) in which microbial challenge experiments can be conducted to evaluate causality and to identify physiological and molecular indicators (biomarkers) of vulnerability to infection.

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