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Anand, Anurag; Crone, Erica; and Zera, Anthony J., "Tissue and stage-specific juvenile hormone esterase (JHE) and epoxide hydrolase (JHEH) enzyme activities and *Jhe* transcript abundance in lines of the cricket *Gryllus assimilis* artificially selected for plasma JHE activity: Implications for JHE microevolution" (2008). *Anthony Zera Publications*. 29.

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Tissue and stage-specific juvenile hormone esterase (JHE) and epoxide hydrolase (JHEH) enzyme activities and *Jhe* transcript abundance in lines of the cricket *Gryllus assimilis* artificially selected for plasma JHE activity: Implications for JHE microevolution

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Abstract

Fat body and midgut juvenile hormone esterase (JHE) and juvenile hormone epoxide hydrolase (JHEH) specific activities, and plasma JHE activity, were measured throughout the last stadium in two pairs (blocks) of lines of the cricket *Gryllus assimilis*, each pair of which had been artificially selected for high- or low-plasma JHE activity. Highly significant differences were observed between high- and low-activity lines of each block on most days for fat body JHE, and on one day for midgut JHE activity. In each block, line differences in developmental profiles for fat body JHE activity paralleled line differences in plasma JHE activity during the early-mid stadium, but not during the latter part of the stadium. The developmental profile of midgut JHE activity differed from that of plasma and fat body JHE activity, exhibiting peaks during the early and latter parts of the stadium. Midgut and fat body JHEH activities exhibited a mid-stadium peak in all lines, but activities were very similar in all lines. Fat body JHE appears to be a more significant contributor to plasma JHE than is midgut JHE. During the middle of the last stadium (day 4), *Jhe* transcript abundance was significantly higher in fat body or midgut of high- vs. low-JHE-activity lines. *Jhe* transcript abundance was positively correlated with JHE enzyme activity in either fat body or midgut, and with plasma JHE activity. Natural populations of *G. assimilis* harbor genetic variation for *Jhe* gene expression which appears to contribute to genetic variation in JHE specific activity in fat body and midgut. These genes appear to have been the targets of artificial selection that resulted in lines that differ dramatically in high- or low-plasma JHE activity. These genes appear to have little, if any, pleiotropic effects on JHEH specific activity.

Keywords: juvenile hormone esterase, JHE, juvenile hormone epoxide hydrolase, JHEH, juvenile hormone gene expression, artificial selection, evolution, microevolution

1. Introduction

The origin of endocrine adaptations is a key issue in evolutionary endocrinology. Endocrine adaptations originate with natural selection differentially favoring genetically-encoded hormonal variants (e.g. for blood titers, receptor characteristics, activities of hormone-regulating enzymes, etc.) segregating within populations of species. However, relatively little is known about the characteristics of this intraspecific endocrine variation, which, in turn, limits our understanding of the mechanisms underlying endocrine microevolution (Zera, 2006; Zera *et al.*, 2007).

Juvenile hormone esterase (JHE) activity in species of *Gryllus* crickets has been intensively studied from an evolutionary-genetic perspective (reviewed in Zera, 2006; and Zera *et al.*, 2007). JHE hydrolyzes the key developmental and repro-

ductive hormone, juvenile hormone (JH), and partially regulates its titer (Hammock, 1985; Roe and Venkatesh, 1990; Goodman and Granger, 2005). Genetically-based variation in plasma (blood) JHE activity has been documented in recently-founded laboratory populations of several cricket species (Zera and Tiebel, 1989; Zera and Zhang, 1995; Roff *et al.*, 1997; Zera and Huang, 1999; Zera, 2006). In wing-polymorphic species, elevated plasma JHE activity during the last nymphal stadium is strongly associated genetically with the subsequent production of the flight-capable (long-winged) as opposed to the flightless (short-winged)/reproductive morph (Zera and Tiebel, 1989; Roff *et al.*, 1997; Zera and Huang, 1999). This strong association implicates selection on genetic variation for JHE activity as an important component of the evolution of flightlessness in *Gryllus* (reviewed in Zera, 2004; Zera, 2006; Zera *et al.*, 2007).

Thus far, our functional-genetic studies of JHE variation have focused almost exclusively on the characteristics, correlations and functional consequences of JHE activity in the plasma of artificially-selected lines of three *Gryllus* species (reviewed in Zera, 2006; Zera *et al.*, 2007; Crone *et al.*, 2007; see Section 4). Results to date collectively indicate that genetic alterations in JHE activity in the plasma during the last nymphal stadium (1) give rise to altered JH metabolism and expression of JH-regulated traits, and (2) likely result from variation in some aspect of JHE gene regulation rather than JHE allozymes that differ kinetically due to variation in primary amino acid sequence or posttranslational modification.

Plasma JHE originates in non-hemolymph tissues such as the fat body, and is secreted into the hemolymph (Hammock, 1985; Roe and Venkatesh, 1990; Goodman and Granger, 2005). Thus, it is essential to investigate JHE in various non-hemolymph tissues, to understand both the causes of hemolymph JHE activity difference between genetic stocks, as well as to understand the function of JHE in non-hemolymph tissues. In the present study, we measured specific activities of JHE, as well as JH-epoxide hydrolase (JHEH), the other main JH-degrading enzyme, in fat body and midgut, throughout the last nymphal stadium in genetic stocks of *Gryllus assimilis* artificially selected for high- or low-plasma JHE activity. These tissues, especially fat body, were chosen since they exhibit high JHE specific activity and are likely major contributors to plasma JHE activity (Wing *et al.*, 1981; Hammock, 1985; Roe and Venkatesh, 1990; Jesudason *et al.*, 1992). In addition, using a recently obtained *Jhe* cDNA sequence from *G. assimilis* (Crone *et al.*, 2007), we measured *Jhe* transcript abundance in both fat body and midgut. The main goal was to begin to directly assess the extent to which genetic variation for *Jhe* gene expression is associated with and contributes to genetic variation in plasma JHE activity in *G. assimilis*.

2. Materials and methods

2.1. Stocks

Studies were conducted on stocks of *G. assimilis* that had been artificially selected for high- or low-plasma JHE activity. These are the same lines in which aspects of JHE activity have been extensively characterized previously (Zera and Zhang, 1995; Zera *et al.*, 1996; Zera *et al.*, 1998; reviewed in Zera, 2006). Stocks had been maintained under the same environmental conditions as those under which the original artificial selection studies had been conducted (*e.g.*, 28 °C; 16 light: 8 dark photoregime; cricket density and diet as reported in Zera and Zhang (1995)). Stocks had been periodically re-selected for high- or low-plasma JHE activity to maintain differences between lines in the activity of this enzyme. The original artificial selection study consisted of three independent selection trials (Blocks, Bks), each of which contained a line selected for high-plasma JHE activity, a line selected for low activity, and an unselected control (Zera and Zhang, 1995; Zera, 2006). In the present study, high- and low-selected lines of two of the three blocks (Bks 1 and 3) were studied. The duration of the last nymphal stadium ranged from 7 to 9 days with an average of 8 days.

2.2. Tissues sampled and preparation

Plasma JHE activity, and fat body and midgut JHE and JHEH specific activities were measured throughout the last nymphal stadium in JHE-selected lines. As mentioned above (Section 1; also see Section 4), fat body and midgut were studied because they exhibit high activities of these enzymes in several insect species, and thus were viewed as the most likely source of he-

molymph JHE activity in *G. assimilis*. JHE transcript abundance was measured in fat body and midgut on day 4 of the last stadium, a day just prior to (midgut) or on which (fat body) JHE specific activity differs considerably between selected lines (see Section 3). Plasma was collected and stored as described previously (Zera and Huang, 1999). Briefly, 2 μ L hemolymph, obtained from cuts in the cerci, were diluted 10-fold with 0.1 M potassium phosphate buffer, pH 7.1. Solutions were centrifuged (14,000 \times g, 10 min), and supernatants (plasma) stored at -86 °C until assayed. Midgut and fat bodies were removed from crickets, tissues were rinsed in saline (0.15 M NaCl), blotted, transferred to preweighed eppendorf tubes, snap frozen in liquid nitrogen, and stored at -86 °C until use. Background experiments demonstrated no loss in enzyme activity in frozen whole tissues or in diluted plasma during the period of storage (up to several months).

2.3. Enzyme assays

Plasma JHE activity was assayed using the radiochemical partition assay of Hammock and Sparks (1977), slightly modified for use in *Gryllus* (Zera and Huang, 1999). Fat body and midgut JHE and JHEH activities were simultaneously measured using a thin-layer chromatographic (TLC) assay similar to that described in Hammock and Sparks (1977). The partition assay of Share and Roe (1988), which also has been used to simultaneously measure JHE and JHEH activities, gave inconsistent results in background studies and was not used. Fat body and midgut samples were homogenized in 0.1 M potassium phosphate buffer, pH 7.1, centrifuged for 15 min at 7000 \times g at 4 °C, and the supernatant was assayed immediately. This gentle centrifugation step increased the duration of linearity of the JHE/JHEH assays, possibly by removing cellular debris that binds added JH, while not removing any JHE or JHEH activity. The pellet, after having been washed twice with homogenization buffer and suspended in homogenization buffer, exhibited <3% of JHE or JHEH activity found in the supernatant. All samples were collected during a 4 h period between 4 h after lights on and 8 h before lights off. No significant diurnal change in JHE activity was observed during this time.

For the TLC assay, 20 μ L of suitably diluted tissue homogenate was added to 178 μ L of 0.1 M potassium phosphate buffer, pH 7.1. The buffer contained 2 μ L of an ethanolic solution of radio-labeled racemic [10-³H]-juvenile hormone III (10–20 Ci/mmol, 370–740 GBq/mmol; PerkinElmer Life and Analytical Sciences) and racemic unlabelled JHE III (Sigma-Aldrich, Inc.) resulting in a final conc. of JH III of 5 μ M and containing 10,000 DPM per assay. Test tubes containing assay solution had been placed in an ice bath for a few minutes before addition of the tissue homogenate. Tubes were transferred from the ice bath to a 30 °C water bath, shaken for 30 s and incubated for 30 min. Reactions were stopped by placing racks of tubes back into the ice bath. To each tube was added 1.5 μ L of 6 M HCl to lower the pH to 6.0 to aid in the extraction of JH acid. Ethyl acetate (200 μ L) was added to each tube, tubes were vortexed for 1 min, and centrifuged for 5 min at 4 °C at 5000 rpm. The organic (top) phase was removed using a pulled Pasteur pipette. The extraction phase was repeated, the organic phases were combined, and solvent was removed under a stream of nitrogen. Diethyl ether (100 μ L) was added to each tube, which was briefly vortexed; solutions were then spotted onto plastic-backed silica gel TLC plates with 254 nm fluorescent indicator (Sigma-Aldrich, Inc.) that had been washed in methanol. JH, JH-diol, JH-acid, and JH-acid-diol standards were added to one lane of the plate. Plates were developed using the following solvent system: hexane:ethyl acetate:acetic acid (55:45:2 drops in 100 mL). Spots corresponding to JH,

JH-acid, JH-diol, and JH-acid-diol were cut out, placed in vials containing Ready Safe cocktail (Beckman Coulter, Inc.) and radioactivity (DPM) quantified using an LKB liquid scintillation counter. Protein concentration was determined for the fat body and midgut homogenates using the Bradford protein assay (Bio-Rad laboratories, Hercules, CA).

Background experiments were conducted to determine the amount of diluted plasma, fat body homogenate, or midgut homogenate required to produce rates that were linear with respect to time or tissue concentration for each of the metabolites of JHE/JHEH (JH-acid, JH-diol and JH-acid-diol). Assays were always run within these limits. Background experiments also demonstrated that it was unnecessary to add unlabelled JH or JH-metabolites ("carriers") during extraction, which significantly reduced the cost of the large number of assays conducted. Addition of unlabelled JH and JH-acid during the ethyl acetate extraction improved the total recovery of radio-labeled JH and metabolites (75% vs. 50% without cold carriers). However, the relative amount of JH and its metabolites extracted was the same whether carriers were used or not. Because reaction rates are estimated in this assay from the relative proportion of JH and its metabolites, reaction rates estimated with or without cold carriers did not differ significantly. In the JHE/JHEH assay of fat body and midgut homogenates, only a small amount (1–2%) of JH acid-diol was produced (2–3% of total JH metabolites produced), which was ignored in computation of JHE and JHEH activities.

JH, JH-acid, JH-diol, and JH-acid-diol standards were produced by incubating 300 µg racemic JH III in 1 mL of potassium phosphate buffer, pH 7.1, containing diluted plasma and fat body homogenate from a day 3–5 last stadium *G. assimilis* from the high-activity line. The solution was incubated for 5 h at 30 °C, and metabolites were extracted as in the JHE/JHEH TLC radiochemical assays described above. Rf values for JH, JH-acid, JH-diol, JH-acid-diol were 0.64, 0.50, 0.19, 0.1, respectively, and were similar to values obtained for chemically-synthesized metabolites (Zera and Tiebel, 1989).

2.4. JHE transcript abundance in fat body and midgut. RNA isolation

Total RNA was isolated from individual tissues using Trizol Reagent (Invitrogen, Carlsbad, CA) following manufacturer's recommendations. RNA was resuspended in RNase free water and stored at –20 °C. 5 µg of total RNA was treated with 5 units of RNase free DNase 1 (Promega, Madison, WI) and reverse transcribed with 200 units of SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) using an oligo dT₁₈ oligonucleotide at 42 °C for 1 h in a final reaction volume of 40 µL.

2.5. Amplification of *G. assimilis* actin sequence (internal control)

The abundance of actin transcript was used as an internal control to standardize JHE transcript abundance in midgut and fat body of *G. assimilis*. Because the actin sequence had not yet been obtained from *G. assimilis*, it was amplified as follows: A consensus sequence generated by a ClustalW alignment of the *Ovis aries*, *Homo sapiens*, *Cavia porcellus*, *Rattus norvegicus*, and *Drosophila melanogaster* β-actin amino acid sequences was used to design oligonucleotide primers to amplify a 959 bp internal region of the *G. assimilis* actin cDNA. 1 µL of cDNA was used as a template in 1 × PCR buffer, 1 mM MgCl₂, 0.2 mM each dNTP, 5 units Taq DNA polymerase (Invitrogen, Carlsbad, CA) and the primers actinF1 5'GC-GTGCTGTTTTCCCGTCCA3' and actinR1 5'GTCGATTTCCT-GTTTGAGA3' at a final concentration of 0.2 µM each. PCR was carried out with an initial DNA denaturing step of 94 °C for 2 min, then 30 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s followed by an extension step of 90 s at 72 °C and a final

extension step of 7 min at 72 °C. 1 µL of a 1/100 dilution of the previous reaction was used as template in a hemi-nested PCR with the same components with the exception that the antisense primer was substituted with actinR2 5'GAGATCCACATCTGCTGGAA3', and the annealing temperature was 55 °C. The hemi-nested amplicon was cloned into the pGem-T Easy vector (Promega, Madison, WI) and sequenced using BigDye terminator chemistry (Applied Biosystems, Foster City, CA). The actin nucleotide sequence obtained (available on request) was 959 bp in length and was 96% identical to *Gryllus bimaculatus* actin nucleotide sequence.

2.6. Real time-polymerase chain reaction (real time-PCR) of JHE and actin

Primer Express software (Applied Biosystems, Foster City, CA) was used to design the following taqman probes and primers to amplify the *G. assimilis* *Jhe* and *actin* cDNA based on the *G. assimilis* *actin* sequence obtained in the present study (see above), and the *G. assimilis* *Jhe* sequence reported in Crone *et al.* (2007): ACTprobe: 5'6-FAM-CCTGTTCTCCTGACTGAAGCCCCTCTG-TAMARA3'; JHE probe: 5'6-FAM-CAGCCGCACGTCCTCCCTCGA-TAMARA3'; Actin primers: 5'GAGTTGCCCTGAGGAGCA3' (actin170 U) and 5'TTCCCTGTTGGCTTTAGGGT3' (actin238L); *Jhe* primers: 5'AATCTCAGACTTCTACTTCCGGCAAC3' (Jhe1098U) and 5'ATGAACGGCTGCGGCTT3' (Jhe1163L). Real time-PCR reactions were performed in a final volume of 50 µL using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). The final concentration of each primer was 900 nM and the final concentration of each probe in the reaction was 250 nM. Reactions were performed using 60 cycles of the manufacturer set cycling conditions on an ABI7700 real-time PCR machine (Applied Biosystems, Foster City, CA). Transcript abundance value of each individual was the mean of three replicates. Separate reactions were performed for *Jhe* and *actin*, and the *Jhe* transcript measurements were normalized using *actin* as the standard. Where the standard deviation of normalized replicate samples was greater than 0.3 the individual was not included in the statistical analysis. To ensure that sample plates could be accurately compared to each other, standard samples were included on each plate. The threshold value for each plate was then set at a consistent value for the standard samples between plates and an allowed standard deviation of no more than 0.3 was maintained between plates. Standard curves were generated using a serial dilution of a mixture of cDNA prepared from Blocks 1 and 3 day-4 samples. Standard PCR using the real time PCR primers yielded amplification products of expected size for both *Jhe* and *actin* and preliminary real-time runs yielded expected amplification plots.

2.7. Statistical analyses

Analyses of differences between traits involving more than one variable (*e.g.*, differences in transcript abundance between Line and Block) were performed using ANOVA (Sokal and Rohlf, 1989), while comparisons between pairs of means for a single variable (JHE or JHEH activity differences between high- and low-activity lines on a particular day of development) were performed using *t*-tests (Sokal and Rohlf, 1989). Associations between JHE and JHEH activity or between *Jhe* transcript abundance and JHE activity were assessed by computing Pearson correlations [Spearman (non-parametric) correlations gave virtually identical results (data not shown)]. In the former case, all variables (*i.e.*, enzyme activities; see Table 1) were tested against each other and the significance of the correlation coefficients was determined by taking into account the number of independent variables tested (*a posteriori* tests) using Table Y of Sokal and Rohlf (1969). Sample sizes and hence statistical power was much lower (about 10-fold) for corre-

lations involving transcript abundance. Hence we only computed selected correlations that tested specific *a priori* hypotheses of interest, which did not require adjusting *P*-values for multiple variables. ANOVAs and correlation coefficients were computed using Systat 8.0, while *t*-tests were computed using Prism 4.0.

Table 1. Correlations between plasma, fat body, and midgut JHE and JHEH specific activities

First variable	Second variable				
	FB-JHE	FB-JHEH	PL-JHE	MG-JHE	MG-JHEH
FB-JHE	-				
FB-JHEH	0.207 ns	-			
PL-JHE	0.670***	0.107 ns	-		
MG-JHE	0.315**	0.062 ns	0.253*	-	
MG-JHEH	0.156 ns	-0.017 ns	0.369**	0.397**	-

****P* < 0.005; ***P* < 0.01, **P* < 0.05, ns: non-significant (*P* > 0.05); correlations were based on 207 individual crickets sampled in the four artificially-selected lines on various days of the last stadium (data in Figure 1 and Figure 2; see Section 2 for additional details of correlational analyses). FB: fat body, MG: midgut, PL: plasma; JHE and JHEH: juvenile hormone esterase or juvenile hormone epoxide hydrolase specific activities, respectively.

3. Results

3.1. Enzyme activity profiles in various tissues and days of the last stadium

Activities of plasma JHE, specific activities of fat body and midgut JHE, and specific activities of fat body and midgut JHEH were measured in approximately 220 individual crickets from two pairs (two blocks) of lines. Each pair consisted of a line that had been artificially selected for high- or low-plasma JHE activity. Developmental profiles of plasma JHE activity, observed in the present study (Figure 1), were similar to profiles observed in previous studies (Zera and Zhang, 1995; Zera, 2006). Activities rose from near zero values at the beginning of the last stadium to a mid-stadium (day 4) peak of about 75 nmol JH-acid/min/mL plasma, and dropped again during the latter part of the stadium. Consistent differences were observed in JHE activity between high- and low-activity selected lines in each of the two blocks: in each case, peak JHE activities were about 7-fold higher in the high- vs. low-activity lines (results of *t*-tests are contained in the legend of Figure 1).

JHE activity in fat body was also significantly higher (5–10-fold at peak) in crickets from the high-selected vs. low-selected

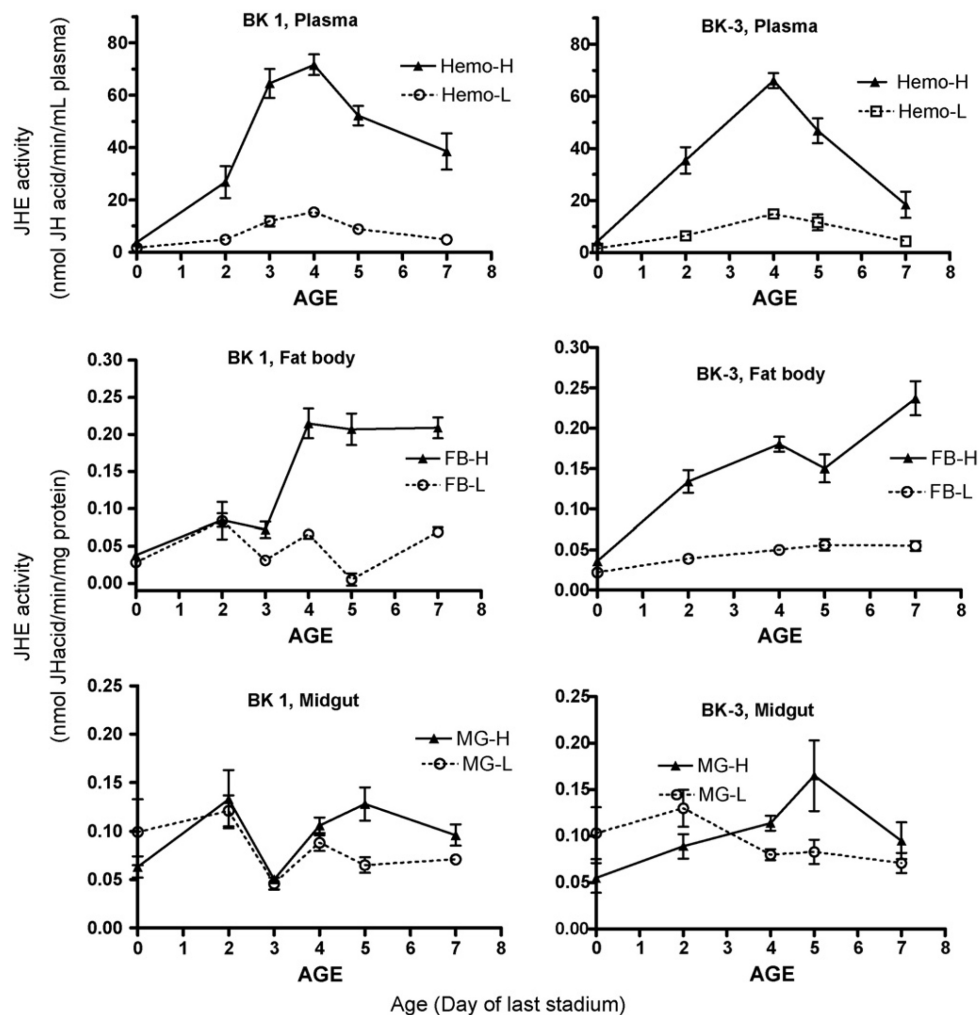


Figure 1. JHE activities (means \pm S.E.M.) in plasma, fat body, and midgut of lines of *G. assimilis* artificially selected for high (filled symbols, solid lines) or low (open circles, dashed lines) plasma JHE activity. Bk (block) refers to independent selection trial (see Section 2). Means were based on assays of 3–9 individuals (median = 4) on days 0–3 and 7–22 (median = 8) individuals on days 4–7. JHE activities differed between high- and low-selected lines in the following cases for Bk-1 (*P* < 0.05; two-tailed *t*-test with unequal variances): plasma: all days except day 0; fat body: days 4, 5, and 7; midgut: day 5. Corresponding results for Bk-3: plasma: all days; fat body: all days; midgut: day 4.

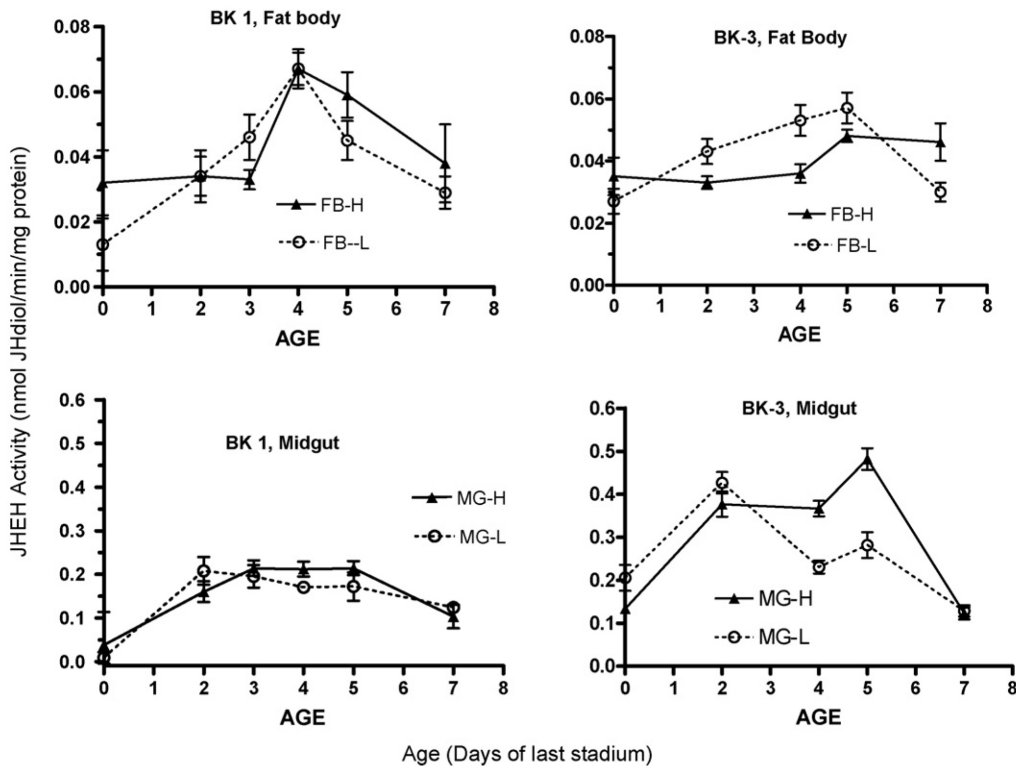


Figure 2. Juvenile hormone epoxide hydrolase specific activity in fat body and midgut of lines of *G. assimilis* artificially selected for high- or low-plasma JHE activity. See Figure 1 for explanation of symbols. Means were based on assays of 3–9 individuals (median = 4) on days 0–3 and 7–22 (median = 8) on days 4–7. JHEH activities differed between high- and low-selected lines on the following days ($P < 0.05$, two-tailed *t*-test with unequal variances): BK-1, fat body: no day; BK-1, midgut: day 4; BK-3, fat body: days 2, 4, and 7; BK-3, midgut: days 4 and 5.

lines during the mid-latter part of the stadium (Bk-1) or during most of the stadium (Bk-3) (Figure 1; results of individual *t*-tests are in figure legend). JHE activity differences between high and low lines in fat body roughly paralleled activity differences in the plasma during most, but not all, portions of the last stadium, and some differences between the blocks were observed. Like plasma JHE activity, JHE activity in the fat body of crickets from the high-selected lines rose from its lowest value at the beginning of the stadium to a mid-stadium (day 4) peak (Bk-1) or near peak (Bk-3). Unlike plasma JHE activity, fat body JHE activity in the high-activity lines did not drop from the mid-stadium peak during the latter part of the stadium; activities either remained on a plateau (Bk-1), or rose slightly higher near the end of the stadium (Bk-3). Only slight changes in fat body JHE specific activity were observed in the low-activity lines during the last stadium, similar to the situation for plasma JHE activity.

The magnitude of peak JHE activity in the midgut was about half that of JHE activity in the fat body (Figure 1), and developmental profiles in the midgut differed from those in the fat body. First, rather than exhibiting a mid-stadium (day 4) peak, JHE activities in the midgut exhibited peaks early (day 2) and slightly later (day 5) in the stadium (Bk-1), or only later in the stadium (Bk-3). Second, JHE activities in the midgut differed to a lesser degree, and mainly on day 5, between the high-activity and low-activity lines (results of *t*-tests are in legend of Figure 1).

JHEH activities in the midgut were much higher (ca. 5-fold) than JHEH activities in the fat body (Figure 2). Developmental profiles were roughly similar to plasma JHE activity (except for activities in the high-selected line of Bk-3). In general, activities rose to a broad mid-stadium peak (days 2–5) and fell near the end of the stadium. Virtually identical JHEH activities in the midgut and fat body were observed between high-activity and low-activity stocks of Bk-1 (results of *t*-tests are in the legend of Figure 2). In Bk-3, JHEH activity

was consistently higher in the midgut of the high- vs. low-activity line during the mid-latter part of the stadium, while the low-activity line exhibited higher JHEH activity than the high-activity line during the mid-stadium in the fat body.

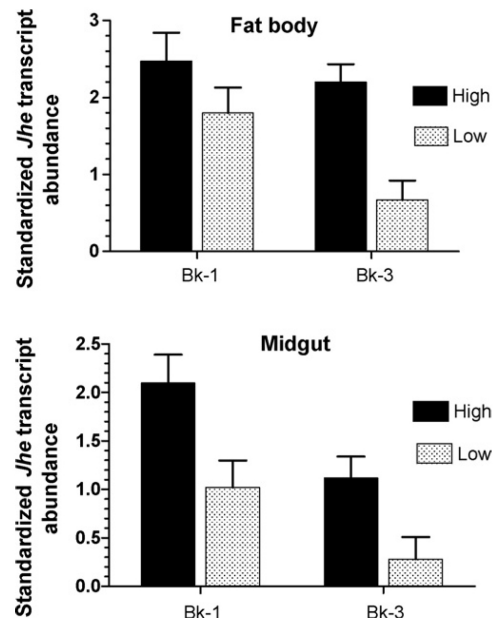


Figure 3. Standardized fat body, or midgut JHE transcript abundance in lines selected for high- or low-plasma JHE activity. Bk refers to independent selection trial (see Section 2). Values are means \pm S.E.M. based on 9–10 (Bk-1) or 4–6 (Bk-3) individuals. Each individual transcript abundance value, in turn, was the average of 3 replicates measured by real-time PCR (see Section 2).

Table 2. Correlations (r) between JHE transcript abundance and JHE activity in fat body, midgut or plasma in lines selected for high- or low-plasma JHE activity

First variable	Second variable	r
FB-JHE-trans vs.	FB-JHE-activity	0.502*
FB-JHE-trans vs.	MG-JHE-trans	0.597**
FB-JHE-trans vs.	PL-JHE activity	0.502*
MG-JHE-trans vs.	MG-JHE activity	0.504*
MG-JHE-trans vs.	PL-JHE activity	0.465*

FB: fat body, MG: midgut, PL: plasma, JHE: juvenile hormone esterase, trans: transcript abundance (mRNA concentration), activity: enzyme specific activity; * $P < 0.05$ and ** $P < 0.01$ (Pearson correlations based on 28 crickets analyzed on day 4 of the last nymphal stadium in high and low lines of Bk-1 and Bk-3). These correlations were measured on the same individuals on which correlations reported in Table 1 were measured. Spearman (non-parametric) correlation coefficients were virtually identical to Pearson correlation coefficients and gave identical P values.

3.2. Correlations between JHE and JHEH activities in various body compartments

Plasma JHE activity was most strongly and positively correlated with fat body JHE specific activity, and to a lesser degree with midgut JHE specific activity (Table 1). Fat body and midgut JHE activities were also positively correlated with each other. Midgut JHEH was correlated with plasma and midgut JHE, while fat body JHEH activity was not correlated with any measured variable.

3.3. JHE transcript abundance in fat body and midgut

Jhe transcript abundance in fat body was measured on day 4, the midpoint of the last nymphal stadium (Figure 3), a time in development just prior to (midgut) or when JHE activity peaks in fat body and plasma. Fat body transcript abundance, averaged over the 2 blocks, was 1.9-fold higher in the lines selected for high- vs. low-plasma JHE activity (ANOVA: $P < 0.005$). When lines were compared within each block, fat body transcript abundance was significantly higher in the high- vs. low-activity lines of Bk-3 ($P < 0.005$), but not in Bk-1 ($P = 0.2$), although rank-order differences between lines were the same for each block. Transcript abundance was also significantly higher in Bk-1 vs. Bk-3 ($P < 0.05$). However, no Line \times Block interaction was observed ($P = 0.17$) indicating that the relative elevation in transcript abundance in high- vs. low-activity lines did not differ significantly between blocks.

Jhe transcript abundance in midgut, measured on day 4 of the last nymphal stadium, was 2.5-fold higher in lines selected for high- vs. low-plasma JHE activity ($P < 0.005$; activities pooled across the two blocks). Transcript abundance was higher in high vs. low lines within each block (ANOVA, Bk-1: $P < 0.025$; Bk-3: $P < 0.025$). Finally, as was the case for fat body, *Jhe* transcript abundance was significantly higher in Bk-1 vs. Bk-3 ($P < 0.005$), and no Line \times Block interaction was observed ($P > 0.6$).

In fat body or midgut, on day 4 of the last nymphal stadium, *Jhe* transcript abundance was positively correlated with JHE enzyme specific activity of the same organ, or with plasma JHE activity (Table 2). *Jhe* transcript abundance in fat body was also positively correlated with transcript abundance in midgut (Table 2).

4. Discussion

Characterizing genetically-based endocrine variation within populations of organisms is one of the most important tasks facing the nascent field of evolutionary endocrinology (Zera *et al.*, 2007).

Information on the types of endocrine variation available for selection to act on, the extent to which endocrine variants are genetically correlated with each other and hence are constrained to evolve in concert, and the effects of endocrine variants on whole organism traits, are key pieces of information for identifying the mechanisms underlying microevolutionary change in endocrine regulation.

In the present study we measured developmental profiles of JHE and JHEH activities in plasma, fat body and midgut, and *Jhe* transcript abundance in fat body and midgut in stocks of *G. assimilis* that differ dramatically in plasma JHE activity due to artificial selection. The main purpose was to identify significant contributors to genetic variation in plasma JHE activity as well as to identify traits that potentially interact genetically (*i.e.*, are correlated) with each other (*e.g.* JHE and JHEH activities, *Jhe* transcript abundance in midgut and fat body). On the one hand, because JHE and JHEH both metabolize the same substrate one might expect to find correlations between organ/tissue-specific activities of these enzymes that co-ordinate metabolism of JH within or between various organs. On the other hand, lack of correlations between JHE and JHEH activities could also be important in the tissue-specific regulation of JH levels. To our knowledge, these data constitute the first published data on population-genetic variation in either JHE activity in non-hemolymph body compartments or in *Jhe* transcript abundance in insects. In addition, JHE and JHEH fat body and midgut enzyme activities reported here are the first reports of activities of these important JH regulators in individual, non-hemolymph organs of a hemimetabolous insect, a member of a major group of insects that has been understudied with respect to *Jhe* regulation. Functional aspects of organ-specific JHE and JHEH activities and *Jhe* transcript abundance will be discussed first, followed by evolutionary aspects.

4.1. Fat body and midgut JHE and JHEH activities: developmental and functional aspects

A major finding of the present study was the dramatic difference in fat body JHE activity between lines artificially selected for high- or low-plasma JHE activity (Figure 1). Line differences in fat body JHE activity, strongly paralleled line differences in plasma JHE activity, while developmental changes in fat body JHE activity within individual lines also strongly covaried with plasma activity (Figure 1, Table 1). An important exception to this pattern occurred in the latter part of the last stadium, when fat body JHE activities remained high after plasma JHE activities had dropped precipitously (Figure 1; discussed below). These results are similar to those reported for lepidopterans where high JHE activity occurs in fat body, and where fat body and plasma JHE activity developmental profiles were, in general, strongly correlated. This was especially the case for *Trichoplusia ni* (Wing *et al.*, 1981; Hammock, 1985), and to a lesser degree for *M. sexta* (Jesudason *et al.*, 1992). In contrast to the single JHE plasma activity peak during development in hemimetabolous insects such as *G. assimilis*, holometabolous insects have two plasma JHE activity peaks. In *T. ni*, JHE activity changed largely in concert in both fat body and plasma during each of these peaks, the exception being the retention of high fat body JHE activity, when plasma JHE activity had dropped after the second peak; by contrast, in *M. sexta*, there was only a single peak in fat body JHE activity that strongly paralleled the first plasma JHE activity peak (Jesudason *et al.*, 1992).

As mentioned previously, plasma JHE activity is produced in non-hemolymph tissues and is secreted into the hemolymph. Several pieces of evidence collectively suggest that fat body JHE, as opposed to midgut JHE, is a greater contributor to the hemolymph JHE activity pool in *G. assimilis*. Developmental changes

in JHE specific activity in fat body more strongly paralleled changes in plasma JHE activity than did midgut activity (Figure 1), and fat body JHE activity was more strongly correlated with plasma JHE activity (Table 1). JHE specific activity in the high-activity lines also was about twice as high in the fat body as in the midgut within the same lines.

A potentially important factor that influences the relative contribution of JHE from various organs to the hemolymph JHE pool, but which has not been extensively studied, is the relative secretion rates of JHE from these organs. Estimates of the contribution of JHE from various organs to the plasma JHE activity pool based on the relative magnitude of JHE activity and the masses of these non-hemolymph organs could give erroneous estimates if the rate of JHE secretion into the hemolymph differs significantly between the organs. We have no data on relative JHE secretion rates from various organs in *G. assimilis*. Although *in vitro* secretion of JHE from the fat body has been reported for several lepidopterans (Hammock, 1985; Jones *et al.*, 1987) relative secretion rates of JHE from various organs has not been determined. As mentioned above, in both *G. assimilis* (high-activity lines; Figure 1) and *T. ni* (Wing *et al.*, 1981; Figure 3) fat body JHE activity remained elevated during the latter part of the last juvenile stadium, when JHE activity in the plasma of both of these species strongly dropped. This suggests that developmental changes in the rates of JHE secretion from fat body may significantly influence the developmental profile of plasma JHE activity.

The non-parallel developmental profiles of plasma, midgut, and fat body JHE activities, during specific periods of the last juvenile stadium of *G. assimilis* also suggest that JH degradation may differ among various organs, genetic stocks, and stages of development in *G. assimilis* as has been proposed for a number of lepidopterans (Hammock, 1985; Jesudason *et al.*, 1992). Degradation of JH by JHE in tissues other than hemolymph has not been well studied in insects but is likely to be an important component of JH titer regulation, especially when the JH titer within specific tissues must differ from that of the hemolymph. For example, JHE activity within imaginal disks of *Galleria mellonella* peaks before plasma JHE activity and may regulate imaginal disk cell proliferation by reducing the JH concentration within the disks (Reddy *et al.*, 1980).

In general, fat body and midgut JH-epoxide hydrolase activities differed to a much lesser degree between lines, which is consistent with the previous observation of no line differences in JHEH activities in whole-body homogenates (Zera and Zhang, 1995; Zera and Huang, 1999; Zera, 2006). The main exception was midgut JHEH in Bk-3, where activity was higher in the high- vs. low-selected lines throughout the mid-stadium. Specific activity of JHEH was about 2–3-fold higher than JHE in the midgut in both blocks, indicating that JHEH may play a more significant role in JH degradation in midgut than in fat body.

4.2. JHE transcript abundance

Several studies of holometabolous insects have demonstrated a strong correlation between developmental changes in *Jhe* transcript abundance in fat body and JHE enzyme activity in fat body, plasma, or whole-body extracts (Hirai *et al.*, 2002; Bai *et al.*, 2007; Munyiri and Ishikawa, 2007). These correlations suggest that developmental changes in *Jhe* gene transcription may be a common cause of developmental changes in JHE specific activity. The present study is the first to report analogous genetic differences in *Jhe* transcript abundance and genetic covariance between *Jhe* transcript abundance and JHE enzyme activity in fat body, midgut or plasma at the same time in development in genetic stocks derived from natural populations (Figure 1, Figure 2, and Figure 3; Table 2).

4.3. Evolutionary implications of tissue and organ specific genetic variation for JHE activity and *Jhe* gene expression

The present study essentially measured correlated (indirect) responses to direct artificial selection on plasma JHE activity (Zera and Zhang, 1995; Zera, 2006). Correlated responses to selection identify traits that are genetically correlated due to two very different causes (Garland and Carter, 1994). The first occurs when traits are mechanistically linked in a chain of causality determining the expression of a particular trait, such as when a specific DNA sequence specifies a high-activity enzyme phenotype; the DNA sequence will increase in frequency and mean enzyme activity will increase in a line selected for high-enzyme activity. The second type results when different traits interact genetically, for example, when they are regulated by the same genes (*i.e.* pleiotropy).

Functional considerations discussed above implicate fat body *Jhe* transcript abundance and fat body JHE enzyme specific activities as important contributors to plasma JHE activity. This information, coupled with the correlated responses in fat body *Jhe* transcript abundance and fat body JHE specific activity (both elevated in lines selected for high plasma JHE activity), identify these two traits as being important contributors to genetic variation in plasma JHE activity, the trait that was directly selected. The cause of the correlated response to selection on midgut JHE activity is less certain, because the extent to which midgut JHE activity contributes to plasma JHE activity is less clear. Thus, midgut *Jhe* transcript abundance and midgut JHE specific activity may have been indirectly altered by selection either due to the contribution of midgut JHE to plasma JHE, or due to midgut *Jhe* and fat body *Jhe* being co-regulated by the same factors. Additional functional and genetic studies will be necessary to distinguish between these two hypotheses.

Because selection was initiated on a laboratory base population, that had been kept for only a few generations after being founded from field-collected individuals (Zera and Zhang, 1995), genetic variation for fat body and midgut *Jhe* transcript abundance and JHE enzyme specific activity were likely present in the field populations from which founding crickets were collected. These findings are significant as there is a paucity of information on the characteristics of standing genetic variation for endocrine traits in either laboratory or field populations. This is especially the case for molecular-genetic aspects of endocrine variation such as variation in *Jhe* transcript abundance. Most current examples of molecular-genetic variation for endocrine traits in natural populations are for traits documented in vertebrates (Zera *et al.*, 2007).

Results of the present study, which directly identified genetic covariation (*i.e.* correlated responses to selection) between *Jhe* gene expression, JHE activity in non-hemolymph tissues, and plasma JHE activity, provide support for earlier arguments that genetic variation in plasma JHE activity in *Gryllus* is more likely due to variation in *Jhe* gene expression rather than to differences in kinetic properties of stock-specific JHE enzyme(s) (Zera and Huang, 1999). No substantial kinetic differences have been identified between JHE enzymes from high- or low-activity selected lines of *G. assimilis* (Zera and Zeisset, 1996; Table 3), or its congener *G. rubens* (Zera *et al.*, 1992). Nor have any nucleotide differences been identified in the coding sequence of a *Jhe* cDNA of *G. assimilis* within or between the same lines that were the subjects of the present study (Crone *et al.*, 2007; Table 3).

At present, relatively few population-genetic studies have investigated correlations between gene expression and enzyme specific activity, and for those that have, no consensus has emerged regarding the extent to which strong correlations exist between these two factors. For example, at one extreme, studies in the kil-

Table 3. Summary of correlated responses to selection (*i.e.* line differences) in lines of *G. assimilis* artificially selected for high- or low-plasma JHE activity on day 3 of the last nymphal stadium

Trait	Value in lines selected for high- vs. low-plasma JHE activity	References
(1) Plasma JHE activity (mid-last stadium)	Significantly higher (6–8 fold)	A, B, D, E, G
(2) Plasma JHE activity in adults	Marginally higher	A, B, D, E
(3) Whole-body JHE activity	2.6-Fold higher	A, E
(4) Proportion of whole-body JHE in plasma	2.6-Fold higher	A, E
(5) JH binding in plasma	3-Fold higher	B, E
(6) JHEH whole specific body activity	No difference	A, E
(7) Whole-body, <i>in vivo</i> rate of JH degradation	1.4-Fold higher	A, B
(8) JHE enzyme kinetics	No difference in Michaelis constant, inhibition constants or thermostability	C
(9) <i>Jhe</i> cDNA nucleotide sequence encoding mature protein	No difference	F
(10) JHE specific activity in fat body	5–10-Fold higher at peak	G
(11) JHE specific activity in midgut	1-Fold higher at day 5 peak; not different on most days	G
(12) <i>Jhe</i> transcript abundance in fat body	1.9-Fold higher	G
(13) <i>Jhe</i> transcript abundance in midgut	2.5-Fold higher	G
(14) JHEH activity in fat body	No consistent difference	G
(15) JHEH activity in midgut	No consistent difference	G

All traits were measured in the last nymphal stadium except for plasma JHE activity in adults. References: A: Zera and Zhang, 1995; B: Zera *et al.*, 1996; C: Zera and Zeisset, 1996; D: Zera *et al.*, 1998; E: Zera and Huang, 1999; F: Crone *et al.*, 2007; G: present study. See Zera and Huang (1999) and Zera (2006) for additional information regarding comparisons between long-wing and short-wing selected lines of *G. firmus* and *G. rubens* for many of these correlated responses.

fish *Fundulus heteroclitus*, have demonstrated that genetic differences in lactate dehydrogenase (LDH) transcript abundance explain a large proportion of genetic variation in LDH specific activity found in natural populations of this species (Crawford and Powers, 1989). At the other extreme, no difference in alcohol dehydrogenase (*Adh*) transcript abundance was identified between genetic stocks of *Drosophila melanogaster* that differ in specific activity of the ADH enzyme, as well as in ADH enzyme protein concentration (Laurie and Stam, 1988). Finally in laboratory selection studies of microorganisms, microevolutionary changes in enzyme activities often are not accompanied by changes in transcript abundance and vice versa (*e.g.* Daran-Lapujade *et al.*, 2004; and references therein).

In the present study we found no correlated responses to selection on midgut or fat body JHEH specific activity when plasma JHE activity was directly selected (Table 3). Thus, alleles that influence fat body and midgut JHE specific activity and, in turn, plasma JHE activity have no observable effect on fat body or midgut JHEH activity. The failure to find correlated responses to selection on either fat body or midgut JHEH specific activity, is consistent with our previous finding of no correlated responses to selection on whole-body JHEH activity (Zera and Huang, 1999; Zera, 2006). These results stand in stark contrast with the strong correlated responses to selection and strong positive genetic correlations between plasma JHE activity and plasma JH binding (see Discussion in Zera *et al.*, 1996; Zera, 2006; Table 3). From an evolutionary perspective, the significance of these findings is that activities of the two main JH degrading enzymes, JHE and JHEH, can evolve independently of each other, while plasma JH binding and JH degradation are constrained to evolve in concert, possibly because they are co-regulated by the same factors. At present it is unclear why certain components of JH regulation are genetically correlated while others are not.

Plasma JHE activity is a complex trait, and there are still many potential contributors to genetic differences in this trait between selected lines of *G. assimilis* that have yet to be investigated. Two especially important foci for future research are rate of secretion of JHE into the hemolymph and the role of variation in expression of additional *Jhe* genes. Previous studies have demonstrated that high- and low-activity lines of several *Gryllus* species differ dramatically in the proportion of whole-body JHE activity that is found in the plasma vs. other body compartments (Table 3). This suggests that (1) genetic variation in JHE secretion into the hemolymph is a likely important contributor to genetic variation in plasma JHE ac-

tivity in *G. assimilis*, and that (2) natural selection on genes regulating secretion rates has been an important aspect of the evolution of high- and low-JHE activity of genetically-specified morphs of *G. firmus* that express different wing-length and life-history traits. Stock-specific rates of JHE secretion into the hemolymph have yet to be directly studied in any *Gryllus* species. Finally, multiple JHE isoforms in *G. assimilis* have been identified that differ in up to 4 residues of the 13–20 N-terminal amino-acid sequences of the mature proteins (Zera *et al.*, 2002; Crone *et al.*, 2007). This suggests the possible existence of multiple *Jhe* genes that encode multiple JHE enzymes in this species. The contribution of differential expression of additional *Jhe* genes to genetic variation in plasma JHE enzyme activity has not yet been investigated.

In summary, we have identified genetic variation in several traits, most notably JHE specific activity in fat body, and *Jhe* gene transcript abundance, that contribute to genetic variation in plasma JHE activity in *G. assimilis*. These findings represent significant contributions to our understanding of the chain of causality the leads from alteration in regulation of *Jhe* gene(s) to alteration in plasma JHE activity for this important experimental model in evolutionary endocrinology.

Acknowledgement

Research reported in this manuscript was supported by NSF grant IBN-0212486 to A. J. Zera.

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