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Transcriptional changes during *Daphnia pulex* development indicate that the maturation decision resembles a rate more than a threshold

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Abstract

Maturation is a critical developmental process, and the age and size at which it occurs have important fitness consequences. Although maturation is remarkably variable, certain mechanisms, including a minimum size or state threshold, are proposed to underlie the process across a broad diversity of taxa. Recent evidence suggests that thresholds may themselves be developmentally plastic, and in the crustacean *Daphnia pulex* it is unclear whether maturation follows a threshold or is a gradual process more akin to a rate. Changes in gene expression across four instars before and during maturation were compared in a cDNA microarray experiment. Developmental stage was treated statistically both as a discontinuous and as a continuous variable, to determine whether genes showed gradual or discrete changes in expression. The continuous analysis identified a greater number of genes with significant differential expression (45) than the discontinuous analysis (11). The majority of genes, including those coding for histones, factors relating to transcription and cell cycle processes, and a putative developmental hormone showed continuous increases or decreases in expression from the first to the fourth instars that were studied, suggestive of a prolonged and gradual maturation process. Three genes coding for a fused vitellogenin/superoxide dismutase showed increases in expression following the second instar and coincided with the posited maturation threshold, but even their expression increased in a continuous fashion.

Introduction

The transition from prereproductive growing juvenile to reproductively mature adult is a critical point in the life history of many organisms (Roff, 2001). The trade-off between growth and reproduction that occurs during maturation determines the age and size at maturity of an organism (Day & Taylor, 1997), which correlates with fitness or reproductive success through effects on fecundity and survival (Roff, 2000, 2002). Life-history theory predicts that organisms will increase fitness by maximizing size and minimizing age at maturity (Stearns & Koella, 1986; Berrigan & Koella, 1994). Yet maturation processes are highly variable, and age and size at maturity differ substantially between species and individuals across environments (Bernardo, 1993) as a result of genetic variation (Roff, 2000; Sgrò & Hoffmann, 2004; Dmitriew *et al.*, 2010) and phenotypic plasticity in responses to environmental variables such as temperature (Atkinson, 1994), resource availability (Berrigan & Charnov, 1994), predation risk (Crowl & Covich, 1990) and season (Nylin *et al.*, 1989; Nylin & Gotthard, 1998). In spite of this variability, it has been hypothesized that certain conserved physiological mechanisms can explain environmentally variable maturation reaction norms across taxa. The maturation threshold proposed by Wilbur & Collins (1973) and formalized by Day & Rowe (2002) hypothesizes that individuals must reach a minimum size or state before maturation can occur. Such a threshold has been

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proposed to explain the often observed negative relationship between age and size at maturity in numerous organisms across a wide variety of taxa (Nijhout & Williams, 1974b; Policansky, 1983; Ebert, 1992; Bradshaw & Johnson, 1995; Denver, 1997; Morey & Reznick, 2004; Plaistow *et al.*, 2004). Although maturation thresholds may provide a theoretical basis for explaining age and size at maturity, in order to understand the evolution of maturation, it is also necessary to consider specific ontogenetic mechanisms that underlie both maturation decisions and subsequent maturation processes (Berner & Blanckenhorn, 2007), as such mechanisms are likely to be idiosyncratic and representative of specific life-history adaptations (Nijhout, 2008).

Maturation decisions and subsequent maturation process may share similar mechanisms, but result in different reaction norms for age and size at maturity. For example, both the tobacco hornworm moth *Manduca sexta* (Nijhout & Williams, 1974b) and the fruit fly *Drosophila melanogaster* (De Moed *et al.*, 1999) must exceed a critical weight or size threshold before pupation occurs. The physiological changes associated with this threshold and the final growth phase before pupation have received further investigation in both species. These studies reveal that although developmental changes associated with maturation and metamorphosis rely on similar endocrine pathways, environmentally dependent differences in their regulation produce different reaction norms for age and size at maturity (Nijhout, 2008). In *M. sexta*, the terminal growth period between initiation and completion of maturation is fixed, resulting in a negative relationship between age and size across growth environments (Nijhout & Williams, 1974a; D'Amico *et al.*, 2001; Davidowitz *et al.*, 2003; Davidowitz & Nijhout, 2004), whereas in *D. melanogaster*, this terminal growth period is plastic, allowing pupation at similar sizes irrespective of food availability (Mirth & Riddiford, 2007; Layalle *et al.*, 2008). It is also possible for patterns of maturation to exist in which the size threshold is not at all fixed with respect to growth environment. Larvae of the dung beetle *Onthophagus taurus* only cease growth and initiate metamorphosis when their food supply is depleted, resulting in a positive reaction norm for age and size at maturity across a growth gradient (Shafiei *et al.*, 2001). Furthermore, the above species are all holometabolous insects, and therefore undergo metamorphosis, ceasing to grow following pupation. Yet for many organisms, growth may continue into adulthood (Heino & Kaitala, 1999), and maturation may not coincide with abrupt metamorphic change but instead involve the gradual attainment of reproductive function during processes such as puberty in vertebrates (Gluckman & Hanson, 2006). Studies of species without metamorphosis and with indeterminate growth, such as collembolans (Van Dooren *et al.*, 2005) and cladocerans (Harney *et al.*, 2013), suggest that the decision to mature may not be fixed with respect to size but is variable and may be better thought of as a rate than as a switch. However, maturation in these systems has not received the same level of investigation, and the mechanisms underlying such patterns of development are unclear. Transcriptomic techniques such as microarrays can help to identify which genes are responsible for developmental change. Furthermore, by investigating changes in gene expression between developmental stages, and modelling development with both discontinuous contrasts (comparing expression between individual developmental stages) and as a continuous process (changes in expression across all developmental stages), it may be possible to identify whether genes are regulated in a discrete fashion between developmental stages, or accumulate or decline steadily over the course of development.

The crustacean *Daphnia* has been a popular system with ecologists and evolutionary biologists for many decades, but its recognition as a genetic model organism is more recent (Ebert, 2011), following the creation of the genomic resource wFleaBase (Colbourne *et al.*, 2005) and the publication of the *Daphnia pulex* genome (Colbourne *et al.*, 2011). We build on physiological and ecological studies of *Daphnia* maturation (e.g. Zaffagnini, 1987; Ebert, 1992; Beckerman *et al.*, 2010; Harney *et al.*, 2013) as well as molecular studies of *Daphnia* (David *et al.*, 2011; Hannas *et al.*, 2011; Jeyasingh *et al.*, 2011), using a microarray to study differential gene expression over four instars prior to and during maturation in one clone of *D. pulex*. In *Daphnia*, the physiological changes associated with maturation have been characterized by cytological observations and suggest that maturation occurs over two moult cycles (Rossi, 1980; Zaffagnini & Zeni, 1986; Zaffagnini, 1987), following a size threshold (Ebert, 1992, 1994). However, maturation processes in *Daphnia* are also environmentally responsive (Beckerman *et al.*, 2010), and so-called maturation thresholds or decisions were themselves phenotypically plastic (Harney *et al.*, 2013). Furthermore, unlike insects, where moulting and development are often tightly coupled (Esperk *et al.*, 2007), *Daphnia* are not constrained by a predetermined number of instars (Barata *et al.*, 2001) and can extend the maturation phase beyond two moult cycles in adverse conditions (Enserink *et al.*, 1995; E. Harney, personal observation). It is therefore unclear whether the cytological changes previously reported are the result of a discrete switch or a maturation rate. However, to explain phenotypic plasticity in maturation decisions (Harney *et al.*, 2013), we hypothesize that maturation is a prolonged process of gradual change, rather than a sudden developmental shift. We might therefore expect to see underlying patterns of gene expression associated with maturation occurring prior to the proposed size threshold; and showing continuous and gradual rates of change, rather than discrete or discontinuous changes between developmental stages.

Materials and methods

Experimental animals

All animals used in this study were of the same laboratory-reared clone belonging to the *D. pulex* complex, named Cyril, originating from a pond in Sheffield, UK (53°24′17″N, 1°27′25″W). Animals were cultured at 21 ± 1 °C with a 14 : 10 light:dark photoperiod. *Daphnia* were maintained individually in 150 mL of hard artificial pond water media (OECD 1984) enriched with a standard organic extract (Baird *et al.* 1989). They were fed a diet of 89 cells μL^{-1} *Chlorella vulgaris* Beijerinck (quantified by haemocytometer) on a daily basis and were transferred to fresh media every other day. To minimize the role of maternal effects on differential gene expression, animals were acclimated to experimental conditions for three generations prior to the experiment. During acclimation, individuals from the third clutch of each generation were used to set up the subsequent generation. To produce sufficient RNA for amplification and hybridization, multiple individuals within a developmental stage sample were pooled. A preliminary study suggested that 40 second instar, 32 third instar, 24 fourth instar and 24 fifth instar individuals would provide sufficient RNA for each sample. Because large numbers of individuals had to be pooled for each RNA sample, samples were generated through 4 staggered cohorts over a 1-month period. Experimental cohorts were set up from the third (or later) clutch of eight synchronous females. Neonates from the eight mothers were mixed and randomly assigned to one of the four developmental stages described below.

Developmental progression in *Daphnia*

Daphnia pulex do not have a fixed number of juvenile instars, but the maturation process is commonly spread across three instars, termed IM-1, IM-2 and IM-3 (Bradley *et al.*, 1991; Enserink *et al.*, 1995; Barata & Baird, 1998), which correspond to the following: the development of oocytes (IM-1); oocyte provisioning (IM-2); and the deposition of eggs in the brood chamber (IM-3). To gain an understanding of the ontogenetic changes that *D. pulex* undergo during maturation, it was decided to sample individuals over four instars: the two instars prior to IM-1, followed by IM-1 and IM-2 (Fig. 1). For simplicity, these instars will henceforth be referred to as developmental stages DS-1, DS-2, DS-3 and DS-4. In *D. pulex* clone Cyril, maturation in good food conditions is most commonly achieved in the sixth instar (data collected in Harney *et al.*, 2013); therefore, we estimated that DS-1 through DS-4 would correspond to instars two through five. To provide a *post hoc* estimate of the developmental stages that were sampled in the study, the numbers of individuals with ovaries sampled in the fourth and fifth instars was recorded at the time they were collected. The appearance of ovaries in the fifth instar would confirm that developmental progression was as expected. For three of the four cohorts, more than 95% of individuals harvested in the fifth instar did indeed have ovaries. However, in the remaining cohort, development occurred in fewer instars, as all individuals harvested in the fifth instar had eggs. Second, third and fourth instar samples from this cohort were therefore treated as being one instar ahead, and fifth instar samples were not used.

Molecular preparation and microarray experimental design

All pooled individuals within a sample were added to a watch glass, and excess artificial pond water was removed. Five hundred micolitre TRIzol (Life Technologies, Carlsbad, CA, USA) was added to the watch glass, and animals and TRIzol were then transferred to a 1.5-mL microfuge tube and stored at −80 °C until all samples had been collected. RNA extractions in TRIzol were completed according to the manufacturer's instructions, and samples were bound, washed and eluted in 30 μL of RNase-free water using an Ambion *Purelink RNA mini kit* (Ambion, Austin, TX, USA).

Thirty samples were generated following RNA extraction. Yield and integrity were assessed with an Agilent

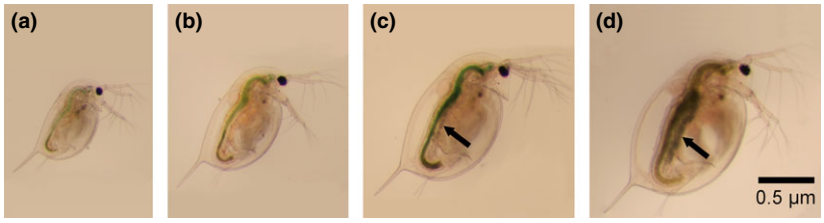

Fig. 1 *Daphnia pulex* phenotypes throughout maturation, corresponding to instars 2–5 or developmental stages 1–4 (DS-1 to DS-4). DS-1 (a) and DS-2 (b), individuals do not undergo observable phenotypic changes. During DS-3 (c), individuals begin 'previtellogenesis' (Zaffagnini, 1987), which in some cases results in the appearance of the ovaries as faint grey/green lines parallel to the gut (arrow in panel c). However, it is not until DS-4 (d) that vitellogenesis causes the ovaries to accumulate large quantities of vitellogenins, visible as a dark grey/green mass adjacent to the gut (arrow in panel d).

2100 bioanalyzer on *RNA 6000 nano labchips* (Agilent, Santa Clara, CA, USA). Rapid development in one of the cohorts reduced the number of suitable RNA samples and prevented a fully dye-balanced interwoven loop design from being selected for the microarray experiment (Wit & McClure, 2004). Using only samples of sufficiently high RNA integrity that could be hybridized with other samples within the same cohort resulted in a design incorporating 22 samples (four DS-1 and DS-3 samples, seven DS-2 and DS-4) in 11 hybrid pairs (see Fig. S1 for experimental design), enough to fill 11 of 12 arrays on the fifth-generation Nimblegen 12 × 135K array (Roche NimbleGen Inc., Madison, WI, USA).

Amplification and labelling of RNA was achieved using Ambion's *Amino Allyl MessageAmp II aRNA amplification kit* (Ambion), and concentrations of RNA and dye were quantified using a Nanodrop ND-1000 Spectrophotometer. Dye incorporation ranged from 28 to 80 dye molecules per 1000 nucleotides for our 22 samples. Pairs of samples to be hybridized together were then combined. Hybrid pairs were incubated at 70 °C for 15 min with 1 μL of Ambion *Fragmentation Buffer* before 1 μL of Ambion *Stop Solution* was added. Samples were then vacuum-dried in the dark for approximately 15 min.

The array used in this study was designed by the *Daphnia* Genomics Consortium (DGC, Centre for Genomics and Bioinformatics: https://wiki.cgb.indiana.edu/display/DGC; see also Supporting Information from Colbourne *et al.*, 2011). Within each of the identical subarrays, most exonic regions were represented by between one and three probes, with a total of 29 569 validated genes. The remaining probes consisted of neighbouring regions, defined as areas of transcription less than the mean intron length (170 base pairs) from a validated gene, transcriptionally active regions (TARs), defined as areas of transcription greater than the mean intron length from a validated gene, and control and random probes that reflected genome nucleotide composition. Hybridization to the array and subsequent washing were carried out according to the manufacturer's instructions. The two-colour array was then scanned at a resolution of 2 μm using Agilent's G2565CA Microarray Scanner System (Agilent), and data were extracted from scanned images using NimbleScan software (Roche NimbleGen Inc.). Microarray data have been deposited in Array Express (www.ebi.ac.uk/arrayexpress/) with accession number E-MTAB-3388.

Statistics and bioinformatics

All statistical analyses were carried out in R (R Development Core Team, 2014). Intensity data for each probe were normalized within and between arrays using the Limma package (Smyth & Speed, 2003), and an intensity score for each feature (exons, TARs and neighbouring regions) was obtained by averaging the intensity scores across probes of the same sequence. Differential expression throughout ontogeny was assessed using linear models and empirical Bayes statistics within Limma (Smyth, 2004) and using Benjamini–Hochberg false discovery rates (B-H FDR; Benjamini & Hochberg, 1995). Changes in gene expression were quantified according to two different analyses. In the first instance, developmental stage was treated as a discontinuous variable, allowing identification of genes that were differentially expressed between any two developmental stages. The second analysis considered developmental stage to be a continuous variable, highlighting genes that were generally up-regulated or down-regulated throughout ontogeny.

Identities and functions of differentially expressed genes were inferred by species-specific searches in wFleaBase (http://wfleabase.org) and the UniProt Protein Knowledgebase (The UniProt Consortium, 2012). Further functional information about putative proteins was obtained by carrying out BLASTs in UniProt of amino acid sequences obtained from wFleaBase (www.uniprot.org/blast/). Blastp was run using standard Uniprot parameters (blosum62 matrix, threshold = 10), and results were ranked according to score. UniProt accession numbers of similar proteins in *D. pulex* and of proteins characterized in other arthropods were retrieved and percentage identity and *E*-values recorded. Gene ontology (GO) terms for each gene/gene product from the UniProt Knowledgebase were recorded, and in addition a single higher order GO term that encompassed the majority of the lower order terms was assigned to each gene/gene product (The Gene Ontology Consortium, 2000).

The packages Biobase (Gentleman et al., 2004) and ggplot2 (Wickham, 2009) were used to create a heatmap and corresponding dendrogram displaying expression for all hybrid pairs, and clustering of co-expressed genes. Limma coefficients estimated from the discontinuous contrasts analysis were used to graphically illustrate whether changing patterns of expression across developmental stages resembled discrete thresholds or continuous rates of change.

Results

Differential gene expression

The limma analysis comparing developmental stages discontinuously found a total of 84 probes differentially expressed between any pair of developmental stages (B-H FDR < 0.05) compared to 264 probes differentially expressed given developmental stage as a continuous variable. Once average intensity scores for each feature had been calculated (transcribed regions were generally represented by between one and three probes), this dropped to 15 features in the discontinuous analysis and 59 differentially expressed features in the continuous analysis (B-H FDR < 0.05; Tables S1 and S2). All but one of the differentially expressed features identified

by the discontinuous contrasts analysis were also identified as being differentially expressed in the continuous contrasts analysis. Furthermore, no features exhibited significant differential expression between consecutive developmental stages (i.e. DS-1 vs. DS-2, DS-2 vs. DS-3 or DS-3 vs. DS-4) in the discontinuous analysis. In total, 15 features were differentially expressed between DS-1 and DS-4, seven between DS-2 and DS-4 and one between DS-1 and DS-3. Even when the B-H FDR was relaxed to 0.1, the discontinuous contrasts analysis only yielded 36 differentially expressed features, with none occurring between consecutive developmental stages (Tables S1 and S2).

Gene descriptions and functions

Of the 59 features that were differentially expressed in the continuous contrasts analysis, 45 were exonic, 11 were neighbouring regions, and three were TARs; this compares with 11 exonic regions, three neighbouring regions and one TAR from the discontinuous contrasts analysis. Because all but one exonic region (an uncharacterized protein) from the discontinuous analysis were the same as in the continuous analysis, we focus on these results. During the course of development, 31 exonic regions were up-regulated and 14 were down-regulated (Tables 1 and 2). Many *D. pulex* genes were either uncharacterized or had been assigned rather general functions (wFleaBase), or were labelled as uncharacterized putative proteins (UniProt). In these cases, and in cases where UniProt accession numbers were absent, protein BLASTs were carried out against the UniProt Knowledgebase on amino acid sequences from wFleaBase. These revealed that, of the seven differentially expressed genes that did not have UniProt IDs, six were extremely similar to other *D. pulex* genes (*E*-values < 1.0E-50), and only one (DAPPU 299589) had an *E*-value > 1.0E-5.

Protein BLASTs were carried out for the majority of differentially expressed genes, as only 12 of 45 *D. pulex* gene products in UniProt had been characterized. In all but one case (DAPPU 110469), protein BLASTs yielded at least one named arthropod protein. Of these 32 proteins, 23 featured functions in the UniProt database. The highest ranking arthropod proteins featuring inferred functions are reported in Tables 1 and 2 for continuously up-regulated and down-regulated genes, respectively. In cases where no functional matches were found, the highest ranking arthropod match is reported. Additionally, a single higher order GO term is provided for each gene. All GO terms are provided in Tables S3 and S4.

Co-expression of genes

The major branches of the hierarchical cluster analysis, based on genes identified by the continuous contrasts analysis, correspond to the regions that were either up-regulated or down-regulated during development (Fig. 2). Within these major clusters, minor clusters with similar patterns of co-expression emerged. Some of these clusters have unifying biological features. For example, all six H3 histone genes fall within the same cluster, along with a gene that is likely to code for geminin (involved in DNA replication). Figure 2 shows molecular functions or biological processes associated with these minor clusters. Amongst the genes up-regulated during development were several coding for lipid-transport proteins. Three of these were vitellogenin fused with superoxide dismutase (*VTG/SOD*) genes, of which two (DAPPU 226068 & 226075) formed a sister group to all other up-regulated genes. These were also the only characterized genes with log fold changes > 1 (Table 1). The remaining *VTG/SOD* formed a cluster with another *VTG* gene and the gene for apolipophorins (*ApoLP*; another lipid-transport protein), along with two H4 histone genes and a cyclin B gene.

Separate limma coefficients for DS-1, DS-2, DS-3 and DS-4 were extracted from the discontinuous contrasts analysis. The majority of significant contrasts in this analysis were between DS-1 and DS-4 (Tables S1 and S2), although three *VTG/SOD* genes showed a significant increase in expression between DS-2 and DS-4. When considering separate limma coefficients for genes identified by the continuous (but not the discontinuous) contrasts analysis, it appears that expression of the remaining lipid-transport genes (*VTG*, and *ApoLP*) together with two H4 histones and a cyclin B gene also increases between DS-2 and DS-4 (Fig. 3a). However, separate limma coefficients for the vast majority of genes show gradual increases (Fig. 3b) or decreases (Fig. 3c) in expression between DS-1 and DS-4.

Discussion

This microarray study found that rather few genes exhibited significant changes in expression during the course of development, but that the majority of observed changes tended to be continuous, rather than associated with a specific moult or developmental stage. Comparison of gene expression between individual developmental stages found no evidence to suggest distinct changes occurring between any two consecutive developmental stages/instars. No indication of changing gene expression between DS-3 and DS-4 implies that the phenotypic changes associated with increased ovary provisioning at this transition (Zaffagnini, 1987) are not the result of a developmental switch, but more likely consequences of a gradual change initiated earlier. The majority of differentially expressed microarray features were contrasts of DS-1 and DS-4, suggesting that developmental changes associated with maturation are occurring earlier and lasting longer than was previously assumed, and although a small number of genes associated with vitellogenesis showed increased expression

Table 1 *Daphnia pulex* genes with significant increases in differential expression during the course of development. Log fold changes of gene expression and false discovery rate (FDR) values from the continuous contrast limma are reported. Where gene products were inferred directly from *Daphnia pulex* annotation, UniProt and organism entries are highlighted in boldface. Where no information about gene products was available, protein BLASTS were performed and the nearest (lowest *E*-value) characterized arthropod protein is reported. With two exceptions (DAPPU 303836 and 263168), *E*-values are lower than 1.0E-5. A single biological process (or in the absence of a process, a molecular function or cellular component) based on the highest order Gene ontology term is reported (see Table S3).

DAPPU gene no.	Log fold change	FDR value	UniProt ID	Organism	Percentage identity	*E*-value	Description	Process/function
308303	0.338	3.82E-02	C1BNJ5	*Caligus rogercresseyi*	44.0	1.00E-43	Peflin	Calcium ion binding
222925	0.631	4.73E-02	**E9G757**	***D. pulex***	–	–	Putative cyclin B, copy D	Cell cycle/cell division
60476	0.371	1.87E-02	Q333R2	*Drosophila sechellia*	38.0	2.00E-72	Alpha 1,3-fucosyltransferase	Fucosylation
303879	0.422	4.43E-03	Q29DG0	*Drosophila pseudoobscura*	42.0	5.00E-23	UPF0389 protein GA21628	Integral to membrane
226075	1.779	7.75E-03	**E9GVW7**	***D. pulex***	96.0	0	Vitellogenin fused with superoxide dismutase (SOD)	Lipid transport
308693	0.654	3.54E-02	D4N2J9	*Paracyclopina nana*	21.0	2.00E-87	Vitellogenin-2	Lipid transport
226068	1.173	1.41E-02	Q1JUB1	*Daphnia magna*	52.0	0	Vitellogenin fused with SOD	Lipid transport/oxidation–reduction process
299677	0.801	3.15E-03	**E9HZI6**	***D. pulex***	–	–	Vitellogenin fused with SOD	Lipid transport/oxidation–reduction process
226761	0.644	5.80E-03	B0WP11	*Culex quinquefasciatus*	33.0	2.00E-83	Asparagine synthetase	Metabolic process
100140	0.360	1.20E-02	E2BIM6	*Harpegnathos saltator*	61.0	4.00E-85	Pre-mRNA cleavage complex II protein Clp1	mRNA processing
220880	0.518	1.10E-02	B4P0Y7	*Drosophila yakuba*	29.0	6.00E-10	Geminin	Negative regulation of DNA replication
43440	0.526	1.55E-02	**E9FUS8**	***D. pulex***	–	–	Histone H3	Nucleosome assembly
43804	0.535	5.80E-03	B4K413	*Drosophila grimshawi*	98.0	1.00E-51	Histone H3	Nucleosome assembly
43863	0.507	2.94E-03	**E9FUS8**	***D. pulex***	–	–	Histone H3	Nucleosome assembly
235586	0.524	2.08E-02	**E9FUS8**	***D. pulex***	–	–	Histone H3	Nucleosome assembly
235631	0.674	4.95E-02	**E9FUS9**	***D. pulex***	–	–	Histone H4	Nucleosome assembly
235802	0.478	3.31E-02	**E9FUS8**	***D. pulex***	–	–	Histone H3	Nucleosome assembly
255862	0.536	2.94E-03	**E9FUS8**	***D. pulex***	–	–	Histone H3	Nucleosome assembly
312260	0.765	5.88E-03	**E9FUS9**	***D. pulex***	–	–	Histone H4	Nucleosome assembly
305707	0.392	8.84E-03	**E9FXL5**	***D. pulex***	100.0	0	Alpha-carbonic anhydrase	One-carbon metabolic process
39705	0.372	2.17E-02	F4WDB8	*Acromyrmex echinatior*	46.0	9.00E-93	S-phase kinase-associated protein 2	Phosphorylation
203760	0.400	3.98E-02	G0ZJA2	*Cherax quadricarinatus*	48.0	7.00E-17	Ubiquitin	Protein binding
303836	0.643	4.95E-03	Q29GT5	*D. pseudoobscura*	26.0	3.80E-01	GA15557, part of the PP2C family	Protein dephosphorylation
46545	0.437	8.63E-03	E0VSK2	*Pediculus humanus*	90.0	7.00E-74	Protein C-ets-1-B, putative	Regulation of transcription
304575	0.540	1.54E-02	E2B862	*H. saltator*	73.0	2.00E-61	U6 snRNA-associated Sm-like protein LSm1	RNA processing
91889	0.463	3.54E-02	E0W3W7	*P. humanus*	27.0	2.00E-07	Gem-associated protein, putative	Spliceosomal complex assembly
229368	0.356	2.17E-02	F4WIP9	*A. echinatior*	41.0	3.00E-43	INO80 complex subunit E	Transcription
304661	0.678	3.23E-02	Q9U943	*Locusta migratoria*	23.0	0	Apolipophorins	Transport
110469	0.651	1.16E-03	–	–	–	–	–	–
263168	0.660	3.23E-02	B4K9R3	*Drosophila mojavensis*	24.0	9.30E-01	GI24314	–
306151	0.539	4.95E-02	**E9GVT8**	***D. pulex***	100.0	1.00E-130	Glycolipid-transport protein	–

Table 2 *Daphnia pulex* genes with significant decreases in differential expression during the course of development. Log fold changes of gene expression and false discovery rate (FDR) values from the continuous contrast limma are reported. No genes or gene products were inferred directly from *Daphnia pulex* annotation. Furthermore, the majority of genes (8/14) had no known function, and in four cases (DAPPU 220921, 328621, 327378 and 312710), the nearest characterized arthropod proteins had *E*-values > 1.0E-5. A single biological process (or in the absence of a process, a molecular function or cellular component) based on the highest order Gene ontology term is reported (see Table S4).

DAPPU gene no.	Log fold change	FDR value	UniProt ID	Organism	Percentage identity	*E*-value	Description	Process/function
107198	−0.423	4.95E-02	F4W8S0	*Acromyrmex echinatior*	62.0	1.00E-160	Septin-4	Cell cycle
227396	−0.408	4.19E-02	E2ARN0	*Camponotus floridanus*	39.0	1.00E-68	Putative RNA exonuclease NEF-sp	Exonuclease activity
219379	−0.509	3.54E-02	Q9XYN0	*Schistocerca gregaria*	65.0	0	Innexin 1	Ion transport
305501	−0.442	4.95E-02	E5L878	*Boophilus microplus*	57.0	1.00E-81	Glutathione *S*-transferase	Metabolic process
305713	−0.260	4.95E-02	P29981	*Blaberus discoidalis*	42.0	1.00E-135	Cytochrome P450 4C1	Oxidation–reduction process
304176	−0.386	3.55E-02	Q1HPW4	*Bombyx mori*	67.0	1.00E-169	Eukaryotic translation initiation factor 3 subunit I	Translation
112957	−0.743	3.55E-02	B4QMT8	*Drosophila simulans*	63.0	5.00E-64	GD12468	–
220921	−1.033	4.72E-05	B4GVT5	*Drosophila persimilis*	27.0	1.30E-01	GL14716	–
228103	−0.396	8.63E-03	A0ND72	*Anopheles gambiae*	38.0	3.00E-12	AGAP002973-PA	–
250400	−0.357	4.56E-02	B4M0F5	*Drosophila virilis*	65.0	1.00E-133	GJ24647	–
299589	−0.678	1.16E-03	B4PHB6	*Drosophila yakuba*	55.0	1.00E-76	GE21946	–
312710	−0.973	4.95E-02	CG4702	*Drosophila melanogaster*	32.0	2.00E-04	CG4702	–
327378	−0.403	4.56E-02	Q71DB3	*D. yakuba*	24.0	3.90E-01	CG9568	–
328621	−0.418	2.41E-02	B3M1V5	*Drosophila ananassae*	40.0	3.00E-04	GF17870	–

between DS-2 and DS-4, even these changes occurred in a continuous fashion, increasing between consecutive developmental stages.

The majority of changes that occurred continuously and gradually between DS-1 and DS-4 were for genes with diverse functions; however, those relating to histone production may be of particular importance. Histones are fundamental components of chromatin, and we observed a gradual increase in gene expression of six H3 histone transcripts between DS-1 and DS-4, suggestive of increased chromatin content in maturing *D. pulex*. Concomitant changes in histone production, regulation and the cell cycle are likely to be the result of endoreduplication: the process of genome replication without nuclear or cellular division (Edgar & Orr-Weaver, 2001; Ullah *et al.*, 2009). Endoreduplication results in endopolyploidy and has been observed across many taxa during oogenic processes (Lee *et al.*, 2009; De Veylder *et al.*, 2011). Endopolyploid nurse cells and ovarian follicles in *D. melanogaster* (Hammond & Laird, 1985) may facilitate increasing metabolic activity (Edgar & Orr-Weaver, 2001) required to meet the needs of developing oocytes (Bastock & St Johnston, 2008; Lee *et al.*, 2009). Sufficient and successful endoreduplication may be a prerequisite for the vitellogenic phase associated with ovarian maturation, as has recently been shown in the migratory locust *Locusta migratoria* (Guo *et al.*, 2014). Endopolyploidy has been observed in *Daphnia* nurse cells (Beaton & Hebert, 1989) with a general increase occurring during ontogeny (Korpelainen *et al.*, 1997). Furthermore, increasing expression of a putative gene for inositol-requiring 80 (*INO80*; DAPPU 229368) throughout development may be required to regulate the increasing amounts of chromatin (Clapier & Cairns, 2009) present in endopolyploid cells, whereas a concurrent decrease in the expression of a putative eukaryotic initiation factor 3 (*eIF3*; DAPPU 304176) and increasing expression of a gene for Sm-like protein (*LSm1*; DAPPU 304575) are likely to be involved in histone translation and transcription (Ling *et al.*, 2002; Marzluff *et al.*, 2008). The changing expression of other transcription factors such as the putative genes for *Clp1* (DAPPU 100140), *ETS1* (DAPPU 46545), gem-associated protein (DAPPU 91889) and RNA exonuclease (DAPPU 227396) may also facilitate and/or result from the observed changes in expression of histones transcripts. Genes involved in the cell cycle including those for SKP-associated protein (DAPPU 39705) and septin-4 (DAPPU 107198) may underlie cell cycle changes associated with endoreduplication, although the elevated expression of geminin (*GMNN*; DAPPU 220880) and *cyclin B* (DAPPU 222925) genes is puzzling, as they are thought to be expressed at lower levels during this process (Narbonne-Reveau *et al.*, 2008; Lee *et al.*, 2009).

An additional reason for increasing histone expression could be the accumulation of histone transcripts within nurse cells and oocytes during oogenesis, as has been observed in *D. melanogaster* (Ruddell & Jacobs-Lorena, 1985; Walker & Bownes, 1998). It is thought that

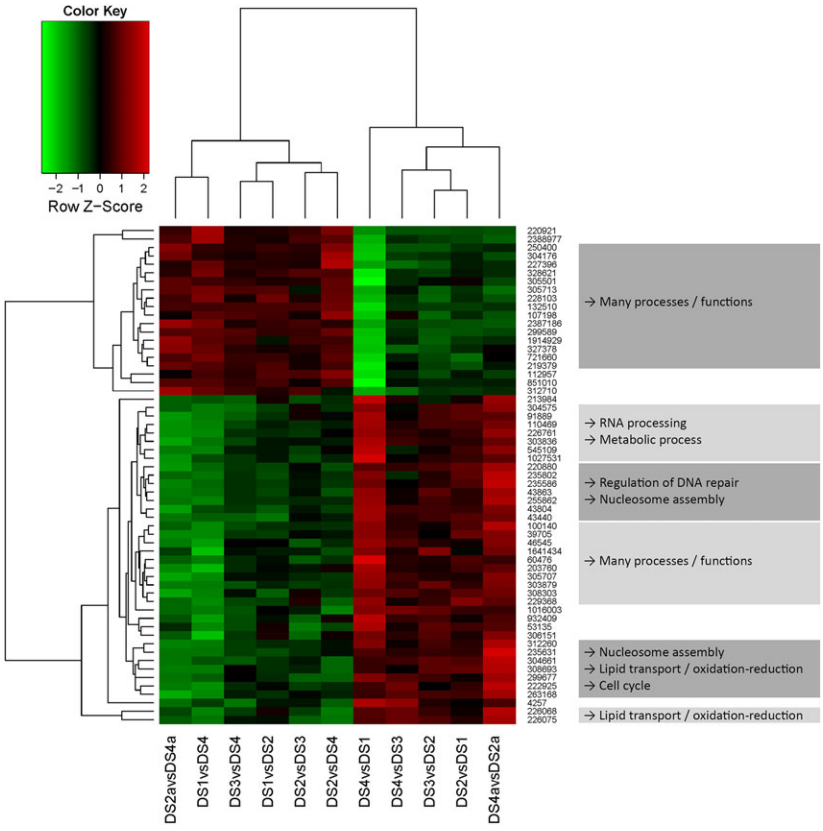

Fig. 2 Hierarchical clustering analysis and log fold changes in gene expression for regions with significant levels of differential expression identified by limma with developmental stage as a continuous variable. All 59 differentially expressed regions are shown. Codes along the bottom margin distinguish between arrays. For example, 'DS1vsDS4' shows levels of gene expression in an array comparing DS-1 to DS-4. Arrays 'DS2avsDS4a' and 'DS4avsDS2a' were comparisons that originated from the cohort with accelerated development (see Materials and Methods: Developmental progression in *Daphnia*). Between arrays, the main branches separate hybrid pairs where the earlier developmental sample was dyed with Cy3 (left hand 6) from those where it was dyed with Cy5 (right hand 5). Thus, the major branching that occurs between features corresponds to regions down-regulated during development (upper 20 rows) and regions up-regulated during development (bottom 39 rows). Within these clusters, levels of co-expression are broadly similar, but lower order clusters are highlighted by boxes on the right. Where genes within a cluster fell into three or fewer categories of biological process or molecular function, these categories are reported.

accumulation of histone transcripts in the oocytes facilitates rapid cell division during embryogenesis and may act as a mechanism for epigenetic inheritance of gene expression (Marzluff *et al.*, 2008). Interestingly, H3 and H4 histones appear to be regulated in different ways; it is possible that the expression of H4 histones, which shows a more pronounced increase between DS-2 and DS-4 than expression of H3 histones, is reflective of oocyte provisioning, as oocytes first appear during DS-2 (Zaffagnini, 1987). However, it is unclear why histones H3 and H4 showed significant increases in expression while other chromosomal histones that were present on the microarray (H2A and H2B) did not.

Gradual changes in gene expression between DS-1 and DS-4 for the putative genes innexin1 (*Inx1*; DAPPU 219379) and glutathione *S*-transferase (*GST*; DAPPU 305501), and changes in expression between DS-2 and DS-4 of *cyclin B*, corroborate previous studies of development in arthropods. We observed declining expression of *Inx1* during development; similarly, *Inx1* is expressed at lower levels in adults of the lobster *Homarus gammarus* than in larvae, where it plays an important role in neural ontogeny (Ducret *et al.*, 2006). *Cyclin B* is known to regulate meiotic maturation of crustacean oocytes (Visudtiphole *et al.*, 2009), and the simultaneous up-regulation of *cyclin B* and down-regulation of *GST* that we observed also occurred during ovarian development in a proteomic study of the swamp crayfish *Procambarus clarkia* (Shui *et al.*, 2012). In their microarray study of *Daphnia magna*, David *et al.* (2011)

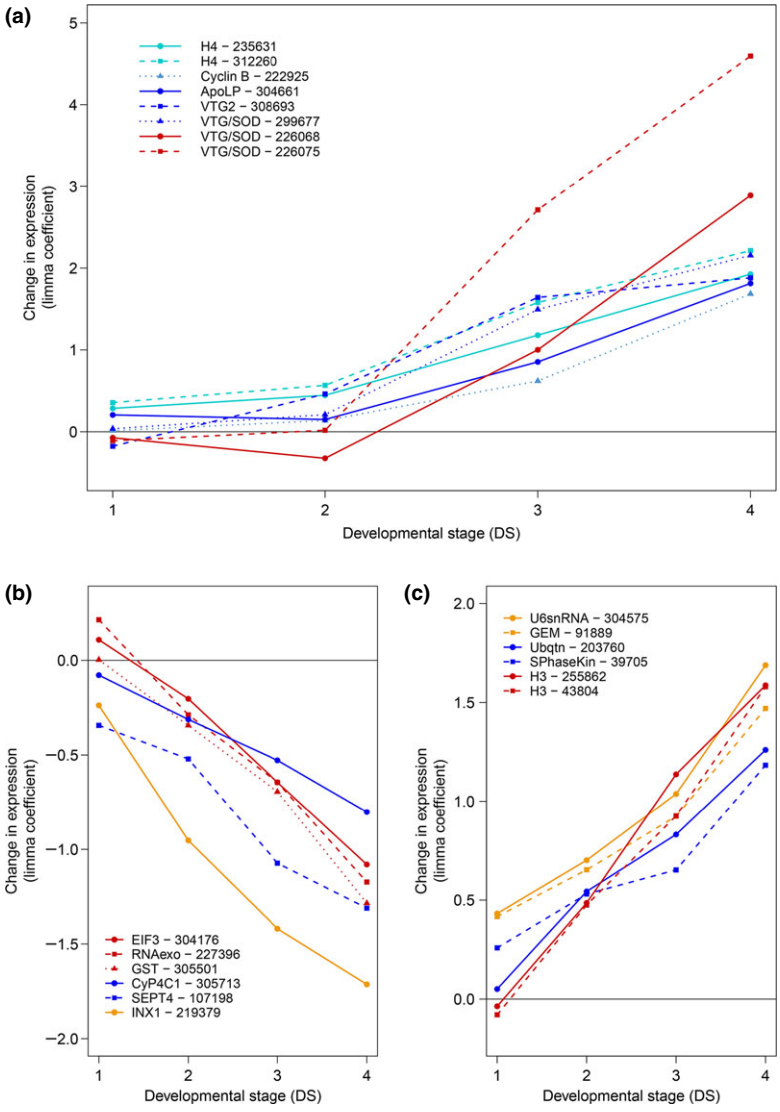

Fig. 3 Changes in expression of selected genes. Lines of the same colour indicate genes that displayed similar levels of co-expression according to cluster analysis: (a) Expression of two clusters appears to increase steeply after DS-2, particularly in two *VTG/SOD* genes (DAPPU 226068 and 226075). (b) Expression for a representative subset of down-regulated and (c) up-regulated genes. Changes in expression of these genes appear continuous. Descriptions of up-regulated and down-regulated genes can be found in Tables 1 and 2, respectively.

found one form of *GST* to be expressed at greater concentrations in neonate *D. magna* compared to adults, and another expressed at lower concentrations; similar results were observed in the silkworm *Bombyx mori*, where two different forms of *GST* were differentially sensitive to juvenile hormone (JH) (Zou *et al.*, 2011), suggesting this expression of this gene may be under neuro-endocrine control.

Neuro-endocrine hormones such as JH, its crustacean equivalent methyl farnesoate (MF) and the moulting hormone 20 ecdysone (20-E) have well-documented roles in development and vitellogenesis (Borst & Laufer, 1990; Laufer & Biggers, 2001; Subramoniam, 2010). Thus, genes involved in the regulation of these hormones may vary in expression during maturation processes. We identified one such gene: a putative cytochrome P450 4C1 (*Cyp4C1*; DAPPU 305713), which gradually declined during development. Arthropod cytochrome P450 4 (*Cyp4*) genes are highly diverse (Feyereisen, 2006) and as many as 38 *Cyp4* genes exist in *D. pulex* (Baldwin *et al.*, 2009). Several proteins within this group could be involved in arthropod developmental regulation (Mykles, 2011) as cytochrome P450 proteins play a wide range of roles in arthropod neuro-endocrine processes (Helvig *et al.*, 2004; Dam *et al.*, 2008; Hansen *et al.*, 2008; Rewitz & Gilbert, 2008). *Cyp4* proteins are down-regulated in response to ecdysone agonists (Davies *et al.*, 2006); *Cyp4C15* is involved in ecdysteroid synthesis in crustaceans (Dauphin-Villemant *et al.*, 1999; Aragon *et al.*, 2002); and *Cyp4C7* serves as a JH-suppressant in the cockroach *Diploptera punctata* (Sutherland *et al.*, 1998). Our finding that *Cyp4C1* expression decreased during the maturation process mirrors that of Sutherland *et al.* (1998), who observed a

decline in *Cyp4C7* during vitellogenesis. Interestingly, a concomitant decrease in expression of *GST* and a different cytochrome P450 4 gene, *Cyp4C9*, was observed in reproductive queens of the termite *Coptotermes formosanus* compared to virgin alates (Husseneder *et al.*, 2012), that is during ovarian maturation; in our study, expression of both *Cyp4C1* and *GST* also declined during maturation. We therefore speculate that both *Cyp4C1* and *GST* may be involved in neuro-endocrine processes during *D. pulex* maturation.

We did not observe significant differential expression of any further enzymes which may feature in the pathways of the hormones. However, both MF and 20-E are tightly linked to moult cycles as well as development (Gilbert *et al.*, 2002; McWilliam & Phillips, 2007). Because individuals within a given sample were not synchronized within moult cycles, we may have failed to detect differential expression (Chang & Mykles, 2011), and it may be essential to compare stages at more precisely timed points within the moult cycle (Gupta, 1990; Davidowitz & Nijhout, 2004; McWilliam & Phillips, 2007). Indeed, the relationship between moult cycle and maturation is a particularly important determinant of plasticity in age and size at maturity in arthropods (Ebert, 1994; Higgins & Rankin, 1996; Higgins, 2000; Oliphant *et al.*, 2013), particularly in holometabolous species, where size at metamorphosis, which determines size at maturity, may be altered either by extending the duration of a final instar (Moczek & Nijhout, 2002) or by increasing the number of instars (Esperk *et al.*, 2007). The strategy chosen may even vary within species (Esperk & Tammaru, 2010), as a result of both environmental and genetic factors (Saastamoinen *et al.*, 2013).

Although most of the genes that were highlighted by the discontinuous analysis were contrasts between DS-1 and DS-4, three genes coding for *VTG/SOD* showed a distinct increase in expression between DS-2 and DS-4. These genes, together with other lipid-transport genes *VTG* and *ApoLP*, two histone H4 genes and *cyclin B* (all identified by the hierarchical cluster analysis to have a pattern of expression similar to *VTG/SOD* isoform DAPPU 299677) were expressed more between DS-2 and DS-4 than between DS-1 and DS-4. The expression of lipid-transport genes and the production of vitellogenin during maturation have been recorded in numerous crustacean species (Okumura *et al.*, 2007; Phiriyangkul *et al.*, 2007; Subramoniam, 2010), including daphniids (Hannas *et al.*, 2011). Vitellogenins are glycolipoprotein precursors of the vitellins that serve as yolk proteins for developing embryos in oviparous species (Hagedorn & Kunkel, 1979; Wahli *et al.*, 1981). Previous cytological studies of *Daphnia* have suggested that IM-2 (DS-4 in this study) is the developmental stage primarily associated with vitellogenesis in *D. magna* (Zaffagnini & Zeni, 1986; Zaffagnini, 1987; Fig. 1d). Although DS-4 was associated with the strongest levels of expression for these proteins, visual inspection of expression coefficients (Fig. 3a) suggests that one transcript (*VTG*) starts to increase between DS-1 and DS-2 and that expression of the remaining lipid-transport genes (*VTG/SOD* and *ApoLP*) increases between DS-2 and DS-3, further increasing between DS-3 and DS-4. However, it is worth noting that this pattern of expression is still more analogous to a rate than a threshold. In *Daphnia*, IM-1 (DS-3) is referred to as being 'previtellogenic' (Zaffagnini & Zeni, 1986; Zaffagnini, 1987; Fig. 1c). However, crustacean vitellogenin may be expressed at previtellogenic stages (Meusy & Payen, 1988; Subramoniam, 2010) in tissues other than the ovaries (Phiriyangkul *et al.*, 2007), and the increase in expression at the transition from DS-2 to DS-3 could explain the proposed maturation threshold in certain *Daphnia* species (Bradley *et al.*, 1991; Ebert, 1994; Barata *et al.*, 2001). Vitellogenin may be one of the first products of maturation to incur a significant energetic cost; thus, the increase in its production between DS-2 and DS-4 could result in a significant decline in juvenile growth rate, coinciding with the maturation threshold that has been described at this developmental stage (Ebert, 1994). However, the process of vitellogenesis itself is dependent on other developmental changes including endoreduplication (Guo *et al.*, 2014) and may be better thought of as one step towards the end of a lengthy maturation process.

One result that bears further investigation is whether increased expression of vitellogenin that is fused with SOD is serving a function beyond its well-described role in lipid transport. SOD-fused forms of vitellogenin have been described in *D. magna* (Kato *et al.*, 2004; Tokishita *et al.*, 2006) and *D. pulex* (Schwerin *et al.*, 2009), as well as the brine shrimp *Artemia parthenogenetica*, a species that also produces both free-swimming and encysted diapausing offspring, suggesting it may play a role in the production of dormant larvae (Chen *et al.*, 2011). It is also possible that expression of SODs during vitellogenesis may help to neutralize free radicals produced during this metabolically active time (Brady *et al.*, 2012). Vitellogenin itself may be serving additional functions, for example as a signalling protein; it has been shown to influence polyphenism induction (Scharf *et al.*, 2005; Azevedo *et al.*, 2011), somatic maintenance (Münch & Amdam, 2010) and immune function (Amdam *et al.*, 2004). The possibility that increased expression of certain forms of vitellogenin serves other physiological and developmental roles (Kang *et al.*, 2008; Havukainen *et al.*, 2011a,b; Kent *et al.*, 2011) presents an interesting avenue for future research in arthropod development.

Overall, our results suggest that certain changes in gene expression associated with maturation are occurring very early in development and that understanding 'when' maturation is initiated requires an appreciation not only of easily observable phenotypic consequences,

such as vitellogenesis, but of underlying prerequisite changes such as endoreduplication. From this perspective, maturation is an ongoing and lengthy process during much of the juvenile period. Because maturation represents the co-ordination of, and trade-off between, growth and reproduction (Zera & Harshman, 2001), gradual changes in resource allocation between these two competing functions during a protracted maturation period provide an explanation for the environmental sensitivity that is frequently observed in final age and size at maturity (Berner & Blanckenhorn, 2007). Maintaining the ability to alter resource allocation in such a manner may be of importance to indeterminate growers, which continue to face the trade-off between growth and reproduction beyond maturity (Heino & Kaitala, 1999), and may invest significantly in growth throughout their adult life (Lord & Shanks, 2012). For both determinate and indeterminate growers alike, an extended and environmentally sensitive maturation period during which the process can be curtailed or extended is necessary to explain phenomena such as reduced size at maturity in response to predation (Rinke *et al.*, 2008), accelerated development to escape ephemeral larval environments (Kulkarni *et al.*, 2011; Gomez-Mestre *et al.*, 2013) and delayed reproduction in favour of growth and future fecundity (Folkvord *et al.*, 2014).

Conclusion

This microarray study identified 45 *D. pulex* genes that showed differential expression over four instars during the maturation process. The majority of these genes showed gradual and continuous changes in expression from the first to the fourth instar under observation, highlighting the fact that maturation can be a protracted process; that certain developmental processes associated with maturation in *D. pulex* are occurring earlier and lasting longer than has previously been considered; and, supporting the idea that maturation is better considered as a rate than as a discrete threshold or switch. Increased expression of H3 histone genes, along with a number of genes involved in the cell cycle and transcription, suggest that processes such as endoreduplication may play an important role in maturation and are initiated very early in development. Furthermore, genes, such as a *Cyp4C1* orthologue, and *GST* represent interesting targets for further study of maturation in *D. pulex*. Although we suggest that maturation is rate like, the expression of genes for vitellogenin and a lipid-transport protein did appear to be 'switched on' between DS-2 and DS-4, a result in line with previous cytological (Zaffagnini & Zeni, 1986; Zaffagnini, 1987) and life-history (Bradley *et al.*, 1991; Ebert, 1992, 1994) studies of *Daphnia* maturation. Along with two histone H4 genes and *cyclin B*, these genes may explain the previously described maturation threshold postulated in the species. However, even expression of these genes increased over the course of multiple instars and is likely to be dependent on earlier developmental changes such as endoreduplication (Guo *et al.*, 2014), lending further credence to the idea that maturation resembles a rate more than a threshold.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Experimental design for microarray study showing numbers of individual juveniles within each pooled RNA sample (white numbers in red and blue squares), across four developmental stages (DS-1 to DS-4), four cohorts (shared great grandmother) and eight sub-cohorts (shared grandmother).

Table S1 Comparison of continuous contrast limma and factorial contrast limma results for features with increasing expression during development.

Table S2 Comparison of continuous contrast limma and factorial contrast limma results for features with decreasing expression during development.

Table S3 Gene Ontology (GO) terms for all *Daphnia pulex* genes/gene orthologs with significant increases in differential expression during the course of development.

Table S4 Gene Ontology (GO) terms for all *Daphnia pulex* genes/gene orthologs with significant decreases in differential expression during the course of development.

Data deposited at Dryad: doi:10.5061/dryad.1rc0v

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