Effects of simulated light regimes on gene expression in Antarctic krill (*Euphausia superba* Dana)

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ABSTRACT

A change in photoperiod has been implicated in triggering a transition from an active to a quiescent state in Antarctic krill. We examined this process at the molecular level, to identify processes that are affected when passing a photoperiodic threshold. Antarctic krill captured in the austral autumn were divided into two groups and immediately incubated either under a photoperiod of 12 hours light: 12 hours darkness (LD), simulating the natural light cycle, or in continuous darkness (DD), simulating winter. All other conditions were kept identical between incubations. After 7 days of adaptation, krill were sampled every 4 hours over a 24 hour period and frozen. Total RNA was extracted from the heads and pooled to construct a suppression subtractive hybridisation library. Differentially expressed sequences were

identified and annotated into functional categories through database sequence matching. We found a difference in gene expression between LD and DD krill, with LD krill expressing more genes involved in functions such as metabolism, motor activity, protein binding and various other cellular activities. Eleven of these genes were examined further with quantitative polymerase chain reaction analyses, which revealed that expression levels were significantly higher in LD krill. The genes affected by simulated photoperiodic change are consistent with known features of quiescence, such as a slowing of moult rate, a lowering of activity levels and a reduction in metabolic rate. The expression of proteases involved in apolysis, where the old cuticle separates from the epidermis, showed particular sensitivity to photoperiod and point to the mechanism by which moult rate is adjusted seasonally. Our results show that key processes are already responding at the molecular level after just 7 days of exposure to a changed photoperiodic cycle. We propose that krill switch rapidly between active and quiescent states and that the photoperiodic cycle plays a key role in this process.

Key words: euphausiid; moulting; overwintering; photoperiod; quiescence; Southern Ocean.

Introduction

Due to their high latitude, Antarctic krill (*Euphausia superba*) must endure extreme seasonality, with phytoplankton blooms filling only a few short summer months. The rest of the year is characterised by food-shortage, an encroaching ice-pack and long periods of darkness. Life-cycle strategies in these environments must include a means of saving energy during the non-productive periods (Dahms 1995). As a result, a number of key marine plankton taxa in the Southern Ocean exhibit some sort of reduced activity during the winter months. The calanoid copepods *Calanoides acutus* and *Rhincalanus gigas*, for instance, enter a classic diapause (Hirche 1996) where they arrest development, lower metabolism, reduce levels of activity and descend to great depths (Schnack-Shiel and Hagen 1994). The strategy of Antarctic krill (*E. superba*) is less clear-cut since, despite the potentially large energy shortfall, they remain within the upper water column during the winter (Godlewska 1996).

Some have proposed that, in the absence of preferred foods such as diatoms, Antarctic krill switch to alternative food sources, such as ice biota (Marschall, 1988), zooplankton (Huntley et al., 1994) and seafloor detritus (Kawaguchi et al., 1986). However, feeding experiments on *E. superba* during autumn in the Lazarev Sea (Atkinson et al., 2002) found that oxygen uptake and clearance rates were three times lower than in summer (Atkinson and Snyder, 1997). Quetin et al. (2003) found that growth rates of larval and juvenile krill were minimal in early winter. Moult rate in adult Antarctic krill has been observed to slow down or even stop during winter-time (Clarke 1976). It therefore appears that krill can potentially employ a strategy of "quiescence" (Hirche, 1996), through retarding feeding activity, metabolism and growth to outlast the winter period.

In line with the definitions used with respect to dormancy in insects (Danks 1987), we use the term quiescence here to mean a type of dormancy in which life-processes are retarded in direct response to a limiting factor (e.g. low temperature, limiting food) without prior acclimation. This distinguishes it from diapause, which is an arrest in development that may be triggered by environmental factors and is compulsory and ultimately genetically determined. Both the onset of quiescence and diapause may be triggered by a variety of cues, including chemical (Slusarczyk and Rygielska, 2004, Pijanowska and Stolpe, 1996), thermal (Jewson et al., 2008) and photoperiodic (Chinnery and Williams, 2003). Others have proposed the involvement of internal clocks, such as Tande and Hopkins (1981), who linked the timing of the re-appearance of the calanoid copepod, *Calanus finmarchicus* in surface waters to the gradual development of the gonads over winter. Nevertheless, internal clocks are frequently synchronised by external cues, or *zeitgebers* (Buchholz 1991). If krill do alternate between distinct physiological states in response to productive and non-productive seasons, it is likely that there is a cue which triggers this change of state.

Teschke et al. (2007, 2008) focused on photoperiod as a potential cue by which key life-cycle processes in Antarctic krill are co-ordinated, based on evidence by Hirano et al. (2003) of its involvement in maturation and spawning. Winter-time krill were exposed to three different photoperiod cycles, continuous darkness (DD), 12 hours light: 12 hours darkness (LD) and continuous light (LL) for three months. Constant temperature was maintained and food was kept abundant across all incubations. Results showed a marked difference in the state of those krill kept in DD compared with those exposed to LL or LD, with individuals maintained in LL or LD showing increased rates of metabolism (oxygen uptake rates, metabolic enzyme activity) and feeding activity (digestive gland size, clearance rate, digestive enzyme activity), and the development of external secondary sexual organs. These experiments suggest, firstly,

93 that krill switch between active and quiescent physiological states and, secondly, that
94 photoperiodic cycles are a major cue in causing this switch.
95
96 Molecular approaches are a useful way of examining the processes involved in switching
97 between physiological states since they foretell the physiological changes that will
98 subsequently occur. In one of the only studies on gene expression during diapause in marine
99 zooplankton, Tarrant et al. (2008) examined the differences in gene expression between
100 active and diapausing *C. finmarchicus*. They found that genes associated with lipid synthesis,
101 transport and storage were more highly expressed in active copepods, while diapausing
102 copepods showed greater expression of the gene ferritin, encoding for an iron sequestering
103 protein that also acts as a molecular chaperone; properties that may help protect cells from
104 oxidative and thermal stress (Chen et al. 2007).
105
106 Underlying the approach of Tarrant et al. (2008) were two key techniques: suppression
107 subtractive hybridisation (SSH) and quantitative PCR (qPCR). SSH is a means of comparing
108 the genes that are expressed by two populations exposed to different conditions and
109 maximising the identification of genes uniquely expressed in one or other population. qPCR
110 is then employed to determine to what degree a gene is differentially expressed between
111 populations. The use of these techniques is now widespread and, in addition to the study of
112 Tarrant et al. (2008), have been used to examine dauer larvae in the nematode *Caenorhabditis*
113 *elegans* (Cherkasova et al. 2000) and diapause in the mosquito, *Culex pipens* (Robich et al.
114 2007).
115
116 In this study, we investigated how gene transcription changes during the transition from
117 summer-time activity to winter-time quiescence in Antarctic krill. Krill caught in autumn

were exposed to either, 12 hours light: 12 hours darkness (LD), simulating autumn, or continuous darkness (DD), simulating winter, for seven days. SSH and qPCR were then used to identify genes that were differentially expressed between the two populations and to characterise the level of up or down regulation. The probable function of these genes within physiological processes was investigated through database searches and comparison to homologues in other organisms. This study represents one the first applications of genomic tools to understand the regulation of physiological processes in Antarctic krill.

Materials and methods

Experimental design

Live *Euphausia superba* were collected using a Rectangular Midwater Trawl net (RMT1+ 8) in the upper 200 m of the water column on 14 March 2004 (65° 15′ S, 4° 45′ W) by RV Polarstern (ANTXXI-4). Immediately after capture, adult krill of mixed sex were transferred into 65 L tanks (30 krill in each) filled with natural sea water, which were located in separate constant temperature rooms at 0 °C. Photoperiod and light intensity were controlled in each room by a PC-controlled timer system. The tanks were exposed to one of the following light regimes to simulate Southern Ocean autumn and winter conditions respectively: experimental tank (1) autumn: 12 hours light and 12 hours darkness (LD) with a maximum of 50 lux light intensity at the surface of the tank during midday; and experimental tank (2) winter: continuous darkness (DD) (Fig. 1). 50% of the incubation water was exchanged daily. Twice a day, moults and a small number of dead animals were removed from the tanks. After 7 days of adaptation to these conditions, three krill were sub-sampled every 4 hours from each experimental tank over a 24 hour time period. In total, 21 krill were extracted from each tank. Sampled krill were immediately frozen in liquid nitrogen and stored at -80 °C for further analyses. Sampling during dark periods was performed in dim red light.

SSH library construction

Total RNA was extracted from the heads of frozen (-80 ºC) *E. superba* using the RNeasy Midi kit (Qiagen) following manufacturer's instructions. RNA was resuspended in DEPC-treated water and the concentration determined using a NanoDrop spectrophotometer (LabTech International). Total RNA was pooled from 21 LD krill and 21 DD krill before isolating mRNA from each pool using a MicroPoly(A) Purist™ kit (Applied Biosystems).

151 Each pool of krill consisted of three krill sampled every four hours, over a 24 hour time
152 period. One microgram of LD and DD mRNA was used in the construction of a SSH library
153 using the PCR-Select™ cDNA Subtraction kit (Clontech) following manufacturer's
154 instructions, in which LD cDNA was the tester and DD cDNA was the driver. In addition to
155 the SSH library, a library was also made of *Rsa I* digested LD cDNA that had not undergone
156 subtraction with DD cDNA to act as a control. Subtracted and non-subtracted cDNAs were
157 cloned into pGEM®-T Easy (Promega) before being transformed to *Escherichia coli* XL2-
158 Blue MRF' cells (Agilent Technologies). Recombinant clones identified by blue/white
159 selection were grown in 100 µl TB medium overnight in 96 well plates and 40 µl 50%
160 glycerol added before storing at -80 ºC.
161
162 **cDNA sequencing**
163 Prior to sequencing, the inserts of each clone were amplified and purified according to the
164 protocol of Purać et al. (2008), with the substitution of Advantage 2 Polymerase mix and
165 buffer (Clontech) for BIOTAQ™ DNA polymerase, buffer and $MgCl_2$ (Bioline). Purified
166 PCR products were then sequenced with M13F (5'-GTAAAACGACGGCCAGTGAAT-3')
167 and M13R (5'-AACAGCTATGACCATGATTACG-3') long sequencing primers using Big
168 Dye® Terminator cycle sequencing kits (version 3.1, Applied Biosystems) and an automated
169 DNA sequencer (MegaBACE™ 1000, GE Healthcare).
170
171 **Sequence analysis**
172 Trace2dbest, (Parkinson et al., 2004) incorporating phred (Ewing and Green, 1998; Ewing et
173 al., 1998) and crossmatch (Green P: unpublished) was used for basecalling and trimming of
174 vectors. Only high quality sequences more than 150 bp in length were used for database
175 searching. In order to examine sequence similarity to known genes, Blastx (Altschul et al.,

1997) was used to search Swissprot, Trembl and non-redundant (nr) sequence databases for significant matches. A significant database search was defined as having an expectation value (E-value) below $1e^{-5}$. All sequences with an E-value below $1e^{-10}$ were further annotated using GO terms and GO slims (The Gene Consortium, 2000). GO enrichment was determined by a one-tailed proportion test at a p value of less than 0.05 that compared the proportion of clones representing a GO slim term in the subtracted library to the proportion of clones representing the same GO slim term in the unsubtracted library. High quality sequences were submitted to dbEST (Boguski et al., 1993) and given the following accession numbers: dbEST: **60125367-60126646**, Genbank: **FL688135- FL689414**.

Quantitative PCR

qPCR sample sets: Two sets of qPCR analysis were performed to compare gene expression between LD and DD krill.

1. Pooled krill analysis: The expression of 11 genes selected from the SSH library based on their known, or proposed involvement in moulting, metabolism and motor activity (Table 1) was compared between the same pools of LD and DD krill (each consisting of 21 krill sampled over a 24 hour period) used in the SSH library construction.

2. Individual krill analysis: qPCR analysis of a subset of the above 11 genes was performed on individual krill from the LD and DD regimes. Each krill was measured for uropod length, sex and maturity where possible (following Makarov and Denys 1980), and moult stage (following Buchholz 1982). Given that some of the largest differences in gene expression from the pooled-krill analysis were in genes proposed to be involved in moulting (see Results), krill were sorted according to moult stage and compared between LD and DD light regimes. Specifically, we focussed on the stages around apolysis (the point at which the cuticle separates from the epidermis). Stage C (or intermoult) occurs immediately before

apolysis and D0 (or 1st stage premoult), at the point of apolysis and just after (Fig. 2). We made a number of comparisons to examine how gene expression alters between these two moult stages and how this is affected by the photoperiodic cycle (Table 2).

Primers were designed around the Expressed Sequence Tag (EST) of interest, using Primer 3 (Rozen and Skaletsky, 2000) to generate PCR products of between 100 and 200 bp (Table 3). To normalise the data, primers were also designed against ESTs with significant homology to the following three potential reference genes; beta actin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and phosphoenolpyruvate carboxykinase (PEP-CK). Beta actin was found to be the most stable in the pooled krill analysis, while PEP-CK changed the least in the individual krill analysis.

qPCR methodology: One microgram of total RNA was used to make first strand cDNA using a Quantitect Reverse Transcription kit (Qiagen) that incorporates genomic DNA removal prior to reverse transcription. The cDNA (20 µl) was diluted to 80 µl and 1 µl was used as template for qPCR. The qPCR mixture consisted of 10 µl 2 x SensiMix*Plus* SYBR mastermix (Quantace), 600 nM of forward and reverse primers, 1 µl cDNA and sterile MilliQ water in a total volume of 20 µl. The qPCRs were performed in duplicate on a Mx3000P QPCR system (Agilent Technologies) with the following cycling conditions: 95 ºC for 10 min, followed by 40 cycles of 95 ºC for 30 sec, 60 ºC for 1 min and 72 ºC for 30 sec. A dissociation curve step was then performed to ensure that only a single product had been amplified in each reaction. Standard curves were performed for each primer pair with a dilution series of cDNA. By plotting threshold crossing cycle (C_t) values against the $\log_{10}$ of the different dilutions, PCR efficiency was calculated as $E = 10^{(-1/slope)} - 1$, using MxPro™ (Agilent Technologies) software. Relative mean expression ratios were statistically compared

between light regimes (LD and DD) and moult stages (C and D0) following normalisation against the reference gene using the relative expression software tool REST (Pfaffl et al. 2002). For statistical analysis of relative mean expression ratios, REST employs randomisation tests with a pair-wise reallocation that make no assumptions about the distribution of observations in populations. The software was used to perform 2000 random allocations to determine if the results were due to chance or to the effects of the treatment (light regime or moult stage), with differences considered to be significant at $P < 0.05$.

Results

SSH library analysis

A krill LD SSH library was constructed along with an unsubtracted LD library in which no subtraction with DD krill was performed. This allowed a comparison to be made between subtracted and unsubtracted libraries that would indicate which genes were enriched by the subtraction with the DD krill. From each library, 960 clones were sequenced that resulted in 595 and 685 high quality sequences longer than 150 bp from the subtracted and unsubtracted libraries respectively (Table 4). A comparison with the ESTs published in the first sequencing study of *Euphausia superba* (De Pittà et al., 2008) showed that 581 of these sequences were novel. Following sequence matching to NCBI databases using Blastx, of the putative mRNAs, 225 (48%) and 232 (40%) could be identified from the subtracted and unsubtracted libraries respectively ($E < 1e^{-5}$). Identified genes with an E-value of less than $1e^{-10}$ were then annotated using GO slims (a database of simplified gene ontology descriptions) from the Gene Ontology Consortium and placed into different functional categories (Table 5). This table shows that metabolism, protein binding and various other cellular activities are enriched in the subtracted library that may correspond to higher expression of genes in these categories in LD krill.

Quantitative PCR analysis

To measure the level of differential gene expression between LD and DD incubated krill, qPCR was first performed on 11 genes selected from the SSH library based on their known, or proposed, involvement in moulting, metabolism and motor activity (Table 1). The list includes five enzymes and three enzyme precursors. Of the enzymes, aldo-keto reductase and fructose-bisphosphate aldolase are involved in carbohydrate metabolism (Gabbay and Tze,

1972; Salvatore et al., 1986; Bagnasco et al., 1987), while β-N-acetylglucosaminidase has been shown to be involved in the degradation of the old cuticle during moulting (Samuels and Reynolds, 1993). CUB-serine protease was included as serine proteases have been found in the moulting fluid of insects (Samuels et al., 1993) and are thought to be involved in the digestion of the old cuticle during the apolysis stage prior to moulting. Trypsin is known to be involved in food protein digestion (Diaz-Mendoza et al., 2005) but it also has protease properties (Muhlia-Almazán et al., 2008) and has been shown to activate other proteases (Tsu and Craik, 1996). As for the enzyme precursors, the activated pancreatic carboxypeptidase A1 precursor is involved in moulting (Ote et al., 2005), while the activated form of the serine collagenase 1 precursor has protease properties (Tsu and Craik, 1996) required for degradation of the old cuticle. Trypsinogen is a precursor of trypsin (Neurath 1964). The other genes in the list encode for cuticle protein CB6, required for formation of new cuticle (Kuballa et al., 2007); myosin light chain protein, involved in motor activity (Poetter et al., 1996) and a low density lipoprotein receptor-related protein involved in both cuticle degradation (Yochem et al., 1999) and protease activation (Krieger and Herz, 1994; Strickland et al., 1995).

Pooled krill analysis: All genes selected for qPCR were found to be up regulated in LD krill, although there were differences in the level of up regulation (Fig. 3). Some of the largest differences in gene expression were in genes proposed to be involved at various stages of the moulting process. In particular, large differences between LD and DD krill were observed with the proteases, serine collagenase, trypsin and CUB-serine protease. These results therefore focussed our further efforts onto the interaction between moult stage (particularly the time around apolysis) and photoperiod cycle within individual krill.

286 *Individual krill analysis*: Three qPCR comparisons were made to reveal the effect of stage of
287 moult and photoperiodic cycles on gene expression around the period of apolysis: A, the
288 effect of apolysis on gene expression (LD/DD intermoult or stage C versus LD/DD premoult
289 stage D0); B, the effect of photoperiod immediately before apolysis (LD stage C versus DD
290 stage C); C, the effect of photoperiod immediately after apolysis (LD stage D0 versus DD
291 stage D0). Comparisons were made for three differentially expressed genes, as identified by
292 the pooled-krill analysis, with suspected involvement in the process of apolysis: serine
293 collagenase, trypsin and CUB-serine protease. A fourth gene, cuticle CB6 was also included,
294 given its function in new cuticle formation:
295 A, there was no significant change in the expression of serine collagenase, trypsin and CUB-
296 serine protease before and after apolysis. However, there was a significant up regulation of
297 cuticle CB6 after apolysis (Fig. 4a).
298 B, serine collagenase, trypsin and CUB-serine protease expression were down regulated
299 significantly ($p<0.05$) in DD stage C krill compared with LD stage C krill. Trypsin showed a
300 200 fold down-regulation in DD krill and serine collagenase, a 140 fold down regulation.
301 There was little difference in the expression of cuticle CB6 (Fig. 4b).
302 C, none of the genes showed a significant change in expression between photoperiod regimes
303 immediately after apolysis i.e. stage D0 (Fig. 4c).
304
305 The results reveal a complex picture of up- and down-regulation of genes depending on both
306 photoperiod and moult-stage relative to the point of apolysis. Photoperiod affected the
307 expression of protease genes (serine collagenase, trypsin and CUB-serine protease) only prior
308 to apolysis (stage C) and not after apolysis has occurred (stage D0). Furthermore, post-
309 apolysis, the formation of a new cuticle (as indicated by the expression of cuticle CB6)
310 continues irrespective of the photoperiodic cycle. Overall, the DD treatment appeared to

311 slow-down or even halt processes leading up to apolysis but had little effect on individuals
312 that were post-apolysis and already making preparations for the next moult.
313
314

Discussion

Analytical approach

The annotation of identified genes with Gene Ontology categories allowed a comparison of biochemical pathways and physiological processes to be made between the subtracted (SSH) and unsubtracted libraries. This comparison showed that the subtracted library was enriched for genes involved in processes such as metabolism, protein binding, signalling, motor activity and gene transcription and translation. Some of the metabolic genes, such as those encoding for trypsin and carboxypeptidase, were also involved in moulting. These results indicate that a number of genes encoding for proteins involved in growth, feeding and swimming activity were expressed at levels greater in krill incubated in a LD photoperiod than in krill incubated in a DD photoperiod. To clarify the differences in gene expression between LD and DD krill further, qPCR was used on 11 genes highlighted by the SSH procedure that were linked to moulting, feeding and motor activity (Table 1). All 11 genes selected from the SSH library for qPCR were found to have expression levels higher in pooled LD krill compared to pooled DD krill. Of these, the expression of eight genes showed more than a two fold difference, which is traditionally used as a significance threshold for expression levels (Leung and Cavalieri, 2003).

Functions of genes

The most striking differential gene expression patterns were shown by the proteases, serine collagenase precursor, trypsin, and CUB-serine protease. Proteases play a major role in the digestive system of invertebrates (Muhlia-Almazán et al. 2008), and have been found in the moulting fluid of insects (Samuels et al., 1993) and crustaceans (Warner and Matheson, 1998) where they have been shown to degrade insect cuticle (Samuels et al., 1993). Although trypsin itself has not been shown to degrade cuticle, Klein et al. (1996) found that both

trypsin mRNA and trypsin enzyme activity were at their highest levels during premoult (stage D_1) of the shrimp *Penaeus vannamei*.

It is known that during premoult in crustaceans (stages D0 to D3), the old cuticle is digested and reabsorbed to recycle nutrients (Buchholz and Buchholz, 1989). The cuticle of *E. superba* consists of epicuticle, exocuticle and endocuticle, with these last two layers built up of stacks of laminae that consist of protein and chitin (Buchholz and Buchholz, 1989). It has been proposed that, in order for the cuticle to be digested during premoult, proteases are needed to degrade the protein protecting the chitin microfibers. Chitinases, such as β-N-acetylglucosaminidase, can then digest the microfibers (Bade and Stinson, 1979; Fukamizo and Kramer, 1985).

The low density lipoprotein receptor-related protein (LRP), found to be expressed at twice the level in LD versus DD krill, is known to be involved in the degradation of the old cuticle (Yochem et al. 1999). LRPs are also known to regulate the activity of extracellular proteases (Krieger and Herz, 1994; Strickland et al., 1995) and so the LRP here may be required for activation of the protease trypsin, that in turn is required for activation of trypsinogen (Neurath, 1964), the serine collagenase precursor (Tsu and Craik, 1996) and pancreatic carboxypeptidase A1 precursor (Bayes et al., 2003).

Other genes found to be up regulated in the LD krill are involved in carbohydrate metabolism and motor activity. Aldo-keto reductase and fructose-bisphosphate aldolase are both involved in glycolysis, the initial process of carbohydrate catabolism (Gabbay and Tze, 1972; Hanson and Garber, 1972; Salvatore et al., 1986), and may indicate that the LD krill require more energy than the DD krill. This is supported by an increase in expression of myosin light chain

365 mRNA, a gene involved in muscle contraction (Poetter et al., 1996), suggesting that the LD
366 krill are more active than the DD krill and so require a greater energy supply.
367
368 The pooled-krill qPCR analysis revealed several effects of photoperiod on physiological
369 processes. Two carbohydrate metabolic genes involved in the glycolysis pathway were less
370 active in DD krill, even though the feeding environment was identical to that of LD krill (i.e.
371 natural seawater exchanged every 24 h). Morris and Priddle (1984) found that the amount of
372 food in alimentary tracts decreased during wintertime, and suggested this was a function both
373 of the level of feeding activity and the availability of food. Similarly, Quetin et al. (2003)
374 concluded that daylength acting on both behavior and primary production explained 74% of
375 the variation in larval and juvenile growth rates. The contrary explanation, that daylength has
376 a direct effect on physiological state, is otherwise supported by Atkinson et al. (2002), who
377 found that late autumn krill ingested at only low rates even when offered high concentration
378 of food. Our results would support that latter explanation in that, on receipt of an appropriate
379 photoperiodic cue, krill no longer maintain physiological processes capable of digesting food.
380 This potentially reduces the cost of maintaining such systems during a time of year when
381 food is traditionally less available. Entry into this state potentially overrides local cues
382 regarding sporadic increases in feeding conditions, at least over short time intervals.
383
384 This analysis also found that gene expression of myosin, which is involved in motor activity,
385 was lower in krill exposed to DD than LD conditions. This may have a more immediate
386 explanation. Krill use light cues in order to synchronise upward and downward vertical
387 migrations. Under DD conditions, there is an absence of such cues and swimming activity
388 could cease. Nevertheless, Gaten et al. (2008) has demonstrated the existence of innate
389 rhythms of activity and inactivity in the absence of any light cues, which persist over a

number of days. What may be more the issue in the present study is that the level of activity is much less under DD conditions, independent of any internal clock.

Interaction between photoperiod and the moult cycle

The individual krill qPCR analysis further highlighted some specific interactions between photoperiod and the moult cycle. In particular, we found that photoperiod affected the expression of protease genes (serine collagenase, trypsin and CUB-serine protease) in certain moult stages only. Specifically, there was a significant down-regulation in expression of protease genes in moult stage C in the DD photoperiod compared to the LD photoperiod, but no such down-regulation in moult stage D0 between photoperiods. Furthermore, cuticle CB6 gene, which is involved in the formation of the new cuticle and only expressed after apolysis (Fig. 3), did not appear to be affected by photoperiod. Serine collagenase, trypsin and CUB-serine protease are all likely to be involved in the break-down of cuticle (Samuels et al. 1993, Klein et al. 1996, Warner and Matheson 1998) and so probably play an important role in the process of apolysis, when the cuticle separates from the epidermis and the first stages of old cuticle degradation begins. Our results indicate that the DD photoperiod slowed-down or even halted processes necessary for apolysis. This, in turn, implies that relatively short-term exposure to continuous darkness is sufficient to slow down or even halt the moult cycle. Apolysis appears to be a gate through which the moult cycle continues under natural activities but at which the cycle is halted when exposed to cues such as continuous darkness (Fig. 2).

The observation that moulting is one of the first processes to be down-regulated after 7 days of darkness is not entirely expected. Moulting continues throughout adult life and is believed to occur in winter as well as summer (Buchholz, 1991, Quetin et al., 2003). However, Clarke

(1976), Morris and Priddle (1984) and Buchholz et al. (1989) all report a lowering of moult rate in Antarctic krill during winter-time, corresponding to period when there is little or no light. Morris and Priddle (1984) also report that there was an increased proportion of krill in intermoult during this time. This agrees with the present study in terms of individuals not progressing through apolysis when entering a stage of winter-time quiescence.

Significance of findings

Overall, our findings indicate:

1) that even 7 days of exposure to different photoperiodic cues during autumn produce a significant change in the transcription of genes underlying a number of physiological processes, including protease activity, cuticle degradation, cuticle formation, protein digestion, glucose metabolism, and muscle contraction
2) that many of these processes are consistent with known features of quiescence, particularly the slowing of moult rate, feeding and swimming activity.

Speculation on the use of cues by krill to alternate between active and quiescent stages was partially addressed by Teschke et al. (2007, 2008) whose incubations in different photoperiodic conditions were carried out over several months. The purpose of the present study was to examine whether a response to photoperiodic conditions could also be initiated over a shorter time interval. At 65 °S, the period of daylight decreases by 7 minutes per day, or 49 minutes per week, as one passes from autumn to winter. It is therefore possible that a set threshold may be passed within the period of a week and physiological cascades of non-essential processes become halted quickly. Although our experiments take krill to the extreme of continuous darkness, we demonstrate that these organisms are capable of halting processes within a matter of days. Although a number of studies have investigated the

process of dormancy in marine zooplankton (Dahms, 1995; Hirche, 1996), this is one of the first to establish that the onset of quiescence in Antarctic krill may be particularly rapid once appropriate cues have been received.

In conclusion, this study has shown that the gene expression underlying activities such as moulting, feeding and swimming is reduced in Antarctic krill after seven days exposure to a winter-time photoperiodic cycle. This reduction in activity levels is consistent with the features of quiescence. We support the view that photoperiod plays an important role in initiating the transition between active and quiescent states. Furthermore, we propose that this transition can be initiated within a few days of crossing a photoperiodic threshold.

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Table 1. ESTs chosen for comparison of gene expression in LD and DD krill by qPCR, showing the top Blastx hit and the associated physiological function.

GenBank accession no	**Top Blastx hit with accession number**	**E-value**	**EST length(bp)**	**Associated physiological function**
FL688501	(AAC47030) Serine collagenase 1 precursor	6e-34	426	Collagenase and protease activity[1]
FL688573	(CAA75311) Trypsin	2e-65	432	Protease activation and activity[1, 2, 3] Food protein digestion[4]
FL688234	(AAK48894) CUB-serine protease	2e-31	455	Degradation of old cuticle[5, 6] Immune response[7]
FL688566	(XP_001648462) Aldo-keto reductase	2e-18	270	Carbohydrate metabolism[8, 9]
FL688449	(BAF33500) Trypsinogen	3e-18	379	Precursor of trypsin[10]
FL688531	(AAL40361) Pancreatic carboxypeptidase A1 precursor	4e-06	457	Degradation of old cuticle[11]
FL688277	(XP_001920116) PREDICTED: similar to low density lipoprotein receptor-related protein 8	1e-30	451	Degradation of old cuticle[12] Protease activation[13, 14]

FL688235	(XP_001608248) PREDICTED: similar to myosin light chain isoform 2	6e-18	535	Skeletal and cardiac muscle contraction[15]
FL688420	(ABM54465) Cuticle protein CB6	1e-19	391	Cuticle formation[16]
FL688711	(XP_001651423) Fructose-bisphosphate aldolase	5e-62	466	Glucose metabolism[17]
FL688434	(ABB86961) β-N-acetylglucosaminidase	5e-12	363	Degradation of old cuticle[18, 19]

The E (Expect) value is the number of Blastx hits that would occur by chance with the same alignment or better: the lower the E-value, the more significant the match. [1]Tsu and Craik (1996), [2]Muhlia-Almazán et al. (2008), [3]Bayes et al. (2003), [4]Diaz-Mendoza et al. (2005), [5]Samuels et al. (1993), [6]Frand et al. (2005), [7]Gorman and Paskewitz (2001), [8]Gabbay and Tze (1972), [9]Bagnasco et al. (1987), [10]Neurath (1964), [11]Ote et al. (2005), [12]Yochem et al. (1999), [13]Krieger and Herz (1994), [14]Strickland et al. (1995), [15]Poetter et al. (1996), [16]Kuballa et al. (2007), [17]Salvatore et al. (1986), [18]Samuels and Reynolds (1993), [19]Nagamatsu et al. (1995).

Table 2. Comparisons carried out on individual krill to examine the effects of light regime and moult stage on the expression of genes involved in moulting

qPCR-comparison experiment	Purpose	Control Group	Test Group
A	Effect of apolysis on gene expression	6 krill moult stage C light regime 3LD & 3DD	3 krill moult stage D0 light regime 3LD & 3DD
B	Effect of light regime on gene expression in moult stage C	6 krill moult stage C light regime LD	6 krill moult stage C light regime DD
C	Effect of light regime on gene expression in moult stage D0	3 krill moult stage D0 light regime LD	3 krill moult stage D0 light regime DD

Table 3. Primer sequences used in qPCR.

Gene name	**Primer sequence 5'-3'**	**Product size (bp)**
Serine collagenase 1 precursor	F: GGTTCATGTGACCACACTCG R: ATCACCATTGCAGGAACCAT	112
Trypsin	F: ATGCCTATGGTGAGGGTGAG R: GGTAGTTGGGTCTGGCACAT	174
CUB-serine protease	F: AGCCCGATGTATTGATGGAG R: TAGAAGAGAGGGCCACCAGA	157
Aldo-keto reductase	F: TTTCAGATTCAACGCAATGTG R: GCTGCACACTCGACCATTAC	147
Trypsinogen	F: ACTTGGAGGAGCACAGGTTG R: GGCAGATCGGAGTTGTGTCT	134
Pancreatic carboxypeptidase A1 precursor	F: GCCTACAAACAGGGTGTTCC R: GCTTCAGGAACAAGTTCACCA	152
Low density lipoprotein receptor-related protein 8	F: ATTTGCCGCTGCTTTTACAT R: AGTGCATACCCGGCAACTAC	123
Myosin light chain isoform 2	F: GTCGATGAGGACACCCAGAT R: TGGTTGAGGCTACTGGAACC	163
Cuticle protein CB6	F: ACCAAAGTCGTTGCCTGAGT R: CGACACGACACACATCATCA	181

Fructose-bisphosphate aldolase	F: AATGTAACACCGGGAACTGC R: TCGCTGATCACAACGTCTTC	160
ß-N-acetylglucosaminidase	F: AGTGTTCTGCCGATTTTGGT R: TCCTCAACAGACCCACTTCC	169
Beta actin	F: GGAGACCGCAAGATTCCATA R: TGAGCAGGAAATGACCACAG	155
Phosphoenolpyruvate carboxykinase	F: TGTTGAAGGTAGTGGCCAAA R: GAAACACGGTGTCATGGTTG	138
Glyceraldehyde 3-phosphate dehydrogenase	F: GATGCCAAAGCTGGCATTAT R: TTGTCCACGACAGCAGAAAA	166

Table 4. General features of subtracted and unsubtracted cDNA libraries.

	LD-DD subtracted	**LD unsubtracted**
Total sequences	595	685
Ribosomal sequences	129	111
Putative mRNAs	466	574
Identified mRNAs	225	232
Unidentified	241	342

Table 5. Significantly enriched GO slim categories in subtracted library compared to unsubtracted library ($p<0.05$).

GO description	**LD-DD subtracted**	**LD unsubtracted**	**p value**
Binding	240	159	1.35E-05
Receptor activity	49	14	0.0003
Transferase activity	55	23	0.0058
Protein binding	196	132	0.0102
Lyase activity	31	10	0.0116
Macromolecule metabolic process	225	160	0.0171
Metabolic process	76	41	0.0263

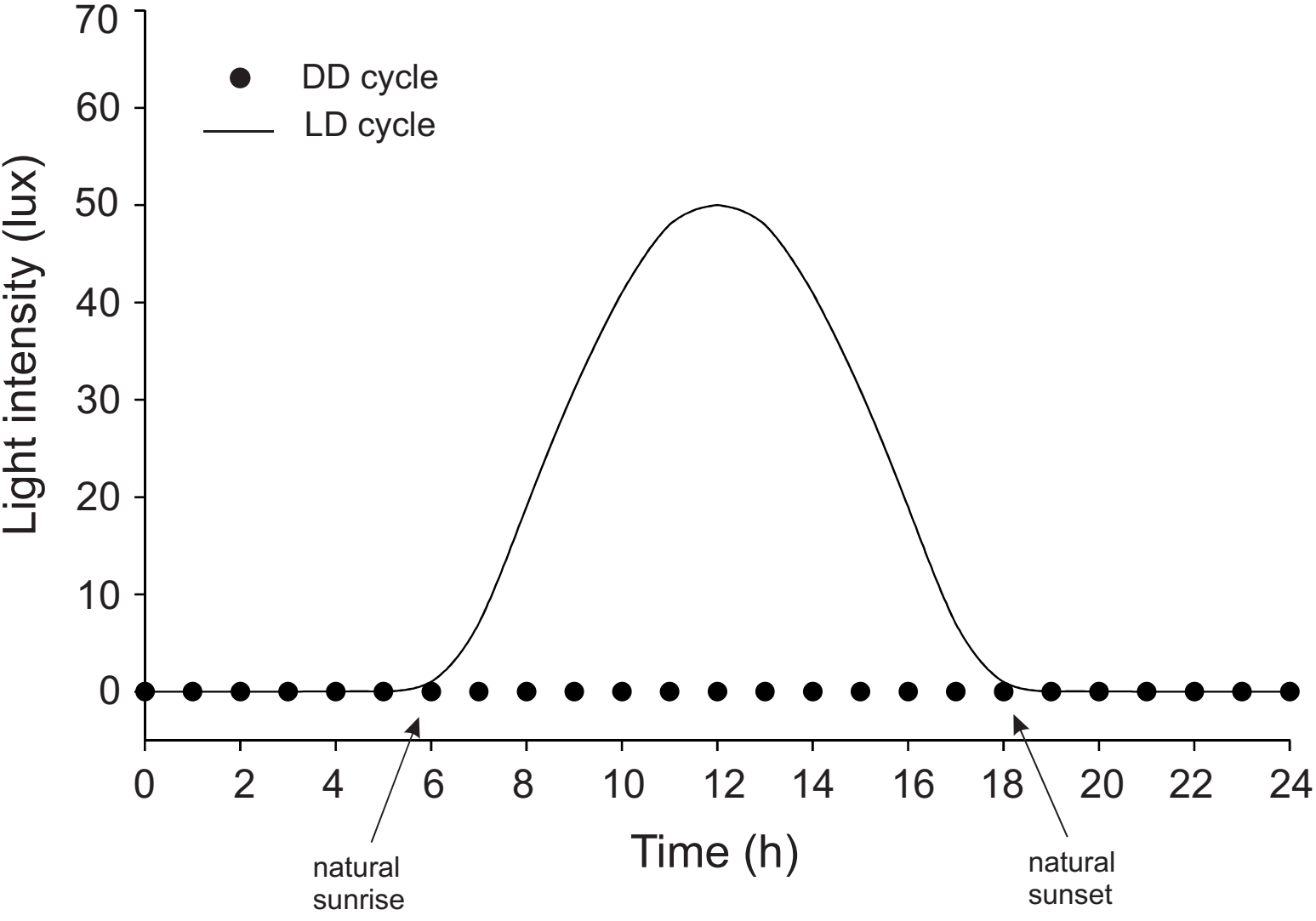
Fig. 1. Schematic representation of 24 hour light regimes in the experimental tanks. LD: 12 hours of light and 12 hours of darkness; DD: continuous darkness. Arrows indicate the approximate time of sunrise and sunset in the natural environment at the time of the experiment

Fig. 2. The moult cycle of *Euphausia superba* showing the specific moult stage (stages A to D3) and moult category (pre-, inter- and post-moult). Individuals in DD photoperiod that are between moult and apolysis do not enter and pass through apolysis. Individuals in DD that are beyond apolysis continue on to moult. Individuals in LD progress normally through the full moult cycle.

Fig. 3. Differential gene expression between pools of LD and DD krill sampled over a 24 hour period, normalised to ß-actin. Data indicate the level of up-regulation of genes in LD krill compared to the level expressed in DD krill.

Fig. 4. Effect of light regime on gene expression in moult staged individual krill normalised to PEP-CK. Gene expression is represented as a $\log_2$ relative expression ratio (means ± SE). (A) LD/DD stage C vs LD/DD stage D_0 (n=6); a positive value indicates the level of up-regulation of krill in moult stage D0 compared to the level of expression in moult stage C krill. (B) LD stage C vs DD stage C (n=6); positive values indicates up-regulation and negative values, down-regulation in DD krill relative to LD krill (C) LD stage D_0 vs DD stage D_0 (n=3); positive and negative values as for (B);. An asterisk indicates significant results ($P < 0.05$).

Figure 1


70
60
50
40
30
20
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0
DD cycle
LD cycle
Light intensity (lux)
0
2
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10
12
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22
24
Time (h)
natural
sunrise
natural
sunset

Figure 2

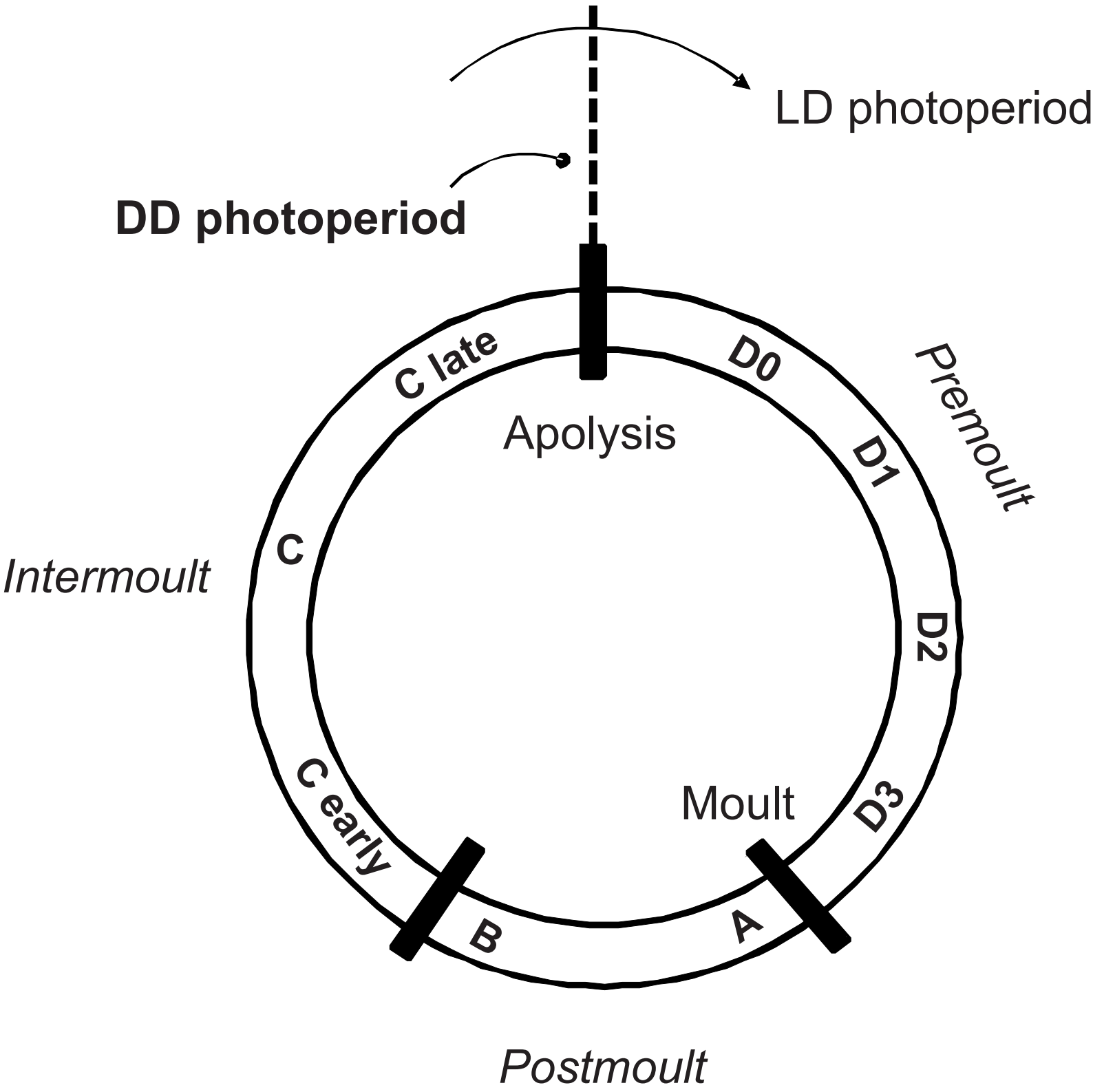
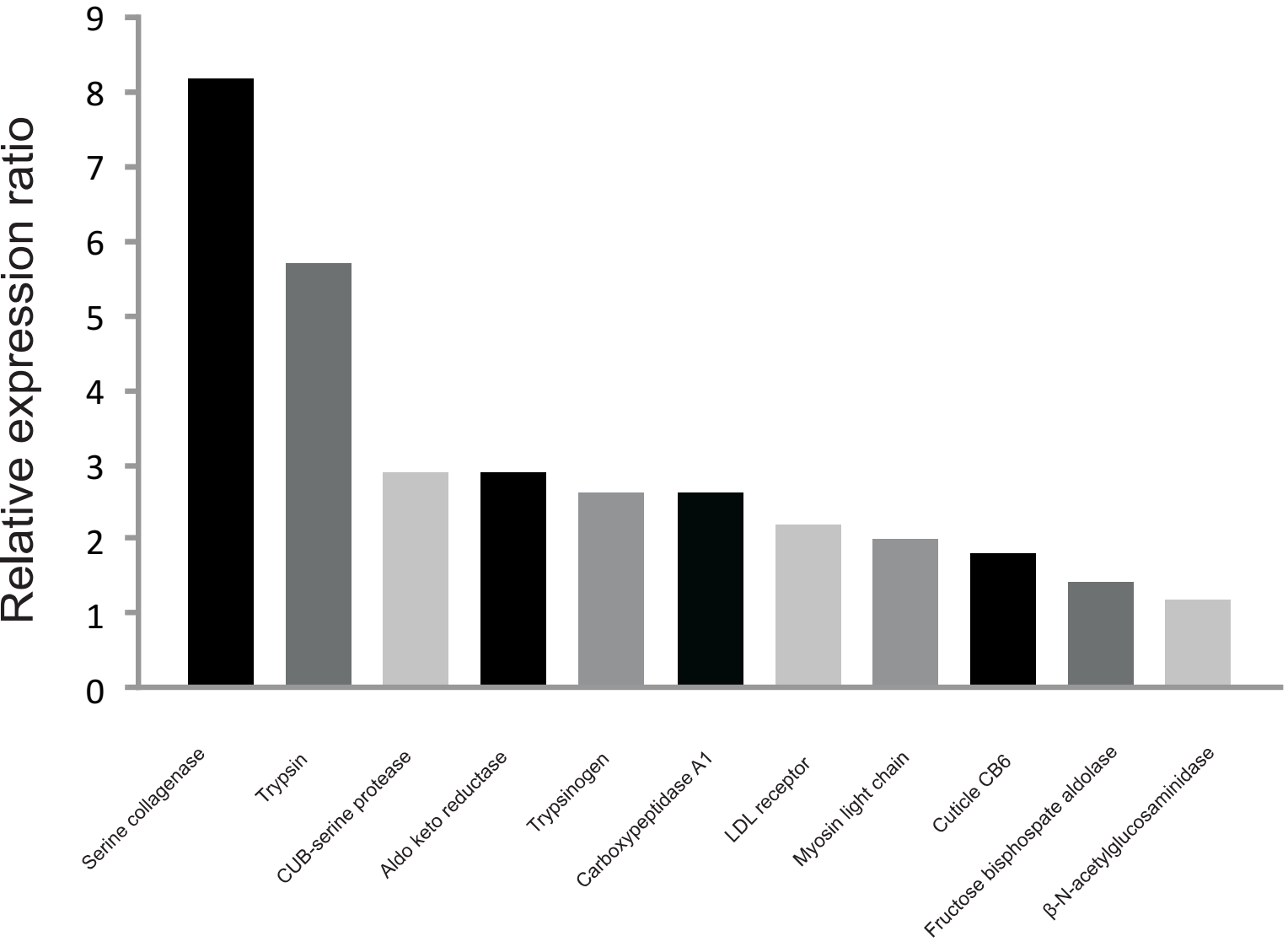

LD photoperiod
DD photoperiod
C late
D0
Premoult
Apolysis
D1
C
Intermoult
D2
C early
Moult
D3
B
A
Postmoult

Figure 3


Relative expression ratio
0
1
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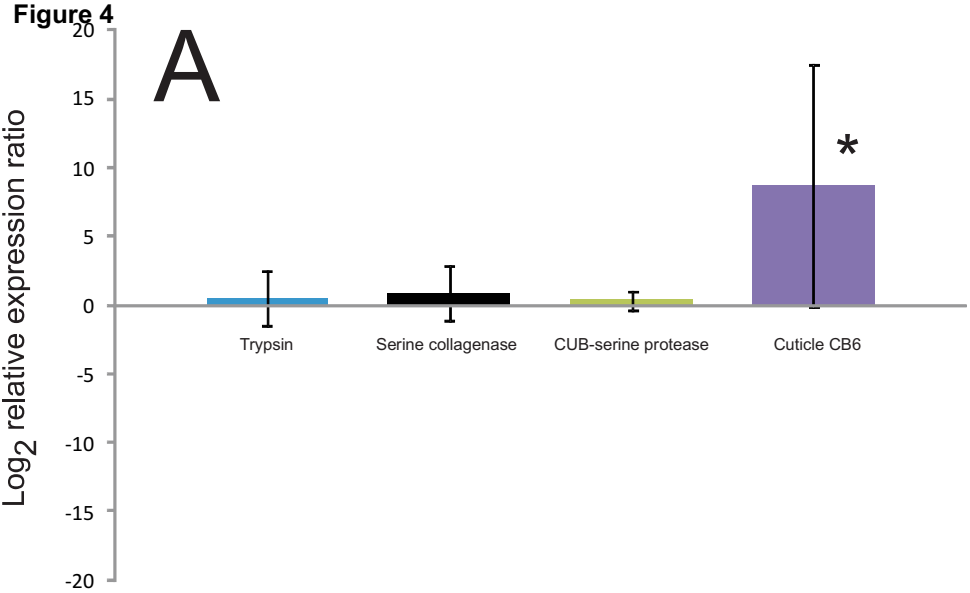
9
Serine collagenase
Trypsin
CUB-serine protease
Aldo keto reductase
Trypsinogen
Carboxypeptidase A1
LDL receptor
Myosin light chain
Cuticle CB6
Fructose bisphospate aldolase
β-N-acetylglucosaminidase

Figure 4


A
Log2 relative expression ratio
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Trypsin
Serine collagenase
CUB-serine protease
Cuticle CB6
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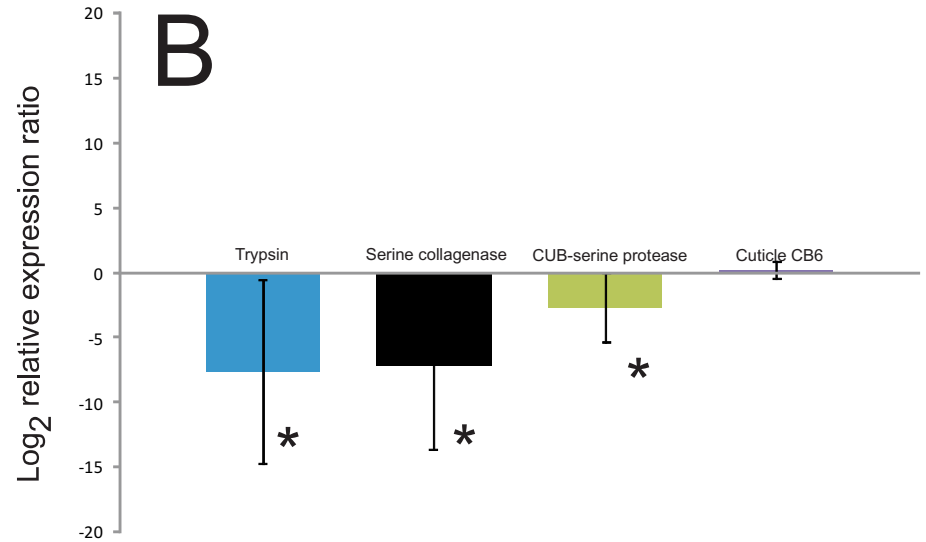
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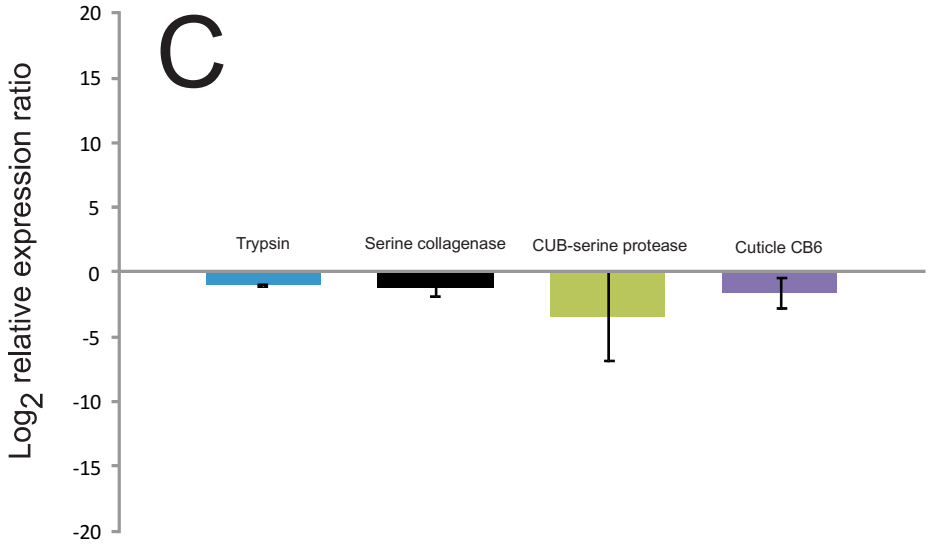
C
Log2 relative expression ratio
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Serine collagenase
CUB-serine protease
Cuticle CB6