Proteomic analysis reveals that COP9 signalosome complex subunit 7A (CSN7A) is essential for the phase transition of migratory locust

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The migratory locust displays a reversible, density-dependent transition between the two phases of gregaria and solitaria. This phenomenon is a typical kind of behavior plasticity. Here, we report that COP9 signalosome complex subunit 7A (CSN7A) is involved in the regulation of locust phase transition. Firstly, 90 proteins were identified to express differentially between the two phases by quantitative proteomic analysis. Gregaria revealed higher levels in proteins related to structure formation, melanism and energy metabolism, whereas solitaria had more abundant proteins related to digestion, absorption and chemical sensing. Subsequently, ten proteins including CSN7A were found to reveal differential mRNA expression profiles between the two phases. The CSN7A had higher mRNA level in the gregaria as compared with the solitaria, and the mRNA amount in the gregaria decreased remarkably during the 32 h-isolation. However, the mRNA level in the solitaria kept constant during the crowding rearing. Finally and importantly, RNA interference of CSN7A in gregaria resulted in obvious phase transition towards solitaria within 24 h. It suggests that CSN7A plays an essential role in the transition of gregaria towards solitaria in the migratory locust. To our knowledge, it’s the first time to report the role of CSN in behavior plasticity of animals.

The migratory locust (Locusta migratoria) is an important pest insect in Asia. When locust disaster breaks out, swarms of locusts gather at very high population densities, and then trigger the migration of whole population towards new areas with more food. The aggregation and migration of locusts definitely result in broader damage. In 1966, Uvarov brought forward the concepts of gregaria and solitaria to describe locust phases with high and low population densities, respectively1. The two phases distinguish each other in many aspects including morphology, behavior, coloration, reproduction, development, endocrine and immunity2. Their behavioral distinction is prominent: the gregaria is more active and easier to be attracted by other individuals, whereas the solitaria exhibits to be more isolated. These differences are usually used as key markers in behavioral assay to distinguish the two phases3-6. Locust phase can shift from one state to another in response to density changes. The phase transition is a continuous, cumulative, and easily reversible process7, and it can take place within a short period (from 4 h to 32 h) in both the migratory locust1 and the desert locust, Schistocerca gregaria8-10.

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In recent years, many fruitful studies have been carried out to elucidate the intrinsic molecular mechanisms of phase transition in locusts from various aspects such as genomics, transcriptomics and metabolomics. A large scale of transcriptomic sequencing was carried out in the migratory locust using an expressed sequence tag (EST) technique in 2004\(^1\), and 532 differentially expressed unigenes were identified between the two phases. The transcriptome dynamics in the same species were further analyzed in 2010 based on a newly emerged next-generation sequencing technology\(^2\). A lot of genes related to neural pathway, such as dopamine receptor, adipokinetic hormone, neurotransmitter synthetase were found to be up-regulated in the gregarious locusts. Another transcriptomic analysis was performed in the desert locust\(^3\). The solitary locusts up-regulate genes related to antioxidant systems, detoxification and anabolic renewal, whereas gregarious locusts have a greater abundance of transcripts for genes involved in sensory processing and nervous system development and plasticity. After monitoring and comparing transcript profiles between the two phases at various developmental stages, Chen et al. found that a sharp rise in phase differences appeared during the 4th instar and the high level difference was maintained in all the following stages. Therefore, the 4th instar stage seems to be a turning point in the process of forming the phase differences in the migratory locust\(^4\). Some neuronal signaling and sensory activity related genes, such as dopamine receptor\(^5\), chemosensory protein (CSP) and takeout\(^6\) were proved to play roles during the phase transition. The successful assembly of the migratory locust genome is a milestone in the study of phase transition of locust\(^7\). The genome is 6.5 giga base pairs (Gb), the largest animal genome sequenced so far. Significant expansion of gene families associated with energy consumption and detoxification were found in the locust genome\(^8\). Besides, small RNA\(^9\) and metabolomics\(^10\) analysis also disclosed a lot of regulators contributing for the phase transition.

Proteomic researches have also been carried out, but few significant progresses have been made till now. In 1999, polypeptide maps were generated from hemolymph of the desert locust and twenty differential spots were identified between the two phases. However, detailed information about these peptides was not available\(^11\). Two proteins, a 6-kDa peptide and a serine protease inhibitor were identified to have different expression patterns between the two phases in the desert locust using a combined approach of high-performance liquid chromatography (HPLC) with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)\(^12\). These proteomic studies moved very slowly because of the lack of locust genome information at that time.

The behavior plasticity makes locusts to be a good model in the epigenetic researches\(^13,14\). Two DNA methyltransferase genes were shown to be phase-specific in certain tissues of the desert locust\(^15\). Further analysis revealed that the methylome of the gregarious desert locust was characterized by CpG- and exon-specific methylation, and the overall methylation levels were substantially higher than other invertebrates\(^16\). These findings suggest that DNA methylation may be involved in the regulation of locust phase transition. Besides, a cAMP-dependent protein kinase (PKA) was reported to play a role in the transition from solitary to gregarious behavior in the desert locust\(^17\). Except for these reports, few studies have been further performed in recent years.

In general, large progresses have been made in exploring the mechanisms of phase transition in the migratory and desert locusts. A lot of differentially expressed genes and pathways have been identified based on DNA sequencing techniques. However, the researches in protein areas, such as protein identification and protein modification, have been largely lagged. One of the most key reasons is the lack of genome sequence information. Fortunately, the genome assembly of the migratory locust was just finished\(^18\), which provides much convenience for protein identification and will do great help for exploring the complex mechanism of phase transition in another viewpoint.

In the present study, we identified 90 differentially expressed proteins between the two phases in the migratory locust by a quantitative proteomic technique. Among them, CSN7A was found to play an essential role in the transition of gregaria towards solitaria.

**Results**

**Proteins identified in the locust head.** A total of 4, 895 peptides were identified by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) from the locust head, and they were finally assembled into 1, 387 proteins. After COG classification, 1, 104 proteins were assigned to 25 COG categories, and the "R" cluster (General function predication) and "O" cluster (Posttranslational modification, protein turnover, chaperones) represent the largest two groups, and their amounts are 18% and 15% of the total identified proteins, respectively (Fig. 1). The "O" cluster proteins are mainly heat shock protein chaperones, ubiquitin-dependent proteins, proteasome-related proteins, peptidase activity-related proteins, glutathione S-transferase, protein disulfide-isomerase, and COP9 signalosome complex subunits (Supplementary Table S1). The top 70 most abundant proteins are listed in Table 1 and Supplementary Table S2. Most are the proteins related to structural construction, such as twitchin, spectrin alpha chain-like and microtubule-actin cross-linking factor 1. Many proteins, including pyruvate kinase (EC 2.7.1.40), malate dehydrogenase (EC 1.1.1.37), aconitate hydratase (EC 4.2.1.3), citrate synthase 2 (EC 2.3.3.1), succinyl-CoA ligase (EC 6.2.1.4), 2-oxoglutarate dehydrogenase E1 component (EC 1.2.4.2), and ATP-citrate synthase (EC 2.3.3.8), revealed high abundance. These proteins are involved in tricarboxylic acid cycle (TCA). It suggests that TCA is very active in the locust head. Besides, several heat shock proteins and hexamerins were also identified to be abundant in the locust head.
Differentially expressed proteins between the two phases. Among the 1,387 identified proteins, 90 proteins were shown to have different expression levels between the two phases. Sixty-four were up-regulated in the gregaria (as compared with the solitaria), and twenty-six were down-regulated (Table 2, Supplementary Table S3). Most of the up-regulated proteins are involved in the processes of structure formation (such as cuticle protein, beta-1 tubulin, profiling, and troponin), energy metabolism (electron transfer flavoprotein subunit alpha [ETFA], dehydrogenase/reductase SDR family member 11-like, 3-ketoacyl-CoA thiolase, V-type proton ATPase subunit B and isocitrate dehydrogenase

Figure 1. COG classification of identified proteins. Based on sequence homology, 1,104 proteins were classified into 25 COG (Clusters of orthologous groups) categories (A). The “R” and “O” clusters represent the largest two groups. Proteins classified in the “O” cluster (Posttranslational modification, protein turnover, chaperones) were further assigned to different categories according to their molecular functions (B). Detailed information about these “O” cluster proteins can refer to Supplementary Table S1.
| No. | ID number in the locust genome database (version 2.0) | Protein name | Species | Accession No. | Peptide |
|-----|------------------------------------------------------|--------------|---------|---------------|---------|
| 1   | LMI_GLEAN_10128031                                   | twitchin     | Cerapachys biroi | EZA52953     | 166     |
| 2   | LMI_GLEAN_10163232                                   | apolipophorin II | Locusta migratoria | Q9U943     | 103     |
| 3   | LMI_GLEAN_10136968                                   | myosin heavy chain, muscle-like | Apis florea | XP_003695415 | 77      |
| 4   | LMI_GLEAN_10142035                                   | spectrin alpha chain-like | Apis mellifera | XP_006558458 | 50      |
| 5   | LMI_GLEAN_10142824                                   | muscle M-line assembly protein unc-89 | Cerapachys biroi | EZA59129 | 49      |
| 6   | LMI_GLEAN_10088460                                   | filamin-A isoform X3 | Tribolium castaneum | XP_008199793 | 48      |
| 7   | LMI_GLEAN_10187850                                   | microtubule-actin cross-linking factor 1 isoform X6 | Nasonia vitripennis | XP_008203191 | 45      |
| 8   | LMI_GLEAN_10135516                                   | alpha-actinin, sarcomeric isoform X2 | Tribolium castaneum | XP_972324 | 36      |
| 9   | LMI_GLEAN_10062054                                   | paramyosin, long form | Zootermopsis nevadensis | KDR08790 | 33      |
| 10  | LMI_GLEAN_10137418                                   | spectrin beta chain | Zootermopsis nevadensis | KDR16227 | 31      |
| 11  | LMI_GLEAN_10140538                                   | titin         | Harpegnathos saltator | EFN83273 | 28      |
| 12  | LMI_GLEAN_10134774                                   | elongation factor 2 | Schistocerca gregaria | AEV89753 | 26      |
| 13  | LMI_GLEAN_10126855                                   | pyruvate kinase | Zootermopsis nevadensis | KDR19430 | 26      |
| 14  | LMI_gi_37993866                                      | heat shock protein 70 | Locusta migratoria | AAP57337 | 25      |
| 15  | LMI_GLEAN_10160912                                   | glycogen phosphorylase-like | Apis florea | XP_003690485 | 25      |
| 16  | LMI_GLEAN_10157178                                   | staphylococcal nucleosome domain-containing protein 1 | Tribolium castaneum | XP_974879 | 23      |
| 17  | LMI_GLEAN_10153094                                   | coracle, partial | Blattella germanica | CCI09964 | 23      |
| 18  | LMI_gi_93278396                                      | heat shock protein 90 | Locusta migratoria | AAS45246 | 21      |
| 19  | LMI_gi_99867354                                      | arginine kinase | Locusta migratoria manilensis | ABF68036 | 20      |
| 20  | LMI_GLEAN_10097368                                   | ATP-citrate synthase | Zootermopsis nevadensis | KDR07798 | 20      |
| 21  | LMI_GLEAN_10043824                                   | clathrin, partial | Locusta migratoria | AHC70342 | 19      |
| 22  | LMI_GLEAN_10164084                                   | heat shock 70 kDa protein cognate 5 | Zootermopsis nevadensis | KDR08641 | 18      |
| 23  | LMI_gi_241997152                                     | ER protein gp78 | Locusta migratoria | ACS57353 | 18      |
| 24  | LMI_gi_256368118                                     | hexamerin-like protein 2 | Locusta migratoria | ACU78069 | 18      |
| 25  | LMI_GLEAN_10109513                                   | tropomyosin-1 | Nasonia vitripennis | XP_001599008 | 17      |
| 26  | LMI_GLEAN_10065878                                   | 60 kDa heat shock protein, mitochondrial | Zootermopsis nevadensis | KDR14060 | 17      |
| 27  | LMI_GLEAN_10001937                                   | myosin heavy chain, non-muscle-like isoform 2 | Bombus terrestris | XP_003394420 | 17      |
| 28  | LMI_gi_225194719                                     | pro-phenoloxidase 2 | Locusta migratoria | ACN81829 | 17      |
| 29  | LMI_GLEAN_10141796                                   | vinculin-like isoform 1 | Bombus impatiens | XP_003493644 | 17      |
| 30  | LMI_GLEAN_10143564                                   | hexamerin-like protein 2 | Locusta migratoria | ACU78069 | 17      |
| 31  | LMI_GLEAN_10196247                                   | beta-actin    | Diabolocatantops pinguis | ACV32627 | 16      |
| 32  | LMI_GLEAN_10097173                                   | tropomyosin-1, isoforms 9A/A/B | Camponotus floridanus | EFN72212 | 16      |

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| No. | ID number in the locust genome database (version 2.0) | Protein name                      | Species                      | Accession No. | Peptide |
|-----|---------------------------------------------------|-----------------------------------|------------------------------|--------------|---------|
| 33  | LMI_GLEAN_10021933                                | tubulin beta-1 chain              | Tribolium castaneum         | XP_967267    | 16      |
| 34  | LMI_GLEAN_10170791                                | fructose 1,6-bisphosphate aldolase | Schistocerca gregaria       | AEV89754     | 16      |
| 35  | LMI_GLEAN_10123052                                | malate dehydrogenase, mitochondrial | Nasonia vitripennis       | XP_001600547 | 16      |
| 36  | LMI_GLEAN_10097518                                | alpha tubulin                     | Schistocerca gregaria       | AEV89775     | 16      |
| 37  | LMI_GLEAN_10142607                                | alpha tubulin                     | Schistocerca gregaria       | AEV89775     | 16      |
| 38  | LMI_GLEAN_10096450                                | alpha tubulin                     | Schistocerca gregaria       | AEV89775     | 16      |
| 39  | LMI_GLEAN_10111205                                | aconitate hydratase, mitochondrial-like | Megachile rotundata     | XP_003705474 | 16      |
| 40  | LMI_GLEAN_10170835                                | nesprin-1                         | Zootermopsis nevadensis     | KDR09330     | 16      |
| 41  | LMI_GLEAN_10104518                                | pyruvate carboxylase, mitochondrial | Zootermopsis nevadensis     | KDR22588     | 16      |
| 42  | LMI_GLEAN_10168000                                | annexin-B9                        | Zootermopsis nevadensis     | KDR08631     | 15      |
| 43  | LMI_GLEAN_10143558                                | hexamerin-like protein 1           | Locusta migratoria          | ACU78068     | 15      |
| 44  | LMI_GLEAN_10126189                                | citrate synthase 2, mitochondrial | Zootermopsis nevadensis     | KDR22581     | 15      |
| 45  | LMI_GLEAN_10189307                                | ubiquitin-like modifier-activating enzyme 1 | Zootermopsis nevadensis | KDR20513     | 15      |
| 46  | LMI_GLEAN_10113091                                | ATPase                             | Homo sapiens                | AAA35578     | 15      |
| 47  | LMI_GLEAN_10165425                                | glycogen debranching enzyme, partial | Zootermopsis nevadensis     | KDR16306     | 15      |
| 48  | LMI_GLEAN_10136778                                | neither inactivation nor afterpotential protein C | Zootermopsis nevadensis | KDR20620     | 15      |
| 49  | LMI_GLEAN_10056004                                | cytoplasmic A3a                    | Helicoverpa armigera        | Q25010       | 14      |
| 50  | LMI_GLEAN_10156223                                | transitional endoplasmic reticulum ATPase TER94 | Zootermopsis nevadensis | KDR08983     | 14      |
| 51  | LMI_GLEAN_10085205                                | transferrin                        | Romalea microptera          | AAQ62963     | 14      |
| 52  | LMI_GLEAN_10071372                                | 14-3-3 protein zeta                | Zootermopsis nevadensis     | KDR15025     | 13      |
| 53  | LMI_GLEAN_10081307                                | glutamate dehydrogenase, mitochondrial, partial | Zootermopsis nevadensis | KDR15400     | 13      |
| 54  | LMI_GLEAN_10127881                                | tubulin alpha-3 chain, partial     | Anas platyrhynchos          | EOQ8266      | 13      |
| 55  | LMI_GLEAN_10183379                                | 14-3-3 protein epsilon             | Schistocerca gregaria       | AEV8977      | 13      |
| 56  | LMI_GLEAN_10181426                                | 2-oxoglutarate dehydrogenase E1 component, mitochondrial | Zootermopsis nevadensis | KDR11185     | 13      |
| 57  | LMI_GLEAN_10042900                                | mitochondrial F1-ATP synthase alpha subunit | Locusta migratoria manilensis | AGOS9887    | 13      |
| 58  | LMI_GLEAN_10122197                                | bifunctional purine biosynthesis protein PURH | Zootermopsis nevadensis | KDR19778     | 13      |
| 59  | LMI_GLEAN_10154078                                | hexamerin-like protein 2           | Locusta migratoria          | ACU78069     | 13      |
| 60  | LMI_GLEAN_10173735                                | phosphoglycerate mutase 2          | Zootermopsis nevadensis     | KDR20387     | 13      |
| 61  | LMI_GLEAN_10053226                                | Rab GDP dissociation inhibitor alpha | Zootermopsis nevadensis     | KDR21130     | 13      |
| 62  | LMI_GLEAN_10105168                                | titin                              | Tribolium castaneum         | XP_008191512 | 13      |

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Table 1. Top 70 most abundant proteins identified in the locust head. Note: Detailed information about these proteins can refer to Supplementary Table S2.

| No. | ID number in the locust genome database (version 2.0) | Protein name | Species | Accession No. | Peptide |
|-----|-----------------------------------------------------|--------------|---------|---------------|---------|
| 63  | LMI_gi_329564865                                    | glutathione S-transferase delta | Locusta migratoria | ADR30117 | 12 |
| 64  | LMI_GLEAN_10096104                                 | calcium-transporting ATPase sarcoplastic/endoplasmic reticulum type-like | Megacile rotundata | XP_003707160 | 12 |
| 65  | LMI_GLEAN_10109919                                 | Hrp65 protein | Zootermops nevadensis | KDR15347 | 12 |
| 66  | LMI_GLEAN_10123367                                 | protein disulfide-isomerase | Schistocerca gregaria | AEY89748 | 12 |
| 67  | LMI_GLEAN_10192650                                 | moesin/exrin/radixin homolog 1 | Riptortus pedestris | BAN21261 | 12 |
| 68  | LMI_GLEAN_10051280                                 | malate dehydrogenase, putative | Pediculas humanus corporis | XP_002424808 | 12 |
| 69  | LMI_GLEAN_10154080                                 | hexamerin-like protein 2 | Locusta migratoria | ACU78069 | 12 |
| 70  | LMI_GLEAN_10108970                                 | succinyl-CoA ligase [ADP-forming] subunit beta, mitochondrial | Tribolium castaneum | XP_970725 | 12 |

NAD subunit beta), and environmental stress response (heat shock protein 60 [Hsp60] and heat shock protein 20.6 [Hsp20.6]). Besides, four hexamerin-like proteins are also abundant in the gregaria. The down-regulated proteins are mainly related to the processes of digestion and absorption (carboxypeptidase A-like, serine protease-like protein, and 1,4-alpha-glucan-branching enzyme-like) and chemical sensing (takeout-like). In addition, the differentially expressed proteins are also enriched in the class of “Regulation of gene expression” in both the gregaria and solitaria (Table 2). For example, wingless protein, 3′-phosphoadenosine 5′-phosphosulfate synthase (PAPSS), COP9 signalosome complex subunit 7A (CSN7A), and juvenile hormone binding protein (JHBP) were highly expressed in the gregaria, and splicing factor 3B subunit, ubiquitin-conjugating enzyme E2 variant 2-like isoform 1, proteasome subunit alpha type-4, and arginine/serine-rich-splicing factor RSP31 (RSP31) showed higher levels in the solitaria.

Differential expression at mRNA levels. To validate the differential expression, fourteen representative proteins were selected according to their function categories in Table 2. Their mRNA expression profiles were examined in the whole head of the two-phase locusts. Nine protein genes, including CSN7A, JHBP, PAPSS, choline transporter-like protein 4 (CTL-4), two hexamerin-like protein 2 (Hexa2 and Hexa2*), cytoplasmic actin A3a (actinA3a), ETF and Arylphorin revealed higher mRNA level in gregaria (Fig. 2). It was in consistent with the protein profiles in Table 2. The brain tissues, the most important part of head, were also studied. Four genes, such as CSN7A, JHBP, PAPSS, CTL-4 and takeout-like, showed similar expression patterns between the mRNA and protein levels. There were still four genes, including V-ATPase subunit B (V-ATPase), ATPsyn-d, RSP31, and NADPH--cytochrome P450 (P450) revealed constant mRNA levels between the two phases (Supplementary Fig. S1).

Time-dependent mRNA expression during phase transition. In order to further narrow target proteins that may play a role in the regulation of locust phase transition, CSN7A was chosen and time-dependent mRNA expression dynamics were examined in brain during the phase transition process. The CSN7A had higher mRNA level in the gregaria (Figs 2, 3), the level decreased significantly at 4, 16 and 32h isolation and was as low as that in the solitaria at 32h (Fig. 3). However, the mRNA level did not change during the crowding of solitary locusts (Fig. 3).

RNA interference (RNAi) and behavioral assay. To validate the function of CSN7A in locust phase transition, RNAi and behavioral assay were carried out. The mRNA level was suppressed by injection of CSN7A dsRNA in the gregaria (Fig. 4A), and the behavioral state shifted from gregaria (dsGFP population) to solitaria (dsCSN7A population) (Fig. 4B). The phase difference between two populations was highly significant \( P_{\text{Mann-Whitney U test}} = 1.61 \times 10^{-12} \). For example, 60% and 0% individuals fall into the \( P_{\text{ggreg}} \) interval of 0.8–1.0 in the dsGFP and dsCSN7A population, respectively. In addition, significant difference existed in the three key behavioral parameters (attraction index, total distance moved, and total duration of movement) between the two populations (Fig. 4C). These results revealed that phase transition did happen by RNAi of CSN7A in the gregarious locust.
| No. | ID number in the locust genome database (version 2.0) | G/S (protein level of gregaria over solitaria) | Protein name | Accession No. | Species | Function category |
|-----|---------------------------------------------------|-----------------------------------------------|--------------|--------------|---------|-------------------|
| 1   | LMI_GLEAN_10019610                                | 16.62 cuticle protein 1                        | XP_970381    | Tribolium castaneum |
| 2   | LMI_GLEAN_10050722                                | 5.56 cuticular protein 49Ae                    | XP_002033546 | Drosophila melanogaster |
| 3   | LMI_GLEAN_10021933                                | 4.48 similar to beta1-tubulin                  | XP_967267    | Tribolium castaneum |
| 4   | LMI_GLEAN_10107594                                | 4.45 cuticular protein RR-1 motif 45 precursor | BAB32485     | Bombyx mori    |
| 5   | LMI_GLEAN_10196247                                | 4.30 beta-actin                               | ACV32627     | Diabolocatantops pinguis |
| 6   | LMI_GLEAN_10170245                                | 4.18 similar to Cuticular protein 62Bc CG1919-PA | XP_967979.1 | Tribolium castaneum |
| 7   | LMI_GLEAN_10056604                                | 3.53 cytoplasmic actin A3b                     | AAL89657     | Helicoverpa zea |
| 8   | LMI_GLEAN_10105042                                | 3.00 Endocuticle structural glycoprotein SpAbd-3 | Q7M4E9       | Apis florea |
| 9   | LMI_GLEAN_10140130                                | 2.31 profilin                                  | NP_001011626 | Apis mellifera |
| 10  | LMI_GLEAN_10061897                                | 2.11 actin-interacting protein 1-like isoform 1 | XP_001943831 | Acyrthosiphon pisum |
| 11  | LMI_GLEAN_10172557                                | 1.97 lambda-crystallin homolog                 | XP_001601340 | Nasonia vitripennis |
| 12  | LMI_GLEAN_10074080                                | 1.51 Troponin I                                | EFN61242     | Camponotus floridanus |
| 13  | LMI_GLEAN_10124366                                | 1.65 troponin t, invertebrate                  | XP_001655223 | Aedes aegypti |
| 14  | LMI_GLEAN_10123370                                | 4.61 electron transfer flavoprotein subunit alpha, mitochondrial-like | XP_003700429 | Megachile rotundata |
| 15  | LMI_GLEAN_10127658                                | 3.53 dehydrogenase/reductase SDR family member 11-like | XP_001947617 | Manduca sexta |
| 16  | LMI_GLEAN_10164303                                | 3.28 3-ketoacyl-CoA thiolase, mitochondrial-like | XP_003488365 | Bombyx mori |
| 17  | LMI_GLEAN_10124634                                | 2.8 V-type proton ATPase subunit B             | P31401       | Acyrthosiphon pisum |
| 18  | LMI_GLEAN_10106737                                | 2.26 probable isocitrate dehydrogenase [NAD] subunit beta, mitochondrial-like | XP_001607423 | Nasonia vitripennis |
| 19  | LMI_GLEAN_10137288                                | 1.71 H+ transporting ATP synthase subunit e    | ABF51335     | Bombus impatiens |
| 20  | LMI_GLEAN_10120585                                | 1.55 ATP synthase, subunit d                    | XP_002096273 | Bombus terrestris |
| 21  | LMI_GLEAN_10088301                                | 1.53 probable pyruvate dehydrogenase E1 component subunit alpha, mitochondrial-like isoform 1 | XP_003399781 | Drosophila yakuba |
| 22  | LMI_GLEAN_10187224                                | 2.02 wingless protein                          | EDS27053     | Culex quinquefasciatus |
| 23  | LMI_GLEAN_10002441                                | 2.02 nucleoplasmn isoform 1-like protein       | ABM55590     | Macanellisocus hirsutus |
| 24  | LMI_GLEAN_10160841                                | 1.93 Four and a half LIM domains protein 2     | EGI61543     | Acromyrmex echinatior |
| 25  | LMI_GLEAN_10099967                                | 1.77 3′-phosphoadenosine 5′-phosphosulfate synthase | XP_970563    | Tribolium castaneum |
| 26  | LMI_GLEAN_10172624                                | 1.75 Putative beta-carotene-binding protein    | P82886       | Drosophila willistoni |
| 27  | LMI_GLEAN_10131973                                | 1.68 COP9 signalosome complex subunit 7A       | EEB19483     | Pediculus humanus corporis |
| 28  | LMI_gi_1710156                                    | 1.66 juvenile hormone binding protein          | AAC47391     | Locusta migratoria |
| 29  | LMI_gi_85816368                                   | 6.21 heat shock protein 20.6                   | ABC84493     | Locusta migratoria |

Continued
| No. | ID number in the locust genome database (version 2.0) | G/S (protein level of gregaria over solitaria) | Protein name | Accession No. | Species | Function category |
|-----|------------------------------------------------------|-----------------------------------------------|--------------|---------------|---------|------------------|
| 30  | LMI_GLEAN_10065877                                   | 3.37                                          | heat shock protein 60 | ACO57619. | Pteromalus puparum |Digestion and absorption |
| 31  | LMI_GLEAN_10110738                                   | 1.54                                          | heat shock protein 60 | AEV89752  | Schistocerca gregaria | |
| 32  | LMI_GLEAN_10190806                                   | 3.43                                          | aspartate aminotransferase | EEB15916  | Pediculus humanus corporis | |
| 33  | LMI_GLEAN_10095175                                   | 1.80                                          | aspartate ammonia-lyase | EAT40064  | Aedes aegypti | |
| 34  | LMI_GLEAN_10116472                                   | 1.56                                          | alpha-amylase          | ABC68516  | Blattella germanica | |
| 35  | LMI_GLEAN_10043969                                   | 1.54                                          | aspartate aminotransferase | AEX97005  | Anopheles stephensi | |
| 36  | LMI_GLEAN_10078839                                   | 1.65                                          | chemoactive protein    | CAJ01464  | Locusta migratoria | Chemical sensing |
| 37  | LMI_gi_484000                                       | 4.23                                          | choline transporter-like protein 4 | NP_001086000 | Xenopus laevis | Transporter |
| 38  | LMI_GLEAN_10154078                                   | 9.96                                          | hexamerin-like protein 2 | ACU78069  | Locusta migratoria |
| 39  | LMI_GLEAN_10057778                                   | 8.50                                          | hypothetical protein TcasGA2_TC001323 | EEE98759  | Tribolium castaneum |
| 40  | LMI_GLEAN_10164915                                   | 5.57                                          | predicted protein       | EEE9602   | Mammal | |
| 41  | LMI_GLEAN_10175054                                   | 5.32                                          | similar to ribosomal protein S28e | CAJ01883  | Tribolium castaneum |
| 42  | LMI_GLEAN_10098678                                   | 5.30                                          | hypothetical protein    | XP_00242276 | Pediculus humanus corporis | |
| 43  | LMI_GLEAN_10126245                                   | 4.82                                          | arylphorin hexamerin-like protein 2 | AAX14951  | Romalea microptera |
| 44  | LMI_GLEAN_10154080                                   | 4.09                                          | hexamerin-like protein 2 | ACU78069  | Locusta migratoria |
| 45  | LMI_GLEAN_10025235                                   | 3.63                                          | peroxiredoxin-like protein | ABV44727  | Phlebotomus papatasi |
| 46  | LMI_GLEAN_10042129                                   | 3.50                                          | similar to conserved hypothetical protein | XP_970222 | Tribolium castaneum | |
| 47  | LMI_GLEAN_10102266                                   | 3.24                                          | AGAP0006260-PD          | EDO63843  | Anopheles gambiae str. PEST |
| 48  | LMI_GLEAN_10042900                                   | 2.80                                          | similar to AGAP005134-PA isoform 1 | EPA00742  | Tribolium castaneum |
| 49  | LMI_GLEAN_10080939                                   | 2.43                                          | GOK3357                | EDW79295  | Drosophila willistoni | Others |
| 50  | LMI_GLEAN_10061914                                   | 2.16                                          | 46 kDa FK506-binding nuclear protein, putative | EEB13519 | Pediculus humanus corporis |
| 51  | LMI_GLEAN_10097536                                   | 2.09                                          | acidic ribosomal protein | CAA72658  | Ceratitis capitata |
| 52  | LMI_GLEAN_10118445                                   | 2.08                                          | major allergen Bla g 1.02 | AAD13531  | Blattella germanica |
| 53  | LMI_GLEAN_10113524                                   | 2.06                                          | c-1-tetrahydrofolate synthase, cytoplasmic-like | XP_00370207 | Megachile rotundata |
| 54  | LMI_GLEAN_10147206                                   | 2.00                                          | hypothetical protein LOCI00160882 | XP_001951692 | Acerosiphon pisum |
| 55  | LMI_GLEAN_10095481                                   | 1.98                                          | myosin 1 light chain    | AAV91412  | Lononma obliqua |
| 56  | LMI_GLEAN_10124558                                   | 1.82                                          | GF22728                | EDV32005  | Drosophila ananassae |
| 57  | LMI_GLEAN_10130423                                   | 1.72                                          | maternal protein exaperantia-like | XP_003697468  | Apis florea |
| 58  | LMI_GLEAN_10154252                                   | 1.61                                          | transketolase-like protein 2-like isoform 1 | XP_003493512 | Bombus impatiens |
| 59  | LMI_GLEAN_10119040                                   | 1.57                                          | pentatricopeptide repeat-containing protein 2-like | XP_001946785 | Acerosiphon pisum |

Continued
| No. | ID number in the locust genome database (version 2.0) | G/S (protein level of gregaria over solitaria) | Protein name | Accession No. | Species | Function category |
|-----|-----------------------------------------------|-----------------------------------------------|--------------|---------------|---------|------------------|
| 60  | LMI_GLEAN_10073596                            | 1.56                                          | hypothetical protein | CAJ01469 | Locusta migratoria |                                |
| 61  | LMI_GLEAN_10181062                            | 1.55                                          | hypothetical protein LOC100169018 | XP_001946070 | Acyrthosiphon pisum |                                |
| 62  | LMI_GLEAN_10135691                            | 1.54                                          | putative leukotriene A4 hydrolase | EFX86132 | Daphnia pulex |                                |
| 63  | LMI_GLEAN_10071089                            | 1.51                                          | hexamerin 4 precursor | NP_001164245 | Tribolium castaneum |                                |
| 64  | LMI_GLEAN_10187958                            | 1.51                                          | conserved hypothetical protein | XP_002416013 | Ixodes scapularis |                                |
| 65  | LMI_GLEAN_10143558                            | 0.106                                         | hexamerin-like protein 1 | ACU78068 | Locusta migratoria |                                |
| 66  | LMI_GLEAN_10175495                            | 0.459                                         | ubiquitin carboxyl-terminal hydrolase isozyme L5 | XP_002431967 | Pediculus humanus corporis | Digestion and absorption |
| 67  | LMI_GLEAN_10051974                            | 0.468                                         | similar to putative carboxypeptidase A-like | EFA05749 | Tribolium castaneum |                                |
| 68  | LMI_GLEAN_10120545                            | 0.557                                         | serine protease-like protein | CAA70820 | Schistocerca gregaria |                                |
| 69  | LMI_GLEAN_10054296                            | 0.567                                         | beta-1,4-endoglucanase 1 | AAF80584 | Panesthia cribrota |                                |
| 70  | LMI_GLEAN_10109880                            | 0.617                                         | similar to cathepsin b | XP_974220 | Tribolium castaneum |                                |
| 71  | LMI_GLEAN_10192264                            | 0.653                                         | 1,4-alpha-glucan-branching enzyme-like | XP_00370245 | Megachile rotundata |                                |
| 72  | LMI_GLEAN_10099118                            | 0.513                                         | similar to pre-mRNA-splicing helicase BRR2 | XP_9705541 | Tribolium castaneum |                                |
| 73  | LMI_GLEAN_10170704                            | 0.588                                         | Splicing factor 3B subunit | EEB16979 | Pediculus humanus corporis |                                |
| 74  | LMI_GLEAN_10196136                            | 0.599                                         | ubiquitin-conjugating enzyme E2 variant 2-like isoform 1 | XP_003398336 | Apis mellifera | Regulation of gene expression |
| 75  | LMI_GLEAN_10002265                            | 0.660                                         | Proteasome subunit alpha type-4 | EFN87452 | Harpegnathos saltator |                                |
| 76  | LMI_GLEAN_10081266                            | 0.666                                         | Arginine/serine-rich-splicing factor RSP31 | EEB16084 | Pediculus humanus corporis |                                |
| 77  | LMI_GLEAN_10133889                            | 0.464                                         | takeout-like | BAH71589 | Acyrthosiphon pisum | Chemical sensing |
| 78  | LMI_gi_311063281-D1                            | 0.490                                         | protein takeout-like | XP_00194737 | Acyrthosiphon pisum |                                |
| 79  | LMI_GLEAN_10133888                            | 0.562                                         | protein takeout-like | XP_001950706 | Acyrthosiphon pisum |                                |
| 80  | LMI_GLEAN_10109545                            | 0.479                                         | NADPH--cytochrome P450, putative | EEB11242 | Pediculus humanus corporis | Immunity and defense |
| 81  | LMI_GLEAN_10117209                            | 0.593                                         | glutathione S-transferase sigma 1 | AEB91973 | Locusta migratoria |                                |
| 82  | LMI_GLEAN_10042954                            | 0.638                                         | similar to DNA-damage inducible protein | XP_969775 | Tribolium castaneum | Transporter |
| 83  | LMI_GLEAN_10138023                            | 0.663                                         | importin subunit beta-1-like isoform 1 | XP_001599381 | Nasonia vitripennis |                                |
| 84  | LMI_GLEAN_10082913                            | 0.663                                         | similar to vesicle docking protein P115 | EFA08682 | Tribolium castaneum |                                |
| 85  | LMI_GLEAN_10168539                            | 0.663                                         | Reticulon-1 | EFN73447 | Camponotus floridanus | Structure formation |
| 86  | LMI_gi_159434                                 | 0.370                                         | conserved hypothetical protein | EEB10389 | Pediculus humanus corporis | Others |
| 87  | LMI_GLEAN_10002280                            | 0.558                                         | 40S ribosomal protein S17 | EEB10115 | Pediculus humanus corporis |                                |
| 88  | LMI_GLEAN_10066930                            | 0.565                                         | similar to eukaryotic translation initiation factor 3 | EFA00209 | Tribolium castaneum |                                |

Continued
Discussion

The “O” cluster proteins are extremely abundant in the locust head. This phenomenon was also found in the antennae of *Battocera horsfieldi* based on cDNA library analysis. However, similar phenomenon did not exist in the whole insect bodies. It seems that “O” cluster proteins are mainly abundant in the head as compared with the other parts of insects. It suggests that the proteins related to post-translational modification, protein turnover and chaperone folding are highly involved in the regulation of head function in insects. Locust phase polyphenism is a typical phenomenon of epigenetics. The existence of high abundant “O” cluster proteins suggests that post-translational modification may play important roles in the locust phase transition.

The two locust phases differ in many aspects, especially in the body color and behavioral activity. The gregaria is darker and more active, while the solitaria is shallower and quieter. The proteomic analysis revealed that proteins related to structure formation, melanism and energy metabolism have significantly higher expression level in the gregaria. This is consistent with the facts that gregarious locusts have stronger muscles, darker color and more frequent activity. As compared with the gregaria, the solitaria owns more abundant proteins related to digestion, absorption and chemical sensing. It’s apparently that the former two characteristics provide the solitary locusts with higher abilities in digestion and absorption, and the latter one gives them stronger olfactory sensation. This makes them have an advantage over the gregarious locusts in feeding and mating, and then results in higher reproductive capacity.

In the present study, hexamerins and JHBP are abundant in the head of gregarious locust. Similar results have been revealed by EST library analysis in the same species. Both hexamerin and JHBP have been suggested to play a role as juvenile hormone (JH) transporters, and even as regulators of JH levels and action. This explains the involvement of hexamerins in JH-dependent differentiation of caste phenotype in some social insects, including termite *Reticulitermes flavipes*, honey bee *Apis mellifera* and wasp *Polistes metricus*. Besides caste-related polyphenism in social insects, JH was also reported to mediate plasticity of aggregation behavior in adult desert locusts. Surgical removal of the corpora allata to terminate JH secretion increased aggregation index and behavioral activity of adult locust. This effect was caused by repressing the responsiveness of olfactory interneurons in the antennal lobe to aggregation pheromone. Thus, hexamerins and JHBP can be involved in the phase plasticity of locust by mediating JH action.

Heat shock proteins (Hsps) are a kind of stress-induced proteins that can be synthesized rapidly in response to various environmental stress signals. Hsps usually function as molecular chaperones and participate in numerous cellular functions such as folding, assembly, intracellular localization, secretion, regulation and degradation of proteins. Gregarious locusts live at high population density. Population density can alter the expression of Hsps. For example, the mRNA levels of five Hsps (*Hsp20.5, Hsp20.6, Hsp20.7 and Hsp90*) are significantly higher in the gregarious locust head as compared with those in the solitaria. The mRNA levels were up-regulated by crowding of the solitary locusts (for 32 h), and down-regulated by isolation of the gregarious locusts. In the present study, *Hsp60* and *Hsp20.6* were identified to have higher protein levels in the gregarious locust head. The over-expression of Hsps in gregaria seems to be a direct response to high-population gather of locust. It’s hard to distinguish whether Hsps play a role to control the phase transition.

In the desert locusts, two phase populations display different sensitivity to aggregation pheromone. Chemosensory protein (CSP) and takeout are important proteins for olfactory sensing. RNA interference combined with olfactory behavioral experiments confirmed that six CSP genes and one takeout gene, *LmigTO1*, are responsible for the formation of gregarious and solitary behaviors, respectively. In our study, another CSP (CSP-7) and three new takeout proteins (TO 4 to 6) were identified from the head of *Locust migratoria* (SupplementaryFig. S2), and the CSPs revealed higher protein level in the gregaria, while the TOs showed higher protein levels in the solitaria. These protein expression patterns are consistent with the early report at mRNA levels, and further confirm that both CSP and takeout are involved in the phase plasticity of locust.
Figure 2. The mRNA expression profiles in the two-phase locusts. The mRNA expression profiles were examined by qRT-PCR in both the head and brain tissues. The mRNA levels were quantified by standard curves generated with serial (10 ×) dilutions of plasmid DNAs. The relative expression level of each target gene was normalized against a house-keeping gene (RP49). Differences between treatments were compared by Student’s t-test, and two levels (P < 0.05 or 0.01) were adopted to judge the significance of difference. Abbreviations: “G”, gregaria; “S”, solitaria. The abbreviation for gene names can refer to Table 3.
The CSN, an eight protein complex (CSN1-8)\(^{48}\) was originally discovered as an essential regulator in light-induced development in *Arabidopsis thaliana*\(^{49}\). In *Drosophila melanogaster*, it also plays an essential role for development. Disruption of one of the subunits caused lethality at the late larval or pupal stages\(^ {50}\). This role of CSN is partly due to its regulation on Hedgehog signaling by mediating proteolysis of some transcription factors\(^ {51}\). In the same species, CSN was also reported to be involved in circadian rhythms by controlling the degradation of two clock proteins\(^ {52}\). Interestingly, our study showed that CSN7A played a role in the phase transition from gregaria to solitaria in the migratory locusts. RNAi of CSN7A triggered the phase shift from gregaria to solitaria within 24 h (Fig. 4). Isolation (gregaria to solitaria) and crowding (solitaria to gregaria) may have different regulation mechanisms. The former takes place within 4 h in the migratory locusts, whereas the latter cannot finish until 32 h\(^ {3}\). In the present study, the mRNA amount of CSN7A in gregaria decreased during the isolation, however, the mRNA level remained constant during the crowding of solitaria (Fig. 3). It suggests that CSN7A may be only involved in one direction transition from gregaria to solitaria rather than in its reverse process.

It is the first time to disclose the role of CSN in behavior plasticity of animals. CSN has been reported to be involved in neural development, and regulates dendritic morphogenesis in *Drosophila* brain through Cullin-mediated protein degradation\(^ {53}\). More and more evidences revealed that CSN plays an important role in protein degradation through Cullin-ubiquitin-proteasome pathway\(^ {54-56}\). Therefore, CSN might be involved in the phase transition of locust by mediating ubiquitin-dependent proteolysis. Further studies need to be carried out to explore the detailed mechanism of CSN in the regulation of phase transition.

In conclusion, a total of 1,387 proteins were identified in the locust head in the present study, and a large proportion of proteins are involved in post-translational modification, especially in protein folding, phosphorylation and ubiquitylation. Ninety proteins were identified to differentially express between two phases in the head of the migratory locust. Gregaria reveals higher expression in proteins related to structure formation, melanism and energy metabolism, whereas solitaria owns more abundant proteins related to digestion, absorption and chemical sensing. This is consistent with their differentiation in morphology and physiology. JHBP, hexamerin, Hsp, CSP and takeout are suggested to play a role in behavior formation according to their differential expression profiles between two phases. The most interestingly, RNAi of CSN7A in gregaria made the behavior shift towards solitaria within 24 h. It is the first time to disclose the role of CSN in behavior plasticity of animals. These results provide important information for further exploration of the complex mechanism of locust phase transition, as well as for the study of behavior plasticity of animals.

**Methods**

**Animals.** The gregarious and solitary populations of the migratory locust are long-term maintained in our laboratory as the early reported method\(^ {5}\). Briefly, gregarious nymphs were cultured in large boxes (40 × 40 × 40 cm\(^3\)) at a density of 500–1000 insects per container. Solitary nymphs were obtained from the gregarious colony and cultured alone in white metal boxes (10 × 10 × 25 cm\(^3\)) supplied with charcoal-filtered compressed air. The gregarious and solitary colonies were maintained under a 14 h light/10 h dark cycle at 30 ± 2 °C and fed on fresh wheat seedlings and bran.
Sample preparation and iTRAQ labeling. When the locusts developed into the second day of 4th instar, the heads of 3 to 5 gregarious or solitary nymphs were collected and thoroughly homogenized in 500 μL cold PBS buffer including 1 mM PMSF, 2 mM EDTA and 10 mM DTT. The samples were centrifuged for 20 min at 25,000 × g, and the supernatant was collected. A total of 100 μg of protein per sample was reduced, alkylated, and then digested by adding 2 μg trypsin (1 μg/μL) at 37 °C overnight. The digested samples were lyophilized and re-suspended in 100 μL of 0.5 M TEAB (triethylammonium bicarbonate). The method of isobaric tags for relative and absolute quantitation (iTRAQ) was adopted for sample labelling according to the protocol of iTRAQ® Reagents—4plex Applications Kit (AB Sciex Pte. Ltd., Foster City, USA). Each sample was labeled with an isobaric tag. The iTRAQ-labeled peptide mixtures were pre-separated by strong cation exchange (SCX) column. For SCX chromatography, the LC-20AB HPLC Pump system (Shimadzu Corporation, Chiyoda-ku, Tokyo, Japan) was used, the peptide sample was reconstituted with 4 mL buffer A (25 mM NaH₂PO₄ in 25% ACN, pH 2.7) and then loaded onto a 4.6 × 250 mm Ultremex SCX column containing 5-μm particles (Phenomenex, Torrance, CA, USA). The peptides was eluted at a flow rate of 1 mL/min with a gradient of buffer A for 10 min, 5–35% buffer B (25 mM NaH₂PO₄, 1M KCl in 25% ACN, pH 2.7) for 11 min, 35–80% buffer B for 1 min. The system was then maintained in 80% buffer B for 3 min before equilibrating with buffer A for 10 min prior to the next injection. Elution was monitored by measuring absorbance at 214 nm, and fractions were collected every 1 min. The eluted peptides were pooled as 12 fractions, desalted by Strata X C18 column (Phenomenex, Torrance, CA, USA) and vacuum-dried. Each fraction was resuspended in certain volume of buffer A (2% ACN, 0.1% FA).

LC-MS/MS Analysis. A total of 5 μg of the above solution was loaded on a LC-20AD nanoHPLC (Shimadzu Corporation, Chiyoda-ku, Tokyo, Japan) equipped with a 2 cm C18 trap column, and the peptides were then eluted onto a resolving 10 cm analytical C18 column. The MS data acquisition was performed with Triple TOF 5600 System (AB SCIEX, Concord, ON) fitted with a Nanospray III source (AB SCIEX, Concord, ON) and a pulled quartz tip as the emitter (New Objectives, Woburn, MA). Data was acquired using an ion spray voltage of 2.5 kV, curtain gas of 30 PSI, nebulizer gas of 15 PSI, and an interface heater temperature of 150°C. The MS was operated with a resolving power of greater than...
or equal to 30,000 FWHM (full width at half maximum). The MS/MS data collection and processing was done on Analyst® software (version 1.6, AB SCIEX, Concord, ON) with the method of Information Dependent Acquisition (IDA) according to the manual.

**Database searching for protein identification.** The resulting MS/MS spectra were searched against the locust protein database generated from the newly assembled genome with MASCOT software (Matrix Science, London, UK; version 2.3.02). The carbamidomethylation of cysteine was considered a fixed modification, and the conversion of N-terminal glutamine to pyroglutamic acid and methionine oxidation were considered variable modifications. The minimal peptide length was seven amino acids, and a single missed cleavage maximum was used. A peptide mass tolerance of 10 ppm was allowed for intact peptide masses and 0.05 Da for fragmented ions. A stringent 0.01 false discovery rate (FDR) threshold was used to filter the candidate peptide and protein. Two thresholds were set up to filter the candidate proteins whose abundances were significantly different from others: <0.05 for a two-tailed P-value test and >1.5 (or <1/1.5) for the fold-change. For gene ontology (GO, http://www.geneontology.org/) mapping, BLAST2GO software (version 2.5.0, http://www.blast2go.org) was employed to deal with the BLASTx results and then to perform the functional annotation by GO vocabularies, enzyme classification codes, KEGG metabolism pathways. The default settings of BLAST2GO were used in every annotation step.

**Quantitative real-time PCR (qRT-PCR).** Total RNAs were extracted from the whole head and dissected brain tissues, respectively using an RNAeasy mini kit (QIAGEN, Hilden, Germany). Three heads or eight brains were used for each RNA isolation, and five biological repeats were performed during sampling. PCR reactions were performed in a 20 μL volume and the final concentration of primers was 250 nM. PCR amplification was conducted on a Roche Light Cycler® 480 system (Roche Applied Science, Fenzberg, Germany) using SYBR green master mix (Roche Diagnostics Ltd. Shanghai, China). The PCR was initiated with a 10-min incubation at 95 °C, followed by 45 cycles of 10 s at 95 °C, 20 s at 58 °C and 20 s at 72 °C. Five biological replicates were performed for each sample. The standard curves for target genes and reference genes (ribosomal protein 49, RP49) were generated with serial (10×) dilutions of plasmid DNAs. Efficiency of qRT-PCR and correlation coefficients were determined for the primers of each gene. The relative expression level of each target gene was normalized against the housekeeping gene RP49. The specificity of amplification was ensured by both melting curve analysis and sequencing of PCR product. The primers for qRT-PCR were listed in Table 3.

**RNAi.** Double-strand RNA (dsRNA) of the target gene and a negative control gene (green fluorescent protein, gfp) were prepared using the T7 Ribomax Express RNAi system (Promega, Madison, USA) according to the manufacturer’s instruction. The primers for dsRNA preparation were listed in Table 3. A total of 35 ng dsRNA was injected directly into eight brains of the 4th instar nymphs using Nanoject II nanoliter injector (Warner Instruments, Hamden, CT, USA). Twenty four hours later, the effects of RNAi on mRNA level were detected by qRT-PCR and behavioral assay. Four biological repeats were performed for qRT-PCR. Four biological repeats were performed for qRT-PCR. For behavioral assay, the same injection was carried out in gregaria, and 30 and 36 individuals were used for dsGFP and dsCSN7A, respectively.

**Phase Transition.** To make gregarious behavior change towards solitaria, the 4th instar gregarious nymphs were individually reared at the same condition as solitary ones. After 2, 4, 8, 16 and 32 h of isolation, the brains were dissected and immediately placed in RNAlater Solution (Ambion, Austin, USA) for qRT-PCR analysis. The gregarious nymphs maintained in normal situation (high population density) were used as controls. To avoid the influences of circadian rhythm and sexual difference, all samples were collected at the same time point of a day with a sex ratio of 1:1. Each treatment included five biological replicates. To make a reverse phase transition (solitaria towards gregaria), ten solitary nymphs were marked and moved into an optic perplex-made box (10 × 10 × 10 cm³), and 20 gregarious individuals were then added to maintain high population density. The sampling, mRNA level detecting and other methods were as same as the isolation of gregaria.

**Behavioral assay.** The behavioral assay was performed in a rectangular arena (40 × 30 × 10 cm³). The wall of the arena is opaque plastic and the top is clear. One of the separated chambers (7.5 × 30 × 10 cm³) contained 20 4th instar gregarious locusts as the stimulus group, and the other end of the chamber with the same dimensions was kept empty. Both ends of the chamber were illuminated equally to prevent the formation of mirror images. The floor of the open arena was covered with filter paper during the behavioral assay. The locust nymphs were gently transferred by a tunnel to the arena. Each individual was recorded for 6 min using EthoVision system (Noldus Inc. Wageningen, the Netherlands). Eleven behavioral parameters (such as attraction index, total distance moved, total duration of movement, etc.) were collected to calculate the possibility of gregaria (Pgreg), which was used for criterion of phase type. Detailed information can refer to the early reported methods. Differences between mRNA levels were compared by Student’s t-test. The relative mRNA levels were presented as mean ± SEM (standard error of the mean). Behavioral data
Gene name | ID number in the locust genome database (version 2.0) | Primer sequence (5′→3′) | Product length (bp)
---|---|---|---
**qRT-PCR**
CSN7A | LMI_GLEAN_10131973 | AGAATCGTGGGCTGAAACATAA | 184
JHBP | LMI_gi_1710156 | AAAGTATTCTGACAGGCAAC | 148
PAPSS | LMI_GLEAN_10099907 | CATCACAAGAGGACACCTTTACA | 135
CTLA4 | LMI_gi_484000 | CTCACAACACAGCTCCAGCAG | 118
Hexa2 | LMI_GLEAN_10154078 | CAAAGGCCTGCACACCTTC | 151
Hexa2* | LMI_GLEAN_10154080 | AGAGGAGATCAGGGACGC | 294
actinA3a | LMI_GLEAN_10056004 | TGAGCGATTCAGGTGCCC | 283
ETFA | LMI_GLEAN_10123370 | ACCTATAACGCAAAATGCCAA | 166
Arylphorin | LMI_GLEAN_10126245 | ACCCCTGTGCGTGCTGAAG | 257
takeout-like | LMI_GLEAN_10133888 | ACTCCGCCAAGACGAAATACA | 166
V-ATPase | LMI_GLEAN_10124634 | TTGCCATCACTCAGTCGTCTCA | 115
ATPsyn-d | LMI_GLEAN_10120585 | AGAAAATCCGCCAATAGGAA | 253
RSP31 | LMI_GLEAN_10081266 | TGCCAGGCTTCAAGTTGAGG | 141
P450 | LMI_GLEAN_10109545 | TGACGAGCCTAAAAAGCATCC | 159
rp49 | LMI_GLEAN_10126536 | CGTAAACCGAAGGGAATTGA | 209

**RNAi**
CSN7A | LMI_GLEAN_10131973 | GCACCCCTACTACCGTTAATGA | 316
GFP | LMI_GLEAN_10131973 | CAGCGGTTGATGTTCT | 420

**Table 3. Primers used for qRT-PCR and RNAi.** Full names: CSN7A, COP9 signalosome complex subunit 7A (GenBank accession NO. KM396884); JHBP, juvenile hormone binding protein; PAPSS, 3′-phosphoadenosine 5′-phosphosulfate synthase; CTLA4, choline transporter-like protein 4; Hexa2, hexamerin-like protein 2; actinA3a, cytoplasmic A3a actin; ETFA, mitochondrial-like electron transfer flavoprotein subunit alpha; Arylphorin, arylphorin hexamerin-like protein 2; V-ATPase, V-ATPase subunit B; ATPsyn-d, ATP synthase, subunit d; RSP31, Arginine/serine-rich-splicing factor RSP31; P450, NADPH—cytochrome P450; rp49, ribosomal protein 49; GFP, green fluorescent protein (cloned from pEGFP-N1 plasmid vector, Clontech, Mountain View, CA, USA; GenBank accession NO. U557622)

were analyzed by the Mann-Whitney U test. Two levels of significance (P < 0.05 or 0.01) were adopted to judge the significance of difference. All the statistics was analyzed using SPSS 15.0 (SPSS Inc., Chicago, USA).
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Author Contributions
L.K., L.H.H. and B.C. designed the study. X.W.T performed the experiments. B.C. and L.H.H. analyzed the data. L.K. and Q.L.F. reviewed the paper. L.H.H. wrote the manuscript.

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