Adaptive evolution of circadian gene *timeout* in insects

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Supplement materials

Sample preparation and experimental design

Field experiments to collect samples, the pollinator wasp species *Ceratosolensolmsi*from *Ficushispida* were demonstrated in Danzhou (19°52' N, 19°52' E), Hainan province, China from July to August 2012. Naturally growing figs were bagged with mesh bags from their very early developmental stages to protect from the interference of any insects including fig wasps. A mated female individual ('mother' wasp) was inoculated into each bagged fig for her to complete the process of oviposition. The developmental process of the offspring lastedfor about 28 days. All the fig fruits were then collected at the same developmental stage several days before ripe and kept in dark condition. Two days later, figs were flash-frozen in liquid nitrogen every 3 hours (3:00, 6:00, 9:00, 12:00, 15:00, 18:00, 21:00, 24:00). Subsequently, we removed female and male pollinators from the syconia and immersed them into Sample Protector (TAKARA, China). In addition, to evaluate the effect of light stimulation on *timeout* expression, we performed another light treatment on adult female pollinators: females that had emerged from the syconia and exposed to natural light for 3 additional hours before flash-freezing. In total, 24 groups of fig wasps were collected.

RNA isolation and cDNA synthesis

For the 24 sampling regimes, total RNA of wasp samples were extracted using EasyPure^{TM} RNA kit (TransGen Biotech, Beijing, China) and dissolved in RNase free water. Because fig pollinators are very small, we used 40 whole body individual wasps for each RNA sample. Genomic DNA was removed by treating with DNase I according to the manufacturer's instruction. A NanoDrop 2000 Spectrophotometer (Thermo, USA) was used to measure RNA purity and concentration. In total, 72 RNA samples (3 biological replicates for each group) with values of A260/A280 between 1.9 and 2.2 and an A260/A230 ratio of more than 2.0 were selected for

further experiments. The integrity of the RNA samples was evaluated by electrophoresis on 1.0% agarose gels stained with ethidiumbromide. Single strand cDNA was synthesized from 1μg total RNA with oligodT per 20μl reaction using TransScript II First Strand cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China).

RT-qPCR expression analysis

Previously, we amplified 9 housekeeping genes from *C.solmsi* that were frequently used as reference genes for qPCR studies in insects and systematically evaluated their expression stability. *RPL13a* and *UBC* were selected as the best reference genes for normalizing the RT-qPCR data[1]. Based on the coding sequences of *timeout* gene, one gene-specific primer pair was designed using Primer Premier 5.0[2](Table S4). Amplification efficiencies and R^2 coefficients of the primer was determined with the slopes of the standard curves generated from plasmid standards. Product obtained via gene-specific primer was cloned with the PEASY-T3 cloning kit (TransGenBiotech, Beijing, China). Clones with appropriate insert size were verified by PCR and sequencing. Plasmids were prepared with EasyPure Plasmid MiniPrep Kit (TransGenBiotech, Beijing, China). We determined the amount of plasmids using a NanoDrop-2000 Spectrophotometer and calculated the copy numbers of the plasmids. Ten-fold serial dilutions to 10^8, 10^7, 10^6, 10^5, and 10^4 copies per 20μl RT-qPCR reaction were made for each plasmid with two technical replicates to generate standard curves. The formula E=10 (-1/slope) was used to calculate amplification efficiencies (Es), which reflected the efficacy of primer pair.

The Stratagene Mx3000p qPCR system (Stratagene, La Jolla, CA) was used to carry out RT-qPCR experiments. A 20 μl PCR mixture containing 1μl of template, 10 μlTransStart Green qPCRSuperMix UDG(2x) (TransGenBiotech, Beijing, China), 0.4 μl Passive Reference Dye II (50x) (TransGenBiotech, Beijing, China), 0.8 μl primer mix (0.2μM), and 7.8 μl sterile water was prepared. A template for no RT-control was prepared for each sample. All cDNA templates were stored at -20 ℃. No-RT controls for all 72 samples were performed to check for gDNA contamination, and a no-template control was also conducted for each run to preclude reagent contamination. Melting curves were constructed for all runs to confirm amplification specificity. RT-qPCR reactions of all

genes for each sample were duplicated (technical replicates) to account for variation between runs. The same suite of thermal conditions was used for all RT-qPCR reactions: 50 ℃ for 2 min, 95 ℃ for 10 min, and then the follwing: 95 ℃ for 10 s, 54 ℃ for 15 s and 72 ℃ for 15 s for 40 cycles.

Several studies indicated that the mean of individual PCR efficiency (Em) gave more reliable results than a standard curve-derived efficiency[3, 4, 5]. Thus, we determined the baseline and calculated an Em of individual reactions for primer pair from the raw RT-qPCR data using LinRegPCR[6,7]. Subsequently, Cq and Em values were used to calculate the relative expression of *timeout* gene with respect to the reference genes *RPL13a* and *UBC* according to the following equation:

$$\frac{R_i}{R_{ref}} = \frac{(1/(1+E_{m\_i})^{Cq\_i})}{\sqrt{(1/(1+E_{m\_RPL13a})^{Cq\_RPL13a})^2 + (1/(1+E_{m\_UBC})^{Cq\_UBC})^2}}$$

R_i/R_{ref} was the expression of each circadian gene normalized to the reference genes *RPL13a* and *UBC*.

Data analysis

Statistical analysis was performed using SPSS v19 (SPSS Inc., Chicago, Ill). ANOVA analysis were used to determine the rhythmic expression.

MIQE guidelines

Herein, we followed the Minimum Information for Publication of Quantitative Real-Time PCRExperiments (MIQE) guidelines[8] to increase the reliability and the integrity of the results and to promote the effort for experimental consistency and transparency between research laboratories. A MIQE checklist was provided in Table S5.

1 Wang, B., Xiao, J. H., Bian, S. N., Gu, H. F. & Huang, D. W. Adaptive evolution of vertebrate-type cryptochrome in the ancestors of Hymenoptera. *Biol. Lett.***9**, 20120958 (2013).

2 Lalitha, S. Primer premier 5. *Biotech Software & Internet Report***1**, 270-272 (2000).

3 Karlen, Y., McNair, A., Perseguers, S. b., Mazza, C. & Mermod, N. Statistical significance of

quantitative PCR. *BMC bioinformatics***8**, 131 (2007).

4 Schefe, J. H., Lehmann, K. E., Buschmann, I. R., Unger, T. & Funke-Kaiser, H. Quantitative real-time RT-PCR data analysis: current concepts and the novel "gene expression's CT difference" formula. *J. Mol. Med.***84**, 901-910 (2006).

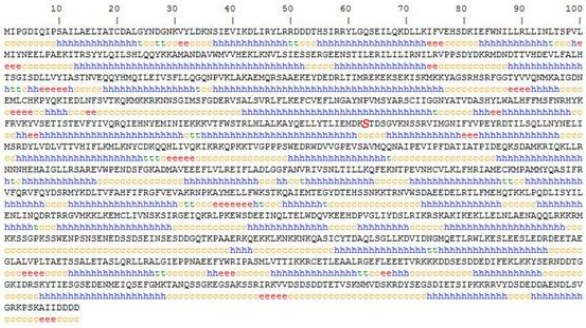
5 Peirson, S. N., Butler, J. N. & Foster, R. G. Experimental validation of novel and conventional approaches to quantitative real-time PCR data analysis. *Nucleic Acids Res.***31**, e73-e73 (2003).

6 Ruijter, J. *et al.* Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Res.***37**, e45-e45 (2009).

7 Ramakers, C., Ruijter, J. M., Deprez, R. H. L. & Moorman, A. F. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci. Lett.***339**, 62-66 (2003).

8 Bustin, S. A. *et al.* The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.***55**, 611-622 (2009).

Fig.S1. The predicted secondary structure of fig wasp TIMEOUT and the mapped positive selection site



Notes: Alpha helix (h), Extended strand (e), Beta turn (t), Random coil (c). The red bold ltalic "*S*" in the Fig.S1 denotes the positive selection sites of fig wasp

Table S1. The exon and intron length of timeout gene in 25 Arthropod species

Species name	Exon-length (bp)	Intron-length (bp)
D.pulex	3291	2187
T.urticae	3162	0
P.humanus	2559	830
A.pisum	3005	6354
T.castaneum	3759	28630
N.longicornis	3345	87177
N.vitripennis	4119	86689
*N.girault*i	3351	87570
A.mellifera	3966	175070
A.florea	3933	184862
C.solmsi	3642	243520
B.terrestris	3951	245598
M.rotundata	3951	308983
S.invicta	3660	116936
A.cephalotes	3507	106542
H.saltator	2949	106179
P.barbatus	3846	97847
A.echinatior	3717	97239
C.floridanus	3780	93141
L.humile	3987	91634
D.melanogaster	4152	70886
A.gambiae	3462	962
B.mori	3756	29258
H.melpomene	4047	15419
D.plexippus	3756	9423

Table S2. The positive sites were detected by both site-model and branch-site model

Models	P	LnL	Parameters	Positive selected sites
timeout:				
One-ratio	49	-22027.490829	ω0=0.08332	None

M1a	50	-21904.067140	p0=0.89689,p1=0.10311, ω0= 0.07136, ω1= 1.00000	N/A
M2a	52	-21904.067140	p0= 0.89689,p1= 0.06118, p2= 0.04193, ω0= 0.07136, ω1= 1.00000, ω2= 1.00000	1227 R 0.992** 1228 K 0.896 1233 H 0.996** 1235 D 0.546 1237 D 0.673 1256 D 0.519
M7	50	-21589.576840	p= 1.09855 q= 9.82809	N/A
M8	48	-21579.792484	p0=0.97883, p=1.20379, q=12.41352, p1= 0.02117, ω=1.10056	242 V 0.901
Branch-site a (Hymenoptera)	52	-21876.996311	site class 0 1 2a 2b proportion 0.78777 0.08997 0.10972 0.01253 background ω 0.07067 1.00000 0.07067 1.00000 foreground ω 0.07067 1.00000 263.97933 263.97933	24 L 0.998** 25 N 0.982* 49 L 0.997** 54 S 0.998** 70 M 0.943 87 K 0.984* 91 Q 0.996** 121 I 0.970* 217 K 0.993** 223 C 0.998** 236 H 0.969* 273 D 0.990* 288 K 0.978* 331 K 0.982* 363 F 0.986* 374 L 0.972* 390 K 0.993** 412 G 0.951* 414 I 0.946 437 N 0.916 443 Y 0.914
Branch-site null a (Hymenoptera)	51	-21887.791679	site class 0 1 2a 2b proportion 0.62728 0.21732 0.11541 0.03998 backgroundω 0.06873 1.00000 0.06873 1.00000 foregroundω 0.06873 1.00000 1.000001.00000	N/A
Branch-site b (wasp)	52	-21899.158781	site class 0 1 2a 2b proportion 0.86444 0.10162 0.03037 0.00357 backgroundω 0.07049 1.00000 0.07049 1.00000 foregroundω 0.07049 1.00000 5.53976 5.53976	222 I 0.905 283 I 0.946 417 K 0.937
Branch-site null b	51	-21904.067136	site class 0 1 2a 2b	N/A

(wasp)			proportion	0.86444	0.10162	0.03037	0.00357	
			backgroundω	0.07049	1.00000	0.07049	1.00000	
			foregroundω	0.07049	1.00000	5.53976	5.53976	
Branch-site c	52	-21898.712653	site class	0	1	2a	2b	45 S 0.970*
(C.solmsi)			proportion	0.88273	0.10062	0.01494	0.00170	
			backgroundω	0.07007	1.00000	0.07007	1.00000	
			foregroundω	0.07007	1.00000	7.03663	7.03663	
Branch-site c null	51	-21900.659340	site class	0	1	2a	2b	N/A
(C.solmsi)			proportion	0.85725	0.09795	0.04021	0.00459	
			backgroundω	0.06933	1.00000	0.06933	1.00000	
			foregroundω	0.06933	1.00000	1.000001	.00000	
Branch-site d	52	-21897.331343	site class	0	1	2a	2b	101 E 0.941
(bee)			proportion	0.83796	0.09386	0.06131	0.00687	133 N 0.984*
			backgroundω	0.06874	1.00000	0.06874	1.00000	
			foregroundω	0.06874	1.00000	3.20212	3.20212	
Branch-site null d	51	-21898.590073	site class	0	1	2a	2b	N/A
(bee)			proportion	0.79208	0.09020	0.10569	0.01204	
			backgroundω	0.06855	1.00000	0.06855	1.00000	
			foregroundω	0.06855	1.00000	1.000001	.00000	
Branch-site e	52	-21902.685822	site class	0	1	2a	2b	126 K 0.957*
(ant)			proportion	0.89282	0.10291	0.00384	0.00044	
			backgroundω	0.07092	1.00000	0.07092	1.00000	
			foregroundω	0.07092	1.00000	14.25637	14.25637	
Branch-site e null	51	-21903.380044	site class	0	1	2a	2b	N/A
(ant)			proportion	0.87226	0.10037	0.02454	0.00282	
			backgroundω	0.07068	1.00000	0.07068	1.00000	
			foregroundω	0.07068	1.00000	1.000001	.00000	
Branch-site f	52	-21886.423459	site class	0	1	2a	2b	10 N 0.993**
(T.castaneum)			proportion	0.80201	0.08719	0.09994	0.01086	111 V 0.963*
			backgroundω	0.07156	1.00000	0.07156	1.00000	163 S 0.958*
			foregroundω	0.07156	1.00000	17.82236	17.82236	226 L 0.950*
								255 P 0.961*
								276 S 0.912
								303 L 0.985*
								306 W 0.943
								363 F 0.929
								392 N 0.970*
								416 N 0.968*
								426 S 0.965*
Branch-site null f	51	-21891.193737	site class	0	1	2a	2b	N/A
(T.castaneum)			proportion	0.79938	0.08740	0.10206	0.01116	

			backgroundω	0.06994	1.00000	0.06994	1.00000		
			foregroundω	0.06994	1.00000	1.000001.00000			
Branch-site g	52	-21900.354815	site class	0	1	2a	2b		
(Hemiptera)			proportion	0.83325	0.09525	0.06417	0.00733	132 Q 0.940	
			backgroundω	0.07184	1.00000	0.07184	1.00000	133 N 0.907	
			foregroundω	0.07184	1.00000	999.00000	999.00000	354 S 0.950	
								356 P 0.948	
								406 G 0.980*	
								410 D 0.900	
								439 D 0.906	
Branch-site null g	51	-21903.388804	site class	0	1	2a	2b	N/A	
(Hemiptera)			proportion	0.83477	0.09589	0.06220	0.00714		
			backgroundω	0.07110	1.00000	0.07110	1.00000		
			foregroundω	0.07110	1.00000	1.000001.00000			
Branch-site h	52	-21883.720514	site class	0	1	2a	2b	15 S 0.943	
(Lepidoptera)			proportion	0.79577	0.08573	0.10698	0.01152	87 K 0.961*	
			backgroundω	0.07146	1.00000	0.07146	1.00000	138 I 0.947	
			foregroundω	0.07146	1.00000	16.62261	16.62261	151 E 0.928	
								156 A 0.997**	
								188 C 0.991**	
								196 I 0.960*	
								204 K 0.937	
								205 E 0.964*	
								215 V 0.996**	
								254 M 0.940	
								366 D 0.948	
								373 S 0.959*	
								414 I 0.996**	
Branch-site null h	51	-21891.093808	site class	0	1	2a	2b	N/A	
(Lepidoptera)			proportion	0.78362	0.08589	0.11760	0.01289		
			backgroundω	0.06958	1.00000	0.06958	1.00000		
			foregroundω	0.06958	1.00000	1.000001.00000			
Branch-site i	52	-21888.373565	site class	0	1	2a	2b	55 R 0.911	
(Diptera)			proportion	0.75397	0.08554	0.14414	0.01635	111 V 0.965*	
			backgroundω	0.07165	1.00000	0.07165	1.00000	137 D 0.937	
			foregroundω	0.07165	1.00000	60.75713	60.75713	138 I 0.975*	
								156 A 0.998**	
								193 N 0.967*	
								230 Y 0.988*	
								238 N 0.997**	
								239 H 0.966*	

									240 C 0.998**
									279 N 0.949
									306 W 0.942
									327 K 0.915
									395 D 0.953*
									406 G 0.997**
Branch-site null i	51	-21894.675893	site class	0	1	2a	2b	N/A	
(Diptera)			proportion	0.76123	0.08697	0.13624	0.01557		
			backgroundω	0.07012	1.00000	0.07012	1.00000		
			foregroundω	0.07012	1.00000	1.000001.00000			
Timeless full:									
one-ratio	15	-9918.024838	ω =0.00925					N/A	

Table S3. Accession numbers of genes in this study

Gene	Species	Accession number
timeout	*Daphnia pulex*	EFX80319
	Apis flora	GL576581:3114281-3114507,3114593-3114697,3114842-3114991,3115222-3115317,3115459-3115473,3116217-3116423,3118873-3119007,3119909-3120008, 3127972–3128069,3128777–3129014,3129212–3129410,3129478–3129568,3129671–3129754,3284494-3284591,3290746-3290826,3290914-3291073,3291141-3291274,3292294-3292448,3292528-3292660,3293076-3293338,3294327-3294444,3295396-3295559,3295702-3295797,3295878-3296009,3296088-3296219,3296347-3296504,3296629-3296715,3303068-3303074
	Apismellifera	XM_003250605
	Bombusterrestris	XM_003394988
	Megachilerotundata	XM_003707786
	Acromyrmexechinatior	GL888331.1:409386-409490,411219-411388,412522-412623,412683-412967,413507-413731,415827-415961,417164-417263,419508-419608,419891-420128,420447-420645,421071-421167,97520-497617,503636-503716,503829-503979,504072-504205,505221-505375,505450-505582,506232-506494,506766-506883,508941-509104,509279-509374,509464-509595,509663-509843,510011-510164,510247-510346.
	Atta cephalotes	GL377341.1:1081473-1081572,1081654-1081807,1081981-1082140,1082215-1082346,1082436-1082531,1082692-1082855,1085548-1085665,1085931-1086193,1087724-1087856,1087931-1088085,1089191-1089324,1089414-1089561,1089685-1089765,1096032-1096129,1180152-1180242,1180667-1180865,1181206-1181443,1181729-1181829,1186005-1186104,1187312-1187446,1189569-1189793,1190336-1190620,1191329 -1191525.
	Harpegnathossaltator	GL450313.1:612-729,919-1181,1726-1858,1939-2093,3217-3350,3478-3628,3792-3872,9306-9403,98878-98977,99639-99837,100227-100464,100799-100899,103459-103558,104810-104944,107236-107460,108193-108477,108565-108678,109326-109421,109516-109

	739.
Linepithemahumile	GL905240:2802119-2802333,2802423-2802518,2802755-2802879,2803183-2803291,2803360-2803644,2804233-2804454,2806458-2806592,2807799-2807898,2810281-2810381,2810678-2810915,2811279-2811477,2811772-2811865,2886173-2886270,2891758-2891838,2891950-2892106,2892188-2892321,2893206-2893360,2893436-2893568,2894276-2894538,2894660-2894777,2896439 -2896602,2896734-2896829,2896912-2897043,2897112-2897554,2897650-2897743.
Camponotusfloridanus	GL437108.1:885907-886000,886088-886241,886336-886516,886820-886951,887047 -887142,887285-887448,889294-889411,889573-889835,890490-890622,890702-890856,891751-891884,891969-892122,892300-892380,897938-898035,972789-972864,973212-973410,973751-973988,974295-974395,976738-976837,978080-978214,980344-980571,981176-981460.982085-982225,982422-982514,982600-982826.
Pogonomyrmexbarbatus	GL738581.1:401072-401165,401267-401420,401932-402112,402178-402309,402587 – 402682,402825-402988,404561-404678,404851-405113,405741-405873,405955-406109,407134-407267,407346-407499,407582-407662,413539-413636,492725-492821,493149-493347,493706-493943,494291-494391,496756-496855,498132-498266,500415-500654,501235-501519,501592-501702,502023-502184,502543-502763.
Solenopsisinvicta	GL771866.1:3436772-3436865,3436949-3437102,3437802-3437982,3438049-3438180,3438948-3439043,3439214-3439377,3445627-3445744,3445885-3446147,3446819-3446951,3447023-3447177,3448142-3448275,3448365-3448515,3448630-3448710,3454362-3454459,3544716-3544803,3545118-3545316,3547010-3547247,3547586-3547686,3551260-3551359,3552569 -3552703,3555133-3555363,3555911-3556195,3556267-3556368,3557141-3557367
Nasoniavitripennis	XM_001604940
Nasoniagiraulti	GL27420.1:2574577-2574691,2574785-2575119,2575191-2575322,2575404-2575499,2575577-2575740,2577243-2577360,2578450-2578709,2579136-2579268,2579343-2579497,2580064-2580182,2580781-2580949,2582037-2582117,2592845-2592942,2659148-2659238,2659307-2659505,2659824 -2660058,2660474-2660574,2663605-2663806,2664005-2664391,2665336-2665496.
Nasonialongicornis	GL277971.1:2573864-2573978,2574072-2574406,2574478-2574609,2574691-2574786,2574864-2575027,2576514-2576631,2577719-2577978,2578406-2578538,2578613-2578761,2579332-2579450,2580043-2580211,2581297-2581377,2592211-2592308,2658004-2658094,2658163-2658361,2658681-2658915,2659365-2659465,2662493-2662694,2662893-2663279,2664224-2664384
Ceratosolensolmsi	Scaffold28: 22699-22850,22950-23138,23816-23911,24825-25106,25990-26199,29033-29164,29284-29383,32077-32177,33157-33394,34243-34441,213353-213450,255828-255908,258229-258364,259527-259645,260638-260792,261012-261144,261816-262075,265854-265971,268368-268531,268611-268706,269420-269551,269619-269799,269874-270027,270115-270229
Triboliumcastaneum	XM_966106

	Anopheles gambiae	XM_001689077
	Drosophila melanogaster	NM_079617
	Danausplexippus	EHJ76705
	Heliconiusmelpomene	HMEL004152 from the http://butterflygenome.org
	Bombyxmori	XM_004925146
	Pediculushumanus	XM_002425891
	Tetranychusurticae	HE:587316:540554-537390
timeless	*Daphnia pulex*	DappuT313767 from the *Daphnia pulex*genome
	Danausplexippus	AY367059
	Bombyxmori	NM_001044157
	Pediculushumanus	XM_002432376
	Tetranychusurticae	Tetur27g02370 from the *Tetranychusurticae*genome
	Drosophila melanogaster	NM_164540
	Triboliumcastaneum	NM_001113465
	Acyrthosiphonpisum	XM_003240268

Table S4.The primerused for real time qPCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon length (bp)	PCR Efficiency (%)	R^2
timeout	AGAGGATGAGAATATGGAA	GGAGTCTGAATCTACAAC	117	93.4	0.997

Table S5. MIQE checklist

Item to check	Importance	Details
Sample		
Description	E	Pollinators (*Ceratosolensolmsimarchali*) of *Ficushispida*
Processing procedure	E	Adult female and male fig pollinators were collected from inside the fig syconia，in addition, we collect the adult female from the outside the fig syconia in light condition.
If frozen, how and how quickly	E	Insect samples were immediately frozen in liquid nitrogen after they were collected
If fixed, with what and how quickly?	E	Stored in sample Protector (TAKARA, China) immediately after frozen
Sample storage conditions and duration	E	Samples were held at -20 °C for less than a week before RNA isolation
Experimental design		
Definition of experimental and control	E	No relative quantification were involved in this work, thus no

groups		control groups were defined
Number within each group	E	3
Nucleic acid extraction		
Procedure and/or instrumentation	E	For each RNA sample, total RNA of 40 individuals was extracted by using an EasyPureTM RNA kit (TransGen Biotech, China)
Name of kit and details of any modifications	E	EasyPureTM RNA kit (TransGen Biotech, China). We exactly followed the protocols of the kit
Details of DNase or RNase treatment	E	Genomic DNA was removed by treating with DNase I according to the standard protocols
Contamination assessment (DNA or RNA)	E	No reverse transcription control (NRC) was performed for each RNA sample to assess the absence of DNA.
Nucleic acid quantification	E	RNA concentration was determined by measuring the abosorbance at 260nm UV light
Instrument and method	E	NanoDrop-2000 Spectrophotometer (Thermo, USA)
RNA integrity: method/instrument	E	RNA integrity was assessed by electrophoresis on 1.0% agarose gels stained with ethidium bromide
RIN/RQI or C_q OF 3' and 5' transcripts	E	N/A
Inhibition testing (C_q dilutions, spike, or other)	E	Standard curve analyses were sufficient to test inhibition
Reverse transcription		
Complete reaction conditions	E	TransScript II First-Strand cDNA Synthesis SuperMix (TransGen Biotech, China) was used to generate single-stranded cDNA total RNA with oligo-dT. For each sample, a template for no reverse transcription-control was prepared.
Amount of RNA and reaction volume	E	Amount of RNA: 1μg; Reaction volume: 20μl
Priming oligonucleotide and concentration	E	oligo-dT: 2μM
Temperature and time	E	50 ^{o}C for 1 hour
qPCR protocol		
Complete reaction conditions	E	PCR reactions were performed in a Mx3000P Real Time Thermocycler (Stratagene, USA). A 20 μl PCR mixture was prepared containing 1 μl of template, 10μl TransStart Green qPCRSuperMixUDG(2x) (TransGen Biotech, China), 0.4μl Passive Reference Dye II(50x) (TransGen, China), 0.8μl primer mix(0.2μM), and 7.8 μl sterile water. The following thermal conditions for RT-qPCR were used: 50^{o}C for 2 min, 95^{o}C for 10 min, and then the follwing: 95^{o}C for 10 s, 54^{o}C for 15 s and 72^{o}C for 15 s for 40 cycles
Reaction volume and amount of cDNA/DNA	E	Reaction volume: 20μl; amount of cDNA: 1μl per reaction volume

Primer, (probe), Mg2, and dNTP concentrations	E	500nM primers; 3mM $MgCl_2$; 0.2 mMdNTP
Polymerase identity and concentration	E	TransStart Green qPCRSuperMix UDG (2x) (TransGen Biotech, China)
Buffer/kit identity and manufacturer	E	TransStart Green qPCRSuperMix UDG (2x) (TransGen Biotech, China)
Additives (SYBR Green I, DMSO, and so forth)	E	Passive Reference Dye II(50x) (TransGen Biotech, China)
Complete thermocycling parameters	E	50°C for 2 min, 95°C for 10 min, and then the follwing: 95°C for 10 s, 54°C for 15 s and 72°C for 15 s for 40 cycles
Specificity (gel, sequence, melt, or digest)	E	Melting curve analysis, gel electrophoresis and sequencing
For SYBR Green I, C_q of the NTC	E	The signal of the amplification plot was late (C_q>30), difference of C_q between NTC controls and cDNA samples was large
Calibration curves with slope and y intercept	E	-3.58~-3.10
PCR efficency calculated from slope	E	Table S4 of Paper
R^2 of calibration curve	E	Table S4 of paper
Linear dynamic range	E	
$C_q$variation at LOD	E	
Evidence for LOD	E	C_q<35 for all samples
If multiplex efficiency and LOD OF each assay	E	N/A
qPCR analysis analysis program (sourse, version)	E	MxPro QPCR Software
Method of C_q determination	E	Cq values were determined using threshold, which is determined using the Amplification-based Threshold method
Outlier identification and disposition	E	None of Cq values was discarded
Results for NTCs	E	The signal of the amplification plot was very late (C_q>35)
Justification of number and choice of reference genes	E	*UBC*; *RPL13a*
Description of normalization method	E	N/A
Number and stage (RT or qPCR) OF technical replicates	E	Duplicate
Number and concordance of biological replicates	E	Triplicate
Repeatability (intraassay variation)	E	ΔCq< 0.5 for all duplicates
Statistical methods for results significnce	E	One way ANOVA
Software (source, version)	E	SPSS v19
qPCR target information		

Gene symbol	E	Text
Sequence accession number	E	
Amplicon length	E	Table S4 of paper
In silico specificity screen (BLAST, and so on)	E	
Location of each primer by exon or intron (if applicable)	E	Primers were designed spanning the splicing sites
What splice variants are targeted	E	N/A

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>Pediculus_humanus
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