Neuropeptides and Peptide Hormones in *Anopheles gambiae*

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The African malaria mosquito, *Anopheles gambiae*, is specialized for rapid completion of development and reproduction. A vertebrate blood meal is required for egg production, and multiple feedings subsequently allow transmission of malaria parasites, *Plasmodium* spp. Regulatory peptides from 35 genes annotated from the *A. gambiae* genome likely coordinate these and other physiological processes. *Plasmodium* parasites may affect actions of newly identified insulin-like peptides, which coordinate growth and reproduction of its vector, *A. gambiae*, as in *Drosophila melanogaster*, *Caenorhabditis elegans*, and mammals. This genomic information provides a basis to expand understanding of hematophagy and pathogen transmission in this mosquito.

The African malaria mosquito, *A. gambiae*, is adapted for rapid development in temporal water and for reproduction cued by blood meals from humans. Successive reproductive cycles in this mosquito require multiple blood meals, thus favoring transmission of malaria parasites, *Plasmodium* spp., among humans. Regulatory peptides acting as neurochemicals and hormones govern these processes in mosquitoes (*1*). In all insects, these peptides are processed, stored, and released within the nervous system as neurotransmitters and from the midgut endocrine system and neurosecretory cells as circulating hormones (*2*). Most regulatory peptides act through heterotrimeric GTP-binding protein (G protein)–coupled receptors to govern homeostatic processes in response to internal and external stimuli and coordinate behaviors.

Here we focus on peptide genes, herein identified, that when expressed likely regulate key physiological and behavioral processes underlying the rapid development and blood lust of *A. gambiae*. There are intriguing hints that insulin-like peptides acting through the insulin signaling pathway, which is conserved in mosquitoes (*3*) and described herein, may affect and even be manipulated by malaria parasites in infected females. Additionally, comparison of the extant gene catalogs of regulatory peptides and their processing and degrading enzymes in *A. gambiae* with those in the fruit fly, *Drosophila melanogaster*, both in the order Diptera, may reveal ones specifically serving hematophagy and pathogen transmission in this mosquito.

Peptide Genes

With bioinformatic approaches, 35 genes encoding putative regulatory peptides were annotated in the *A. gambiae* genome (Table 1; see supplemental table S1 for amino acid sequences), including five insulin-like peptides, AgamILP1–5 (Table 2; see table S1 for amino acid sequences). Potential orthologs were obtained by tblastn searches of the National Center for Biotechnology Information (NCBI) nucleotide database and analyzed for open reading frames, splice sites, signal sequences, and necessary processing sites (see Supporting Online Material). Among highly scored peptide sequences, a distinctive subset exhibited amino acid motifs requisite for precursor processing (*2*), typically Gly-Lys-Arg for cleavage and subsequent COOH-terminal amidation, from the preproproteins determined. Only one *D. melanogaster* peptide gene, *amnesiac* (CG11937), was not found in *A. gambiae*. Two previously unknown genes/peptides were identified in *A. gambiae*: *AgamNPFF* encodes a neuropeptide FF, an opioid peptide in vertebrates, and *AgamMY*, a locust myotropin-like peptide. Neither was found in the *D. melanogaster* genome. Orthologs were identified in *A. gambiae* for five of six peptide genes structurally and functionally characterized in yellow fever mosquito, *Aedes aegypti* (table S1), as described below. This gene catalog of putative regulatory peptides in *A. gambiae* is doubtless incomplete, given the potential problems of identifying peptides with short sequences.

Insulin-Like Peptides and the Insulin-Signaling Pathway

Discovery of genes for proteins associated with the insulin signaling pathway in *A. gambiae* (Table 2) shows that this pathway probably has pivotal roles in the life history of this mosquito, as in the nematode *Caenorhabditis elegans* and fruit fly *D. melanogaster* (*4*, *5*). Although five AgamILPs were identified, only one insulin receptor, AgamINR, was found in *A. gambiae*, as is typical for other invertebrates but not for vertebrates. *AgamINR* encodes a receptor tyrosine kinase that is most closely related to the AaegINR (*3*), both lacking the extended COOH-terminus of cognate receptors in *C. elegans* (*daf*-2) and *D. melanogaster*. AaegINR is expressed only in ovaries of *Ae. aegypti* during reproductive arrest and the first 24 hours of a reproductive cycle when ecdysteroids are being synthesized (*3*). This receptor likely activates the insulin receptor substrate, IRS, which is the node for activation of the mitogen-activated protein kinase pathway and the pathway consisting of phosphoinoside 3-kinase (PI3K) and a serine/threonine protein kinase, Akt.

For a range of organisms, life-span is increased when reproduction and caloric intake are reduced (*5*). Longevity and diapause in *C. elegans*, in response to a lack of food, are regulated by a hormonal signal through the PI3K/Akt pathway. Mutations in this pathway result in dauer formation, increased longevity, and occasionally reduced fecundity (*5*). In *D. melanogaster*, this pathway links fecundity and longevity. Female flies with mutations in DmelINR or IRS exhibit reduced body size, increased longevity, and sterility (*4*, *5*). Similarly, this pathway may be the key regulatory switch between arrest/longevity and reproduction in female *A. gambiae*, thus contributing to its vectorial capacity for malaria, which is largely dependent on the probability of daily survival of the mosquito and the extrinsic incubation period of the parasite (*6*). During the 10 to 14 days required for *Plasmodium* development, the infected female *A. gambiae* could complete at least three cycles of egg maturation. A strategy to prevent *Plasmodium* transmission by *A. gambiae* after an infected blood meal could be devised that would perturb expression of a target gene in this pathway to allow completion of a single gonotrophic cycle, with subsequent senescence or death, thus assuring fitness of female mosquitoes while preventing parasite transmission.

Effects of *Plasmodium* infection on the reproductive physiology of *A. gambiae* females also may be mediated through the insulin signaling pathway. In female *A. stephensi*, *Plasmodium* infection initiates ap-

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optosis in ovarian follicles, leading to resorption and reduced brood size (*7*). Resorption of follicles leads to a greater proportion of the blood meal's nutrients being available for parasite development and less for the females, resulting in a longer life to transmit *Plasmodium*. Apoptosis (*8*) and nutrient metabolism (*4*) also are regulated similarly in *D. melanogaster*. Even more intriguing is the observation that *Plasmodium* appears to use insulin from either the female mosquito or the vertebrate blood meal for its own development (*9*). Female *A. gambiae* and *A. stephensi* ingesting a *Plasmodium*-infected blood meal and given access to sugar solution supplemented with human insulin both exhibited a marked increase in the number of oocysts on the midgut compared with females given an infective blood meal and sugar solution lacking insulin. This pathway may even regulate innate immunity in *A. gambiae*, because Akt regulates immune response (*10*) and nitric oxide production in mammals (*11*).

Ecdysteroid Hormone Secretion

Ecdysteroid hormones govern gene expression during female reproduction and larval development. A blood meal releases an endocrine cascade that switches female mosquitoes from an arrested to a reproductive state in which as many as 200 eggs are produced in less than 3 days (*1*). In *Aedes aegypti*, ovary ecdysteroidogenic hormone (AaegOEH) released from brain neurosecretory cells promptly after blood ingestion stimulates ovaries to end the arrested state and begin secreting ecdysteroids (*12*), which drive secretion of yolk proteins by the fat body. These proteins are taken up by oocytes to begin egg maturation and are used by embryos. *AgamOEH* (Table 1) is the ortholog for *AaegOEH* (table S1) (*12*). Both genes encode a single peptide that was localized in neurosecretory cells in brains and ventral nerve cords and midgut endocrine cells of *A. gambiae* and *Ae. aegypti* (*13*). Localization of OEH in female *A. gambiae* is the first step to demonstrating its conserved function in mosquito females, and the absence of an ortholog in *D. melanogaster* is suggestive of a unique role in blood-feeding female dipterans.

AaegOEH activates ovarian ecdysteroidogenesis through either the insulin signaling pathway (*3*) or a G protein–coupled receptor/cyclic adenosine monophosphate (GPCR/cAMP) pathway (*14*). Ecdysteroidogenesis in mosquito development may be comparable to that of moths, where prothoracicotropic hormone (PTTH) stimulates prothoracic glands (PTGs) by way of GPCR/cAMP to secrete ecdysteroids, which in turn regulate larval and pupal molts (*2*). These glands degenerate in pupae, and PTTH has no known function

Table 1. Regulatory peptide genes identified in the *A. gambiae* genome with abbreviation, genome scaffold accession numbers, predicted nucleotide sequences for the open-reading frame (ORF), NCBI numbers for predicted protein and gene transcript, and information for *D. melanogaster* orthologs. ND, gene not detected.

Peptide gene–symbol	Chromosome	Scaffold	ORF bp range	Protein	Transcript	*Drosophila* gene
Adipokinetic hormone–AKH	3	AAAB01008984	490675–491033	ND	ND	AKH CG1171
Allatostatin–AST	2	AAAB01008888	3324922–3325683	agCP7920	agCT44355	AST CG13633
Allatostatin 2–AST2	3	AAAB01008980	13439599–13446185 13439599–13442771	agCP8174	agCT55991	Ast2 CG14919
Allatropin 1-AT	3	AAAB01008986	4006038–4005660	agCP5165	agCT42442	ND
Allatropin 2-AT2	2	AAAB01008987	12762080–1276213	agCP9503	agCT53755	ND
	2	AAAB01008807	4814789–4814839			
Bursicon-BSN	2	AAAB01008859	9069946–9070242	ND	ND	CG13419
capa/CAP-2b-CAPA	X	AAAB01008846	5171770–5172289	ND	ND	Capa CG15520
Corazonin-CRZ	2	AAAB01008888	2834504–2834258	agCP10813	agCT42609	Crz CG3302
Crustacean cardioactive peptide-CCAP	3	AAAB01008980	5708118–5708674	agCP8078	agCT48460	Ccap CG4910
Diuretic hormone/Corticotropin releasing factor-DH	2	AAAB01008794	452072–452242 471243–471413	ND	ND	Dh CG8348
Diuretic hormone 31/ Calcitonin-like peptide-DH31	2	AAAB01008987	12154886–12155318	agCP12293	agCT51113	Dh31 CG13094
Ecdysis-triggering hormone-ETH	2	AAAB01008807	7238795–7239016	ND	ND	Eth CG18105
Eclosion hormone-EH	3	AAAB01008849	909629–909966	ND	ND	Eh CG5400
FMRFa peptides-FMRF	2	AAAB01008960	2640522–2641514	ND	ND	Fmrf CG2346
IFa peptide-IF	2	AAAAB01008807	7459232–7458932	ND	ND	Ifa CG4681
Ion transport peptide/ Crustacean hyperglycemic hormone-ITP	2	AAAB01008900	356740–359312	agCP15037	agCT45013	CG13586
Leucokinins-KIN	2	AAAB01008987	14661486–14661854	ND	ND	Leucokinin CG13480
Myoinhibitory-like peptide/ Allatostatin B-MIP	X	AAAB01008963	340616–340043	agCP9104	agCT47826	MIP CG6456
Myosuppressin-MS	2	AAAB01008987	10850719–10850298	agCP12084	agCT55889	Dms CG6440
Myotropin-MY	3	AAAB01008964	4711486–4711253	ND	ND	ND
Neuropeptide F-NPF	2	AAAB01008952	195191–215964	agCP4465	agCT55467	Npf CG10342
Neuropeptide FF-NPFF	3	AAAB01008933	2221002–2221730	ND	ND	ND
Neuropeptide-like precursor 1-NPP1	3	AAAB01008849	2198137–2200736	agCP10689	agCT45844	CG3441
Ovary ecdysteroidogenic hormone/Neuroparsin-OEH	X	AAAB01008846	9519863–9520455	agCP13249	agCT55373	ND
Pyrokinins-PK	2	AAAB01008799	336457–337184	agCP14788	agCT48399	Hug CG6371
Pigment dispersing hormone-PDH	2	AAAB01008960	6558256–6557667	agCP6031	agCT54005	Pdf CG6496
Proctolin-PT	2	AAAB01008978	1880379–1879669	ND	ND	
Prothoracicotropic hormone-PTTH	X	AAAB01008963	794586–795008	ND	ND	CG13687
Short Neuropeptide F/ LRLRFa-SNPF		AAAB01047054 (not mapped)	2551–1937	ND	ND	SNPF CG13968
Sulfakinin-SK	3	AAAB01008984	11735357–11735941	ND	ND	Dsk CG18090
Tachykinin-TK	3	AAAB01008980	11498154–11497029	agCP8706	agCT52596	Tk CG14734

Table 2. Genes for insulin-like peptides and proteins associated with the insulin signaling pathway identified in the *A. gambiae* genome with abbreviation, genome scaffold accession numbers, predicted nucleotide sequences for the open-reading frame (ORF), NCBI numbers for predicted protein and gene transcripts, and information for *D. melanogaster* orthologs. ND, gene not detected; S1, supplemental table S1.

Protein gene–symbol	Chromosome	Scaffold	ORF bp range	Protein	Transcript	*Drosophila* gene
Insulin-like peptide–ILP1	3	AAAB01008933	2144119–2143580	ND	ND	S1
ILP2	3	AAAB01008933	2132657–2133093	agCP11852	agCT43360	IRP CG8167
ILP3	3	AAAB01008933	2143225–2142664	agCP11855	agCT43363	S1
ILP4	3	AAAB01008933	2137767–2137175	agCP11853	agCT43361	S1
ILP5	2	AAAB01008978	317505–317978	ND	ND	S1
Insulin receptor–INR	3	AAAB01008986	39223–24510	agCP9384	agCT52486	InR CG18402
Insulin receptor substrate–IRS	3	AAAB01008984	883077–1018948	ND	ND	Chico CG5686
p110 Phosphoinositide 3-kinase-P110 PI3K	X	AAAB01008846	11060170–11057027	agCP13380	agCT43608	Pi3K92E CG4141
p60 PI3K-P60 PI3K	2	AAAB01008960	3465275–3466438	agCP6161	agCT51158	Pi3K21B CG2699
Protein kinase B–AKT	2	AAAB01008799	2377298–2372388	agCP14714	agCT44125	Akt1 CG4006
Phosphatase and tensin homolog–PTEN	3	AAAB01008980	1144802–1155044	agCP8091	agCT51705	Pten CG5671
3′-Phosphoinositide-dependent kinase-1–PDK1	2	AAAB01008987	10864984–10866450	agCP12532	agCT55867	Pk61C CG1201
Target of Rapamycin–TOR	3	AAAB01008964	9986100–9993859	ebi7283	ND	Tor CG5092
S6 Kinase–S6K	2	AAAB01008960	12661343–12664900	agCP6029	agCT50817	S6k CG10539

in adult moths. In *Ae. aegypti* larvae and pupae, thorax and abdomen body walls are de novo sources of ecdysteroids, not the PTG (*15*). With identification of *AgamPTTH* (Table 1), it will be of interest to determine whether these phenomena are common to *A. gambiae*.

Molting

Precisely regulated behavioral and physiological processes associated with molting allow *A. gambiae* to grow and complete development rapidly in temporary water pools. Juvenile hormones (JHs) maintain larval characteristics between molts and guide tissue differentiation in female mosquitoes after eclosion (*1*). Peptides originating in brain control JH secretion by paired glands (corpora allata). Allatostatin A–type peptides (ASTs) inhibit JH secretion and hindgut contractions in insects. *AgamAST* encodes five peptides in the allatostatin-A family, the same number as encoded by *AaegAST* (*16*), which in females is expressed in neurosecretory and midgut endocrine cells (*17*). Two genes identified in *A. gambiae* encode allatotropins (ATs), which stimulate JH secretion in adult moths, but neither was identified in the *D. melanogaster* genome. *AgamAT* is the ortholog for *AaegAT* (*18*) that is expressed in abdominal ganglia of female *Ae. aegypti*. *AgamAT2* is tentatively identified as an allatotropin gene because it encodes two peptides having limited sequence similarity to AgamAT1 (table S1). The gene for a mosquito eclosion hormone, which regulates molting behaviors, is now known from *AgamEH*; previously, only a partial AaegEH was reported (*19*). Recently, the gene encoding bursicon, which promotes cuticle tanning and wing expansion after molting, was identified in *A. gambiae* (*AgamBSN*) and *D. melanogaster*.

Water and Ion Balance

After blood feeding, female *A. gambiae* engage in rapid diuresis to decrease the volume/weight of the meal to allow flight. Water and ion homeostasis is maintained by the midgut, Malpighian tubules (MTs), and hindgut, as exemplified in *Ae. aegypti* (*20*). Genes for all four diuretic hormones known to increase diuresis or fluid secretion by MTs in insects were identified in *A. gambiae*: diuretic hormone (*AgamDH*), calcitonin-like peptide (*AgamDH31*), leucokinins (*AgamKIN*), and a CAP-2b peptide (*AgamCAPA*) (Table 1). *AgamKIN* and its ortholog, *AaegKIN* (table S1) (*21*), each encode three related peptides that stimulate fluid secretion by MTs and hindgut contractions in *Ae. aegypti*. *AgamDH* encodes a single peptide related to one isolated from *Culex salinarus*, which effects ion transport in *Ae. aegypti* MT (*22*).

Host-Seeking and Feeding

Both *A. gambiae* and *Ae. aegypti* females use chemical cues to find vertebrate hosts from which to take blood meals, but ignore these cues for more than 40 hours after initiation of a gonotrophic cycle (*23*). *Ae. aegypti* Head Peptide (AaegHP, pERPhPSLKTRFa; coisolated with pERPPSLKTRFa) inhibited host seeking when injected into non–blood-fed female *Ae. aegypti* (*24*). The timing of this behavioral inhibition coincided with a high hemolymph titer of AaegHP in blood-fed females. *AaegHP* encodes three AaegHPs (*24*) and is expressed in brain, terminal ganglion, and midgut. Surprisingly, no *AaegHP* ortholog was identified in *A. gambiae* or *D. melanogaster*, but a gene encoding "short neuropeptide Fs" (SNPFs) similar to AaegHP was found in both species. *AaegHP* is not an *AgamSNPF* ortholog, because an authentic AaegSNPF (APQLRLRFa) isolated from *Ae. aegypti* (*25*) is absent from *AaegHP*. This may indicate loss of *AaegHP* in *A. gambiae* and *D. melanogaster* or its duplication in *Ae. aegypti* from a gene common to all three species, i.e., the SNPF gene.

The "long" form of NPF likely affects feeding behavior and digestive processes in mosquitoes, as demonstrated in other invertebrates and mammals for the neuropeptide Y (NPY) superfamily (*26*). *AgamNPF* and orthologs in *Ae. aegypti* (*26*) and *D. melanogaster* encode a single peptide (table S1). Concordantly, NPF gene expression occurs in brain and midgut of female *Ae. aegypti* and *D. melanogaster* (*26*). Furthermore, the NPF receptor has been identified in *D. melanogaster* (*27*), and now in *A. gambiae* (*28*). When *DmelNPFR1* was expressed in mammalian cells, activation by DmelNPF inhibited cAMP production, the signature action of NPY GPCRs (*27*).

Peptide Processing and Degradation

Genes for enzymes that process or degrade regulatory peptides have been identified in *D. melanogaster* (*2*) and now *A. gambiae* (Table 3). Amino acid motifs pivotal to identifications of regulatory peptide genes (above) are required by processing endopeptidases, glutaminyl cyclase, and amidation enzymes (*2*). For degradation, insulinases may target AgamILPs specifically. Genes for metalloendoproteases with astacin domains identified in *A. gambiae* (Table 3) warrant further investigation because they play an important role in development of *D. melanogaster* and also activate and degrade peptide hormones in the digestive tract of vertebrates (*29*).

Perspectives

With key elements now revealed in the genome of *A. gambiae*—for which no regulatory peptide sequences were previously known—understanding the regulation of growth, development, and reproduction will require study of peptide secretion, signal transduction, and degradation. Pairings of peptide ligand and receptor can be accomplished now that 40 peptide GPCRs have been annotated for *A. gambiae* (*28*).

Table 3. Genes for peptide processing and degrading enzymes identified in the *A. gambiae* genome with abbreviation, genome scaffold accession numbers, predicted nucleotide sequences for the open-reading frame (ORF), NCBI numbers for predicted protein and gene transcripts, and information for *D. melanogaster* orthologs. ND, gene not detected.

Enzyme gene	Chromosome	Scaffold	bp range	Protein	Transcript	*Drosophila* gene
Processing enzymes						
Prohormone convertases						
Furin 1	2	AAAB01008987	13047880–13042014	agCP12685	agCT47565	Fur1 CG10772
dfurin 2	2	AAAB01008859	2175454–2211578	agCP1407	agCT46736	Fur2 CG4235
Amontillado	2	AAAB01008799	2103919–2096916	agCP14799	agCT44132	Amon CG6438
Angiotensin-converting enzymes	3	AAAB01008980	6212654–6220875	agCP8603	agCT48990	Acer CG10593
	3	AAAB01008980	6221421–6223518	agCP8177	agCT48991	Ance CG8827
	3	AAAB01008980	6225014–6227085	agCP8519	agCT48977	Ance-2 CG16869
	2	AAAB01008898	3781136–3785682	agCP14039	agCT53190	Ance-3 CG17988
	3	AAAB01008980	6235356–6238125	agCP8223	agCT49000	Ance-4 CG8196
Carboxypeptidases						
Silver	2	AAAB01008859	10489068–10499817	ebiP1195	ND	Svr CG4122
Silver2	X	AAAB01008847	2437513–2435016	agCP7325	agCT49939	CG4678
COOH-terminus amidation enzymes						
Peptidylglycine Alpha-hydroxylating Monooxygenase	2	AAAB01008807	2829113–2813898	agCP3647	agCT55264	Phm CG3832
Peptidyl alpha-Hydroxyglycine alpha-Amidating lyases	2	AAAB01008960	6656396–6638648	agCP5453	agCT53988	Pal CG5472
		AAAB01008807	1189935–1188994	ebiP9346	ND	CG12130
Glutaminyl cyclases	3	AAAB01008980	2103488–2104658	agCP8088	agCT47152	CG10487
	2	AAAB01008960	20784078–20786429	agCP5619	agCT53125	
Degrading peptidases						
Neprilysins/ Enkephalinases	3	AAAB01008980	7169972–7172717	agCP8372	agCT53623	Nep1 CG5894
	2	AAAB01008987	5709618–5706139	ebiP8439	ND	Nep2 CG9761
	3	AAAB01008964	11325892–11328388	agCP11334	agCT52312	Nep3 CG9565
	2	AAAB01008879	898821–896524	ebiP3181	ND	
	2	AAAB01008859	10098863–10102974	ebiP1161	ND	
Dipeptidyl peptidase III	2	AAAB01008898	1754302–1756833	agCP14130	agCT46116	CG7415
Prolyl oligopeptidase	3	AAAB01008984	9323132–9320042	agCP4638	agCT46526	CG5355
Metalloendoproteases	2	AAAB01008979	1194670–1193696	agCP9011	agCT46878	CG15255
	2	AAAB01008952	465936–464338	agCP4419	agCT55491	CG6763
	3	AAAB01008966	701722–702541	agCP14375 ebiP141	agCT50193	CG6696
	2	AAAB01008979	1315048–1315837	agCP9016	agCT46881	CG5715
	2	AAAB01008979	1324116–1324857	agCP8971	agCT46903	CG10280
Intracellular degrading enzymes						
	3	AAAB01008849	2502247–2505641	agCP10680	agCT45808	Ide CG5517
Insulinases	?	AAAB01008985	168715–148271	ebiP1808	ND	CG2025
Tripeptidyl peptidase II	2	AAAB01008905	376184–371624	ebiP1178	ND	TppII CG3991

Because most peptide types exist as single-copy genes, each is a target for genetic interference, both to unravel regulatory functions and in the long-term quest to engineer this mosquito so that it is less hospitable for *Plasmodium*. Further, knowledge of genes known to affect the processing, signal transduction, or degradation of regulatory peptides is likely pivotal in development of truly new pesticides that may provide cost-effective and strategic reductions in this mosquito's populations.

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30. We thank L. Dewey and H.-Willi Honegger (Vanderbilt University) for identifying bursicon genes; P. H. Taghert (Washington University) for providing the *Adv. Genet.* manuscript before publication; and F. H. Collins (University of Notre Dame) and A. O. Lea for reviews. Supported by NIH grant AI33108 (M.R.B.). Financial support to the *Anopheles gambiae* Genome Consortium was provided by NIH grant U01 AI50687 to Celera Genomics, NIH grant U01 AI48846 to the University of Notre Dame, and French Government funds to Genoscope.

Supporting Online Material

www.sciencemag.org/cgi/content/full/298/5591/172
SOM text
Table S1
References

31 July 2002; accepted 10 September 2002