1 S6K activates juvenile hormone and vitellogenin production in the
2 cockroach *Blattella germanica*

3

4 MARC ABRISQUETA, SONGÜL SÜREN-CASTILLO and JOSÉ LUIS MAESTRO

5 Institut de Biologia Evolutiva (CSIC-Universitat Pompeu Fabra), Passeig Marítim de la
6 Barceloneta 37-49, 08003 Barcelona, Spain.

7

8

9

10 **Short title:** *S6K activates JH and Vg in B. germanica*

11

12

13

14

15 Correspondence: Dr. José Luis Maestro, Institut de Biologia Evolutiva (CSIC-
16 Universitat Pompeu Fabra), Passeig Marítim de la Barceloneta 37-49, 08003 Barcelona,
17 Spain. Tel.: +34 932309639; e-mail: joseluis.maestro@ibe.upf-csic.es.

18

19 **Abstract:**

20 Nutritional status regulates different processes, such as growth and development,
21 through TOR (target of rapamycin) and insulin receptor signalling pathways. The
22 ribosomal S6 protein kinase (S6K) is a downstream element of both pathways. Using
23 cDNA from the German cockroach *Blattella germanica* (L.) two S6K isoform
24 sequences (BgS6K) were cloned. The long isoform differs from the short one by the
25 insertion of a 22-amino acid duplication, involving the key phosphorylation position
26 Thr³⁹⁰. As a result of this, the long isoform presents a new, potentially regulatory
27 phosphorylation site. RNAi knock down of both BgS6K isoforms induces an increase in
28 the length of the last nymphal instar, together with a reduction in the mRNA levels of a
29 number of enzymes of the juvenile hormone biosynthetic pathway in the corpora allata,
30 vitellogenin mRNA levels in the fat body and basal oocyte length. Thus, BgS6K is
31 important for nymphal development and is necessary for full induction of juvenile
32 hormone synthesis and vitellogenin production in adult females.

33

34

35

36

37

38

39

40

41 **Keywords:** *Blattella germanica*, insulin, juvenile hormone, nutritional signalling, S6K,
42 TOR, vitellogenin

43 **Introduction**

44 Nutrient availability is a key regulator of critical processes at both cellular and
 45 organism levels. The protein kinase TOR (target of rapamycin) is a key mediator of
 46 cellular nutrient sensing. TOR can sense information from cellular amino acids levels
 47 and energy, and regulates the activity of processes involved in cell growth, such as
 48 protein synthesis and autophagy. On the other hand, insulin receptor (InR) signalling is
 49 the main mechanism of systemic nutrient sensing and works largely through a
 50 sequential protein kinase pathway. Both pathways contribute to control and modulate
 51 the organism response to the nutritional situation and crosstalk at different points. For
 52 example, TOR is regulated by InR pathway through the modulation of tuberous
 53 sclerosis 2 (TSC2) activity via Akt phosphorylation (Ma & Blenis, 2009).

54 The ribosomal protein S6 kinase (S6K), a protein belonging to the AGC family
 55 of protein kinases that are characterized by sharing a similar catalytic domain
 56 (Jastrzebski *et al.*, 2007), is one of the main targets of TOR, which phosphorylates and
 57 activates S6K when the pathway is active. S6K can then phosphorylate several proteins,
 58 for example, the ribosomal protein S6, the eukaryotic translation initiation factor 4B
 59 (eIF4B) and the eukaryotic elongation factor-2 kinase (eEF2K). S6K participates in the
 60 regulation of mRNA translation, ribosome biogenesis, cell size and cell cycle
 61 progression, lipid synthesis, glucose homeostasis, energy balance, and others processes
 62 (Fenton & Gout, 2011; Magnuson *et al.*, 2012). An inhibitory role of S6K on Insulin
 63 Receptor Substrate (IRS) activity has also been described, creating a negative feedback
 64 that stops nutritional signalling when it lasts too long or as consequence of a mutation
 65 (Shah *et al.*, 2004).

66 Full activation of S6K requires a sequential, multi-site phosphorylation.
 67 According to the most conventional model, the process is divided into four steps

(Magnuson *et al.*, 2012). The first step is calcium-dependent, and breaks the interaction between C-terminal and N-terminal domains of the protein (Hannan *et al.*, 2003). This allows the phosphorylation of four proline-directed Ser/Thr sites in the C-terminal autoinhibitory domain (Ferrari *et al.*, 1992). Then, TOR has access to the critical phosphorylation site, which corresponds to T³⁸⁹ in mammal p70-S6K1, which is located in the hydrophobic motif of the protein (Isotani *et al.*, 1999). Finally, phosphoinositide-dependent kinase-1 (PDK1) phosphorylates the T-loop site in catalytic domain (T²²⁹ in mammal p70-S6K1), stabilizing the active protein and driving it to its maximal activation (Pullen *et al.*, 1998).

Mammals have two similar S6 protein kinases, named S6K1 and S6K2, encoded by two different genes. Both genes can produce two different isoforms (p70S6K and p85S6K for S6K1; p54S6K and p56S6K for S6K2), through the use of two alternative translational start sites (Grove *et al.*, 1991; Lee-Fruman *et al.*, 1999; Pardo & Seckl, 2013). In insects, all studies point to the existence of only one gene that encodes S6K.

In *Drosophila melanogaster*, S6K loss through mutation produces smaller flies as a consequence of a cell-autonomous decrease of cells size, but not cell number.

Moreover, mutant flies have decreased viability and delayed development (Montagne *et al.*, 1999). In the mosquito *Aedes aegypti*, S6K phosphorylation at T³⁸⁸, homologous to mammalian T³⁸⁹, is stimulated by amino acids in the fat body in vitro, and by a blood meal in vivo, and this stimulation is sensitive to the TOR inhibitor rapamycin (Hansen *et al.*, 2005). In addition, mosquito S6K knockdown by RNAi reduces amino acid stimulation of vitellogenin expression (Hansen *et al.*, 2005). Furthermore, insulin promotes S6K phosphorylation in mosquito fat body, and rapamycin treatment reduces S6K phosphorylation and vitellogenin gene stimulation induced by insulin (Roy *et al.*,

2007). In the Oriental fruit fly, *Bactrocera dorsalis*, S6K knockdown reduces yolk protein expression and ovary size (Suganya *et al.*, 2011).

The aim of the present work is to analyse S6K function and its role in the regulation of reproduction in the German cockroach, *Blattella germanica* (L.). The results show that *B. germanica* has two S6K isoforms which differ in a 66 nucleotides (22 amino acids) insertion/deletion. The insertion, which is consequence of a duplication of the adjacent sequence, includes a potential new target for TOR phosphorylation. Simultaneous RNAi knockdown of both isoforms is undertaken to analyse the role of S6K in development and reproduction.

Material and methods

Insects

Specimens of *B. germanica* were obtained from a colony reared on dog food and water, at $30 \pm 1^\circ\text{C}$ and 60-70 % relative humidity. Dissections of corpora allata and abdominal fat body were carried out on carbon dioxide-anaesthetized specimens. For starvation experiments, animals received only water after the imaginal moult.

Cloning of BgS6K cDNA

Degenerated primers based on conserved regions of insect and mammalian S6K sequences were used to obtain a *B. germanica* homologue fragment. Primer sequences can be found as Supplementary data, Table S1. As template for the PCR amplification a cDNA generated by reverse transcription from UM-BGE-1 cells (derived from a cell culture of *B. germanica* early embryos) was used. The amplified fragment was cloned into the pSTBlue-1 vector (Novagen, Merck Millipore) and sequenced. 3'-RACE and 5'-RACE methodologies (Invitrogen, Carlsbad, California) were used to complete the sequence.

116

117 *RNA extraction, cDNA synthesis and real-time PCR analyses*

118 The corpora allata and fat body mRNA levels of the different studied genes were
119 analysed using quantitative real-time PCR. cDNAs were synthesized from total RNA as
120 previously described (Abrisqueta *et al.*, 2014; Maestro *et al.*, 2009). In the case of fat
121 bodies, 500 ng of total RNA was used, whereas for corpora allata, the whole RNA from
122 one pair of glands was used. The absence of genomic contamination was confirmed
123 using a control without reverse transcription. The cDNA levels were quantified using iQ
124 SYBR Green supermix in an iQ cycler and iQ single colour detection system and the
125 iQ5 optical system software v. 2.0 (Bio-Rad Laboratories, Hercules, California), as
126 previously described (Maestro *et al.*, 2010). Primer sequences to amplify BgS6K can be
127 found in Table S1. Primers used to amplify HMG-CoA synthase-1 and -2, HMG-CoA
128 reductase, vitellogenin (BgVg) and BgActin 5C (used as a reference) have been already
129 reported (Maestro *et al.*, 2010; Suren-Castillo *et al.*, 2012). The total reaction volume
130 was 20 μ L. All reactions were run in duplicate or triplicate. Results were expressed as
131 copies of a specific mRNA per copies of BgActin 5C.

132

133 *RNA interference*

134 Systemic RNAi in *B. germanica* females was performed as previously described
135 (Maestro *et al.*, 2009). A 519-bp fragment spanning positions 402 to 920 of the BgS6K
136 cDNA was used to synthesize a dsRNA (dsS6K). A heterologous 307-bp fragment from
137 the gene sequence of the polyhedrin of *Autographa californica* nucleopolyhedrovirus
138 was used as control. One microlitre of a 2 μ g μ L⁻¹ dsRNA solution was injected into the
139 abdomen of freshly emerged penultimate (fifth) nymphal instar females, and a second

140 injection of 2 μL of a 1 $\mu\text{g } \mu\text{L}^{-1}$ dsRNA solution was injected once they moulted into
141 the last (sixth) instar. Dissections were carried out 5 days after the imaginal moult.

142

143 *Western-blot analysis*

144 Fat bodies from control (fed and starved) and dsS6K-treated females were
145 dissected in saline solution and homogenized using a plastic pestle in the breaking
146 buffer described by Hansen *et al.* (2005). After centrifugation (10 min, 13,000xg, 4 °C)
147 to remove insoluble material and quantification of protein concentration, 40 μg of the
148 homogenates were separated in a 10 % SDS/PAGE gel. Proteins were transferred to a
149 nitrocellulose membrane (Protran; Schleicher and Schuell Bioscience GmbH, Dassel,
150 Germany) and incubated with a polyclonal primary antibody made at the Custom
151 Antibody Service of the ciber-bbn (iQAC, CSIC, Barcelona, Spain) against a synthetic
152 peptide encompassing the first 18 amino acids of the BgS6K protein. Primary antibody
153 serum was purified by affinity purification using SulfoLink Immobilization Kit for
154 Peptides (Thermo Fisher Scientific, Waltham, Massachusetts) and used at a
155 concentration of 1:2000. Primary antibody was detected using SuperSignal West Femto
156 detection kit (Thermo Fisher Scientific). To check the antibody specificity, one lane of
157 an extract from control fed females was revealed with the primary antibody blocked
158 (overnight, 4 °C) with the antigenic peptide (5 μg of peptide / μg antibody).

159

160 **Results**

161 *Cloning of BgS6K and sequence analysis*

162 By means of degenerate primers designed in conserved regions of an alignment
163 of S6K sequences from different organisms and cDNA from *B. germanica* UM-BGE-1
164 cells as a template, a 528 bp fragment sequence of a presumed S6K homologue from *B.*

165 *germanica* (BgS6K) was obtained. Using the sequence identified in this fragment,
166 specific primers were designed for cloning the full-length of the sequence using 3'-
167 RACE and 5'-RACE methodologies.

168 During the cloning process, when the amplification by PCR of a region was
169 checked, the occurrence of two bands in the agarose gels was consistently detected. The
170 cDNA fragments corresponding to both bands were cloned and it was observed that one
171 of them has a single 66 nucleotides insert compared to the other. When analysing the
172 two sequences, it was found that the 66 additional nucleotides sequence was very
173 similar to the 66 nucleotide sequence that followed, with only seven different
174 nucleotides (Fig. S1). When translated into amino acids, to yield the 22 amino acids
175 corresponding to these fragments, only three amino acids were different (Fig. 1). Thus,
176 the results indicated the occurrence of two different *B. germanica* S6K isoforms:
177 BgS6K short (2410 nt, GenBank accession number: LN901440) and BgS6K long
178 (2476nt, GenBank accession number: LN901441), which code, respectively, for two
179 putative 468 and 490 amino acids proteins. The BgS6K short and long proteins show 68
180 % and 65 % similarity, respectively, when compared to *D. melanogaster* S6K, and 60 %
181 and 58 % similarity, respectively, compared with *Homo sapiens* p70-S6K1.

182 Both BgS6Ks showed the typical organization of S6K proteins, maintaining the
183 characteristic S/T residues whose phosphorylation by diverse kinases (PDK1, TOR, and
184 some still unidentified kinases) (Magnuson *et al.*, 2012) is necessary for the activation
185 of the protein. In particular, T³⁹⁰ in BgS6K short, homologous to T³⁸⁹ in human p70-
186 S6K1 and *D. melanogaster* T³⁹⁸, provides a characteristic phosphorylation target for
187 TOR (Magnuson *et al.*, 2012; Pardo & Seckl, 2013). The 22 amino acids insertion of
188 BgS6K moves this Thr to position 412 and, in addition, affords a putative additional
189 TOR phosphorylation target in S³⁹⁰.

190 *BgS6K expression studies*

191 Using PCR approaches, the expression of both BgS6K isoforms was examined
 192 in different tissues. Due to the similarity between the two BgS6K sequences, it was not
 193 possible to design isoform-specific primers to quantify their expression using real time-
 194 quantitative PCR. Instead, one pair of primers (Table S1) flanking the duplicated region
 195 was used, which produces two bands of 154 and 220 bp when amplifying the short form
 196 and long form, respectively, and then separating them using gel electrophoresis. With
 197 this approach, the differential expression of both isoforms was compared in different
 198 tissues of 5-day-old adult females (corpora allata, brain, fat body, ovary and midgut).
 199 Results showed that the short isoform was the most abundant in all the studied tissues,
 200 whereas the highest proportion of the long isoform with respect to the short isoform was
 201 found in corpora allata (Fig. 2).

202

203 *RNAi of BgS6K*

204 To study BgS6K function, the BgS6K mRNA levels were depleted using
 205 systemic RNAi. A dose of 2 µg of a 519 bp fragment of dsRNA against a common
 206 region to the two isoforms (see Table S1 for primers sequences) was injected in newly
 207 emerged fifth (penultimate) nymphal females and the treatment was repeated when they
 208 moulted into the sixth (last) nymphal instar (dsS6K females). Control females were
 209 treated with a heterologous dsRNA. The effect of dsRNA treatment on BgS6K mRNA
 210 reduction was checked by quantifying its levels in the corpora allata and fat body from
 211 5-day-old adult females. BgS6K mRNA levels were reduced in dsS6K group compared
 212 to control by 90 % in corpora allata and 94 % in fat body (Fig. S2).

213 Using a polyclonal antibody designed against the first 18 amino acids of BgS6K
214 the presence of the BgS6K protein was analysed by Western-blot analysis in extracts
215 obtained from fat body of control (fed and starved) and dsS6K females. Results showed
216 the presence of BgS6K protein in fat body from fed and starved control females,
217 whereas BgS6K protein could not be detected in samples from dsS6K-treated females
218 (Fig. S3).

219

220 *Effect of BgS6K RNAi on nymphal instar length*

221 The first phenotype observed in the dsS6K-treated females was a delay in the
222 imaginal moult. Thus, whilst no difference was detected in the duration of the fifth
223 nymphal instar, the sixth instar lasted on average 7.94 days for control females and 8.83
224 days for dsS6K-treated females (Fig. 3).

225

226 *Effect of BgS6K RNAi on the expression of JH biosynthesis enzymes and Vg*

227 The mRNA levels of key enzymes in the JH biosynthesis pathway, BgHMG-
228 CoA synthase-1, BgHMG-CoA synthase-2 and BgHMG-CoA reductase, were
229 quantified in corpora allata from control and dsS6K 5-day-old adult females. Results
230 showed a reduction in mRNA levels of all three enzymes (55 % for BgHMG-CoA
231 synthase-1, 35 % (although non-statistically significant) for BgHMG-CoA synthase-2
232 and 46 % for BgHMG-CoA reductase) in CA from dsS6K-treated females compared to
233 control (Fig. 4).

234 In addition, fat body BgVg mRNA levels were measured in control and dsS6K-
235 treated females. Results showed a 60 % reduction in BgVg mRNA levels in dsS6K-
236 treated females, concomitantly with a reduction on basal oocyte length (Fig. 5).

237

238

239 **Discussion**

240 The present study reports the cloning of the *B. germanica* orthologue of the
 241 ribosomal protein S6K (BgS6K), and the analysis of its functions related to reproductive
 242 processes. During the cloning procedure, two forms of the sequence were obtained,
 243 differing by the presence of an additional 66 nucleotides sequence fragment in the long
 244 form, which appears to have arisen by duplication of the adjacent fragment, and
 245 differing from it only in 7 nucleotides and 3 amino acids encoded (Fig. 1; Fig. S1).
 246 After getting these sequences, a draft version of the *B. germanica* genome became
 247 available (<https://www.hgsc.bcm.edu/arthropods/german-cockroach-genome-project>).
 248 Searching in this database revealed that the sequence corresponding to the 66 bp
 249 duplication is segregated in exon 11, making possible a differential mRNA splicing
 250 which would produce both isoforms. Both of the BgS6Ks show the characteristic
 251 organization of S6K proteins and a high similarity to other S6Ks. An exhaustive search
 252 in the NCBI database found no other insect, not even within the Dictyoptera, showing a
 253 similar duplication that would produce two S6K isoforms.

254 S6K activation is produced by a complex serial multi-site phosphorylation
 255 (Jastrzebski *et al.*, 2007; Magnuson *et al.*, 2012). One of the critical phosphorylation
 256 steps involves TOR phosphorylation of a Thr residue placed in a region belonging to the
 257 kinase extension domain known as the hydrophobic motif. This threonine corresponds
 258 to T³⁸⁹, T³⁹⁸ and T³⁸⁸ in *H. sapiens*, *D. melanogaster* and *Ae. aegypti*, respectively and
 259 lies in a conserved sequence (F-X-X-F/Y-S/T-F/Y) found in many AGC kinases
 260 (Fenton & Gout, 2011). The homologous residue in the BgS6K short isoform is T³⁹⁰. In
 261 the BgS6K long isoform, the duplication places a homologous Ser residue in position

262 390, whereas that Thr is moved to position 412. In addition, duplication places a Lys
263 (instead of Phe) four residues before T⁴¹² (Fig. 1). The effect of this change on TOR
264 kinase activity upon T⁴¹² of the BgS6K long isoform should deserve further analysis.
265 Furthermore, both TOR kinase activity upon S³⁹⁰ and the effect of a putative
266 phosphorylation of this amino acid (together or not with T⁴¹²) on BgS6K activity is also
267 worth of analysis in future work.

268 Tissue expression studies of both isoforms in different adult female tissues
269 (corpora allata, brain, fat body, ovary and midgut) show that BgS6K short mRNA is
270 more abundant than BgS6K long in all the tissues (Fig. 2). Nevertheless, the proportion
271 between both isoforms is not equal in the different tissues, with the corpora allata being
272 the tissue where the long isoform is proportionally more expressed (Fig. 2).

273 Treatment of penultimate and last instar nymphs of *B. germanica* with dsRNA
274 against BgS6K (dsS6K) produces a strong reduction of BgS6K, at both mRNA and
275 protein levels (Fig. S2 and S3). In the case of the Western-blot, the antibody against the
276 N-terminus of BgS6K shows a strong and specific signal, which is completely
277 eliminated when the antibody is blocked with an excess of the antigen (Fig. S3). The
278 aim with the Western-blot technique was to analyze the phosphorylation level of
279 BgS6K at the residue homologous to mammalian T³⁸⁹ or *D. melanogaster* T³⁹⁸ which,
280 as indicated above, is one of the main regulatory sites in the molecule. This would be
281 even more interesting in the case of BgS6K because of the 22 amino acid duplication,
282 which can provide an additional phosphorylation site. Nevertheless and unfortunately,
283 none of the commercial antibodies against phosphorylated mammalian T³⁸⁹ or *D.*
284 *melanogaster* T³⁹⁸ that were tested in the current study, even those that have been
285 proved to work in other insect species, showed any signal in the *B. germanica* fat body
286 extracts.

287 The dsS6K-treated females show a delay of approximately one day in the
288 duration of the last nymphal instar compared to control, whereas there is no effect on
289 the duration of the penultimate instar (Fig. 3). A similar treatment of *B. germanica*
290 nymphs with dsRNA against insulin receptor (InR) produces an increase in the length of
291 both penultimate and last instars (Abrisqueta *et al.*, 2014), indicating a stronger effect of
292 the knockdown of InR upon this process. *Drosophila melanogaster* mutants for S6K
293 also show a severe delay in development (Montagne *et al.*, 1999). By contrast, in the
294 case of the cricket, *Gryllus bimaculatus*, RNAi depletion of S6K reduces body size but
295 not nymphal instar duration (Dabour *et al.*, 2011).

296 A possible explanation for the extension of the nymphal instar produced by
297 dsS6K treatment in *B. germanica* would be a reduction or a delay in the synthesis of 20-
298 hydroxyecdysone (20E), which would produce a delay in the moult. In *Bombyx mori* it
299 has been reported that prothoracicotrophic hormone (PTTH), which stimulates 20E
300 synthesis in prothoracic glands, also stimulates S6K phosphorylation, and that
301 rapamycin treatment reduces both PTTH stimulation of 20E synthesis and S6K
302 phosphorylation (Gu *et al.*, 2012). Nevertheless, the existence of an authentic PTTH
303 (i.e. structurally and functionally equivalent) has not been described in *B. germanica* or
304 any other hemimetabolous species.

305 The dsS6K treatment produces a decrease of mRNA levels of HMG-CoA
306 synthase-1 and -2 and HMG-CoA reductase, key enzymes of the JH biosynthetic
307 pathway (Fig. 4). Previous reports from this laboratory have shown that the knock down
308 of both TOR and InR also produces a reduction of the expression of these enzymes
309 (Abrisqueta *et al.*, 2014; Maestro *et al.*, 2009). This reduction, especially in the case of
310 TOR knockdown, is higher than the reduction shown for dsS6K treatment.

311 The dsS6K females also show a reduction in fat body BgVg mRNA levels
312 concomitant with a reduction in basal oocyte length. Again, previous reports from this
313 research group have shown that both TOR and InR knockdown also produce a reduction
314 in BgVg mRNA levels and ovary growth, and again, the effects of TOR or InR
315 knockdown are higher than those produced by dsS6K treatment (Abrisqueta *et al.*,
316 2014; Maestro *et al.*, 2009).

317 The present results show that knockdown of BgS6K produces similar
318 phenotypes to those produced by knockdown of TOR and InR in similar experiments.
319 This is, in fact, an expected result because S6K has been reported to be downstream
320 TOR and InR in their signalling pathways (Magnuson *et al.*, 2012). Nevertheless,
321 BgS6K knockdown, even when this knockdown is extremely significant at both mRNA
322 and protein levels, produces milder effects on the measured parameters (nymphal instar
323 length, expression of genes of the JH biosynthesis, BgVg and oocyte growth). A
324 possible explanation for these lower effects is that another protein could be taking on
325 some of the roles of S6K in absence of it. In fact, a persistence of ribosomal protein S6
326 phosphorylation upon knockout of both S6K1 and S6K2 has been shown in mice (Pende
327 *et al.*, 2004), as a result of the action of 90kDa RSK (ribosomal 6 kinase), a mitogen-
328 activated protein kinase (MAPK)-dependent kinase, which has been reported to
329 phosphorylate some of the substrates of mammalian S6K (Magnuson *et al.*, 2012; Pende
330 *et al.*, 2004). From examination of the *B. germanica* genome, a homologue of the
331 mammalian 90kDa RSK can be found (results not shown), and this molecule could be
332 responsible for the lower effect of BgS6K knock down compared to TOR and InR
333 knockdown.

334 In summary, the results indicate that BgS6K is involved in nymphal
335 development and in the regulation of JH biosynthesis and Vg production in the

336 cockroach *B. germanica*. The different regulation and activities of the two isoforms of
 337 BgS6K, together with the significance of its differential tissue expression can be a
 338 relevant physiological and evolutionary question that will be properly addressed in
 339 future work.

340

341 **Acknowledgements**

342 This work was supported by grants BFU2006-01090/BFI (Spanish Ministry of Science
 343 and Innovation (MICINN) and FEDER), BFU2010-15906 (MICINN) and 2014-SGR-
 344 619 (*Secretaria d'Universitats i Recerca*, Catalan Goverment) to J.L.M. M.A. and S. S.-
 345 C. were recipients of a pre-doctoral fellowship (MICINN) and a post-doctoral contract
 346 (CSIC, JAE program co-funded by the European Social Fund), respectively. Thanks are
 347 due to the Human Genome Sequencing Center of the Baylor College of Medicine for
 348 the access to the German Cockroach Genome Project. Thanks are due to Xavier Bellés
 349 for critical reading of the manuscript.

350

351 **References**

352 Abrisqueta, M., Süren-Castillo, S. & Maestro, J.L. (2014) Insulin receptor-mediated
 353 nutritional signalling regulates juvenile hormone biosynthesis and vitellogenin
 354 production in the German cockroach. *Insect Biochemistry and Molecular Biology*,
 355 **49**, 14-23.

356 Dabour, N., Bando, T., Nakamura, T., *et al.* (2011) Cricket body size is altered by
 357 systemic RNAi against insulin signaling components and epidermal growth factor
 358 receptor. *Development, Growth & Differentiation*, **53**, 857-869.

- 359 Fenton, T.R. & Gout, I.T. (2011) Functions and regulation of the 70kDa ribosomal S6
360 kinases. *International Journal of Biochemistry & Cell Biology*, **43**, 47-59.
- 361 Ferrari, S., Bannwarth, W., Morley, S.J., *et al.* (1992) Activation of p70s6k is associated
362 with phosphorylation of four clustered sites displaying Ser/Thr-Pro motifs.
363 *Proceedings of the National Academy of Sciences of the United States of America*,
364 **89**, 7282-7286.
- 365 Grove, J.R., Banerjee, P., Balasubramanyam, A., *et al.* (1991) Cloning and expression
366 of two human p70 S6 kinase polypeptides differing only at their amino termini.
367 *Molecular and Cellular Biology*, **11**, 5541-5550.
- 368 Gu, S.H., Yeh, W.L., Young, S.C., *et al.* (2012) TOR signaling is involved in PTTH-
369 stimulated ecdysteroidogenesis by prothoracic glands in the silkworm, *Bombyx mori*.
370 *Insect Biochemistry and Molecular Biology*, **42**, 296-303.
- 371 Hannan, K.M., Thomas, G. & Pearson, R.B. (2003) Activation of S6K1 (p70 ribosomal
372 protein S6 kinase 1) requires an initial calcium-dependent priming event involving
373 formation of a high-molecular-mass signalling complex. *Biochemical Journal*, **370**,
374 469-477.
- 375 Hansen, I.A., Attardo, G.M., Roy, S.G., *et al.* (2005) Target of rapamycin-dependent
376 activation of S6 kinase is a central step in the transduction of nutritional signals
377 during egg development in a mosquito. *Journal of Biological Chemistry*, **280**, 20565-
378 20572.
- 379 Isotani, S., Hara, K., Tokunaga, C., *et al.* (1999) Immunopurified mammalian target of
380 rapamycin phosphorylates and activates p70 S6 kinase alpha in vitro. *Journal of*
381 *Biological Chemistry*, **274**, 34493-34498.

- 382 Jastrzebski, K., Hannan, K.M., Tchoubrieva, E.B., *et al.* (2007) Coordinate regulation of
383 ribosome biogenesis and function by the ribosomal protein S6 kinase, a key mediator
384 of mTOR function. *Growth Factors*, **25**, 209-226.
- 385 Lee-Fruman, K.K., Kuo, C.J., Lippincott, J., *et al.* (1999) Characterization of S6K2, a
386 novel kinase homologous to S6K1. *Oncogene*, **18**, 5108-5114.
- 387 Ma, X.M. & Blenis, J. (2009) Molecular mechanisms of mTOR-mediated translational
388 control. *Nature Reviews Molecular Cell Biology*, **10**, 307-318.
- 389 Maestro, J.L., Cobo, J. & Bellés, X. (2009) Target of rapamycin (TOR) mediates the
390 transduction of nutritional signals into juvenile hormone production. *Journal of*
391 *Biological Chemistry*, **284**, 5506-5513.
- 392 Maestro, J.L., Pascual, N., Treiblmayr, K., *et al.* (2010) Juvenile hormone and
393 allatostatins in the German cockroach embryo. *Insect Biochemistry and Molecular*
394 *Biology*, **40**, 660-665.
- 395 Magnuson, B., Ekim, B. & Fingar, D.C. (2012) Regulation and function of ribosomal
396 protein S6 kinase (S6K) within mTOR signalling networks. *Biochemical Journal*,
397 **441**, 1-21.
- 398 Montagne, J., Stewart, M.J., Stocker, H., *et al.* (1999) Drosophila S6 kinase: a regulator
399 of cell size. *Science*, **285**, 2126-2129.
- 400 Pardo, O.E. & Seckl, M.J. (2013) S6K2: The Neglected S6 Kinase Family Member.
401 *Frontiers in Oncology*, **3**, 191.
- 402 Pende, M., Um, S.H., Mieulet, V., *et al.* (2004) S6K1(-/-)/S6K2(-/-) mice exhibit
403 perinatal lethality and rapamycin-sensitive 5'-terminal oligopyrimidine mRNA
404 translation and reveal a mitogen-activated protein kinase-dependent S6 kinase
405 pathway. *Molecular and Cellular Biology*, **24**, 3112-3124.

- 406 Pullen, N., Dennis, P.B., Andjelkovic, M., *et al.* (1998) Phosphorylation and activation
407 of p70s6k by PDK1. *Science*, **279**, 707-710.
- 408 Roy, S.G., Hansen, I.A. & Raikhel, A.S. (2007) Effect of insulin and 20-
409 hydroxyecdysone in the fat body of the yellow fever mosquito, *Aedes aegypti*. *Insect*
410 *Biochemistry and Molecular Biology*, **37**, 1317-1326.
- 411 Shah, O.J., Wang, Z. & Hunter, T. (2004) Inappropriate activation of the
412 TSC/Rheb/mTOR/S6K cassette induces IRS1/2 depletion, insulin resistance, and cell
413 survival deficiencies. *Current Biology*, **14**, 1650-1656.
- 414 Suganya, R., Chen, S.L. & Lu, K.H. (2011) CDNA cloning and characterization of S6
415 kinase and its effect on yolk protein gene expression in the oriental fruit fly
416 *Bactrocera dorsalis* (Hendel). *Archives of Insect Biochemistry and Physiology*, **78**,
417 177-189.
- 418 Sören-Castillo, S., Abrisqueta, M. & Maestro, J.L. (2012) FoxO inhibits juvenile
419 hormone biosynthesis and vitellogenin production in the German cockroach. *Insect*
420 *Biochemistry and Molecular Biology*, **42**, 491-498.
- 421

422 Figure legends

423 **Fig. 1.** Amino acid sequences of the duplicated region for the short and long isoforms of
 424 ribosomal S6 protein kinase (BgS6K) of the cockroach *Blattella germanica*. Underlined
 425 sequence corresponds to the duplicated region in the long isoform. Amino acids
 426 highlighted in grey in the long isoform are the ones that are different between the
 427 original and the duplicated sequences. Amino acids highlighted in black are outside of
 428 the duplicated region. Numbers indicate the position of the amino acids in the protein
 429 sequences.

430

431 **Fig. 2.** Analysis of the mRNA levels of the BgS6K isoforms in different tissues of
 432 *Blattella germanica*. The PCR bands of the long (220 bp band) and short (154 bp band)
 433 BgS6K isoforms were separated in an agarose gel. CA: corpora allata, Br: brain, FB: fat
 434 body, Ov: ovaries, Md: midgut. Actin 5C was used as reference.

435

436 **Fig. 3.** Effect of BgS6K RNAi on developmental time. Two μg of dsRNA targeting
 437 BgS6K (dsS6K) or a heterologous dsRNA (Control) were administered on the first day
 438 of the penultimate (fifth) and last (sixth) nymphal instars. Graphs show cumulative
 439 percentages of female *Blattella germanica* of the indicated treatment that moulted into
 440 the next stage (last nymphal instar or adult). The penultimate nymphal instar lasts $5.88 \pm$
 441 0.13 (mean $\pm$ S.E.) days for Control group ($n = 24$) and 6.04 ± 0.14 days for dsS6K
 442 group ($n = 24$) (not significant differences in Student's t test). The last nymphal instar
 443 lasts 7.94 ± 0.17 days for Control group ($n = 18$) and 8.83 ± 0.17 days for dsS6K group
 444 ($n = 18$) ($P < 0.001$ Student's t test).

445 **Fig. 4.** Effect of BgS6K RNAi on mRNA levels of enzymes in the biosynthesis
446 pathway of juvenile hormone. Two μg of dsRNA targeting BgS6K (dsS6K, $n = 6$) or a
447 non-homologous dsRNA (Control, $n = 4$) were administered on the first day of the
448 penultimate (fifth) and last (sixth) nymphal instars of *Blattella germanica*. Dissections
449 were performed five days after the moult to the adult stage. Graphs show mRNA levels
450 of HMG-CoA synthase-1 (HMG-S1), HMG-CoA synthase-2 (HMG-S2) and HMG-
451 CoA reductase (HMG-R). The Y-axis indicates copies per copy of BgActin 5C. Results
452 are expressed as the mean $\pm$ S.E. Asterisk represents significant differences between
453 Control and dsS6K (Student's t test, $*P < 0.05$).

454

455 **Fig. 5.** Effect of BgS6K RNAi on BgVg mRNA levels and basal oocyte length in
456 *Blattella germanica*. The experimental procedure was the same as in Fig. 4. (Left) Fat
457 body BgVg mRNA levels expressed as copies per copy of Actin 5C ($n = 9-10$). (Right)
458 Basal oocyte length ($n = 34-35$). Results are expressed as the mean $\pm$ S.E. Asterisks
459 represent significant differences between Control and dsS6K (Student's t test, $*P <$
460 0.05 ; $***P < 0.0001$).

```

BgS6K short:1291-CTTGTGTTCCAG-----
BgS6K long :1291-CTTGTGTTCCAGSGTTTCTCGTATGTGGCTCCATCTGTCTTGGAGGAGA

-----GGTTTCACATATGTGGCTCCATCTGTGTTGGAAGA
TGTATAAACCTCCTCACATGGTTAAAGCTGGTTTCACATATGTGGCTCCATCTGTGTTGGAAGA

GATGTATAAACCTCCTCGTATAGTTAAAGCTCGCTCTCCAAGA-1380
GATGTATAAACCTCCTCGTATAGTTAAAGCTCGCTCTCCAAGA-1446

```

Fig. S1. Nucleotide sequences of the duplicated region for the short and long BgS6K isoforms. Underlined sequence corresponds to the duplicated region in the long isoform. Nucleotides highlighted in grey in the long isoform are the ones that are different between the original and the duplicated sequences. Nucleotides highlighted in black are outside of the duplicated region. Numbers indicate the position of nucleotides in the cDNA sequences.

Fig. S2. Effect of BgS6K RNAi on BgS6K mRNA levels. 2 μ g of dsRNA targeting BgS6K (dsS6K) or a heterologous dsRNA (Control) were administered on the first day of the penultimate (fifth) and last (sixth) nymph instars. Dissections were performed five days after the adult moult. (Left) CA BgS6K mRNA levels (Control, $n = 5$; dsS6K, $n = 6$). (Right) Fat body BgS6K mRNA levels (Control, $n = 11$; dsS6K, $n = 14$). Y-axis indicates copies per copy of BgActin 5C. Results are expressed as the mean $\pm$ S.E. Asterisk represents significant differences between Control and dsS6K (Student's t test, ** $P < 0.01$; *** $P < 0.001$).

Fig. S3. Western-blot analysis of BgS6K. Protein was extracted from fat bodies of females treated accordingly to the procedure described in Fig. S1. Starved corresponds to Control females maintained with only water since the imaginal moult. Additionally, an equivalent sample of protein from Control females was incubated with the primary antibody previously blocked with the antigen (see Materials and Methods).

Table S1

Cloning

S6K-F: TAYGGNAARGTNTTYCARGT

S6K-R1: GCNACRTANGTRAANCCYTG

S6K-R2: CCACCARTCNACNGCYTTNCC

Isoform analysis

S6K-F: TGACTCACCTGATGAGTCAACACT

S6K-R: AGCGGGTGTAGGATAAGCTGTTTT

Actin-F: TCGTTCGTGACATCAAGGGAGAAGCT

Actin-R: TGTCGGCAATTCCAGGGTACATGGT

dsRNA synthesis

S6K-F: CAAAGTCACAGGCCAAGATGCAG

S6K-R: AGTGCTCCTAATGACCACCAGTC

qPCR:

S6K-F: AAGGCATCAATTGTGCGAAAC

S6K-R: TGAAACGCGTAGATCAGGTCC