# Immune stimulation reduces sleep and memory ability in *Drosophila melanogaster*

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**Abstract**

Psychoneuroimmunology studies the increasing number of connections between neurobiology, immunology and behaviour. We establish *Drosophila melanogaster* as a tractable model in this field by demonstrating the effects of the immune response on two fundamental behaviours: sleep and memory ability.

We used the Geneswitch system to upregulate peptidoglycan receptor protein (PGRP) expression, thereby stimulating the immune system in the absence of infection. Geneswitch was activated by feeding the steroid RU486, to the flies. We used an aversive classical conditioning paradigm to quantify memory and measures of activity to infer sleep.

Immune stimulated flies exhibited reduced levels of sleep, which could not be explained by a generalised increase in waking activity. The effects on sleep were more pronounced for day compared to night sleep. Immune stimulated flies also showed a reduction in memory abilities.

These are important results as they establish *Drosophila* as a model for immune-neural interactions and provide a possible role for sleep in the interplay between the immune response and memory.

**Keywords**

immune-neural interactions, imd, geneswitch, PGRP-LCa

## Introduction

Psychoneuroimmunology, in vertebrates, studies the connections between neurobiology, immunology and behaviour (Ader *et al.* 1991). These neural-immune interactions have also been found in invertebrates (Demas *et al.* 2011). For example, immune response negatively affects learning and memory in bees (Mallon *et al.* 2003; Riddell & Mallon 2006; Gegear *et al.* 2006; Iqbal & Mueller 2007; Alghamdi *et al.* 2008). A tractable invertebrate model of these immune-neural links would provide a stimulus to this field (Aubert 2007). The fruit fly, *Drosophila melanogaster*, has been tremendously helpful to the analysis of associative learning (Kim *et al.* 2007) and immunity (Lemaitre & Hoffmann 2007). In this paper we demonstrate immune-memory links in *Drosophila* and further expand the paradigm by showing immune-sleep interactions in flies.

Sleep is a resting state where the sleeper exhibits inattention to the environment and is usually immobile (Siegel 2003). *Drosophila melanogaster* like vertebrates have been shown to have a distinct sleep state. In flies, a sleep episode is defined as a period of immobility lasting five minutes or longer (Hendricks *et al.* 2000; Shaw *et al.* 2000). Such intervals are associated with reversible increases in arousal threshold, which can be further augmented following sleep deprivation (Huber *et al.* 2004), are associated with changes in brain electrical activity (Nitz *et al.* 2002; Alphen *et al.* 2013), and are reduced by several drugs like caffeine and modafinil and are increased by antihistamines (Hendricks *et al.* 2000; Shaw *et al.* 2000). As in mammals, sleep deprivation leads to a rebound in quantity of sleep (Shaw *et al.* 2000).

Infections increase sleep in humans, most likely through induction of proinflammatory cytokines (Bryant *et al.* 2004). Fruit flies infected with gram-negative bacteria also

show increased sleep (Kuo *et al.* 2010). On the contrary, Shirasu-Hiza infected flies with gram-positive bacteria and observed that they slept less (Shirasu-Hiza *et al.* 2007). The latter agrees with findings of increased immune gene transcription and resistance to disease in sleep-deprived flies or in reduced sleep phenotype transgenic flies (Cirelli *et al.* 2005; Williams *et al.* 2007).

Here, we activated the immune system non-pathogenically (Moret & Schmid-Hempel 2000; Mallon *et al.* 2003; Riddell & Mallon 2006; Alghamdi *et al.* 2008; Richard *et al.* 2008). This has the advantage that it separates the effect of the immune response from any direct effect of the pathogen, for example, parasite manipulation of the host (Adamo & Webster 2013). We used Geneswitch (Osterwalder *et al.* 2001) to up-regulate peptidoglycan receptor protein LCa (PGRP-Lca) in adult flies. PGRP-Lca is a pattern recognition protein that recognizes gram-negative bacteria, setting off the IMD immune pathway and leading to the expression of antimicrobial peptides (Gottar *et al.* 2002). Geneswitch is activated in the presence of the steroid RU486. We used an aversive classical conditioning paradigm to measure memory abilities of flies (Mery & Kawecki 2005). Sleep was measured using the *Drosophila* Activity Monitoring System 2 (DAMS2, Trikinetics, Waltham, MA).

## Methods and Materials

The Geneswitch line $w^{1118}$;P{w$^{+mW.hs}$=Switch1}$bun^{Switch1.32}$ (hereafter referred to as *GS1.32*) drives expression of RU486-activated GAL4 in adult fat bodies (Gottar *et al.* 2002) (http://flystocks.bio.indiana.edu). The three genotypes used were GS1.32>PGRP-Lca($w^{1118}$;*GS1.32*/+; *UAS-PGRP-Lca*/+), and the control genotypes GS1.32/+($w^{1118}$; *GS1.32*/+; +/+) and +/PGRP-Lca (*+/+;UAS-PGRP-Lca/+)*.

Flies were maintained in vials containing agar, sugar, and Brewer's yeast media in a 12 h: 12 h light: dark cycle at 25°C. Males and females were selected at eclosion and flies were 1–3 days old at the beginning of the experiment. Both sexes were used for the memory assay. As is common in fly research, only males were used for the sleep assay as they sleep for over twice as long as females (Isaac *et al.* 2010).

### Geneswitch

In the Geneswitch system, the DNA binding domain of the GAL4 protein is fused to the activation moiety of p65 through a mutant progesterone receptor ligand binding domain. Thus, Geneswitch is a chimeric ligand-stimulated activator of transcription. In the absence of ligand, the Geneswitch is in the "off" state. In the presence of the antiprogestin RU486 the Geneswitch molecule changes to an active conformation, in which it binds, as a dimer, to UAS sequences and activates transcription of downstream genes. In flies, Geneswitch mediated expression can be detectable 3–5 hr after feeding on RU486, reaching maximal levels 21–48 hr later (Roman *et al.* 2001; Osterwalder *et al.* 2001).

20 ml of RU486 (Sigma Aldrich) 10mM stock solution (0.13 g of RU486 in 32 ml of 80% ethanol) was mixed with 980 ml molten *Drosophila* food (200 μM final concentration). For the memory assay, flies were fed for two days with RU486 before the start of the training and returned to the RU486 food after training. For the sleep assay, flies were placed in vials containing RU486 food for two days to allow feeding. After two days flies were immediately loaded into tubes containing more of the RU486 food. For all lines we have flies fed with RU486 and genetically identical animals cultured on fly medium supplemented with an equal amount of vehicle (80% ethanol) that lacked RU486.

Memory assay

Each sample was a single sex group of 50 adult flies. This memory assay was described previously (Mery & Kawecki 2005). Conditioning consisted of 5 training sessions separated by 20min intervals. In each training session flies were first exposed for 30s to one odorant simultaneously with mechanical shock delivered every 5s. This period was followed by a 60s rest period (no odour and no shock). Then, for 30s another odorant was delivered, without shock. Flies were either conditioned against 3-octanol or 4-methylcyclohexanol (both 0.6ml/l of paraffin).

24 hours after the conditioning period flies were transported to the choice point of a T-maze, where they were allowed to choose between the two odors for 60s. The memory score was the proportion of individuals choosing the correct odour, i.e. not the one they were trained against. One hundred and fifteen replicates were carried out, distributed between the genotype, sex, RU486 (presence/absence) and odour used. The data was normalised using a box-cox transformation.

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134 Sleep assay

135 Fly locomotor activity was monitored by the *Drosophila* Activity Monitoring System 2
136 (DAMS2, Trikinetics, Waltham, MA), at 25°C, continuously for seventy-two hours
137 under a 12:12 light:dark cycle. Output from DAMS2 was the number of times a fly
138 crossed an infrared beam in a given 1 min period (bin). A sleep episode (bout) was
139 defined as 5 or more consecutive bins of immobility. 384 flies were tested, divided
140 between genotype and RU486 (presence/absence).

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142 Data analysis for sleep assay

143 The DAMS2 output was converted to three measures; 1) Sleepbins per hour: number of
144 minutes when a fly is asleep in an hour, 2) Mean waking activity: the mean activity
145 taking into account only those bins that are classified as 'waking' and 3) Bouts of sleep:
146 the number of sleep episodes.

147 Flies sleep differently during the day and night (Ishimoto *et al.* 2012). Therefore for
148 each dependent sleep variable, two ANOVAs one for day and one for night was run.
149 The independent variables were genotype and RU486 (presence/absence). The
150 important term here is an interaction term between genotype and RU486. If this was
151 significant, the genotypes responded differently to the treatments. To discover which
152 genotypes were significantly different two further ANOVAs were performed, one for
153 genotypes GS1.32>PGRP-Lca vs GS1.32/+ and one for genotypes GS1.32>PGRP-Lca
154 vs +/ PGRP-Lca. If the interaction terms in both these ANOVAs are significant
155 GS1.32>PGRP-Lca (the immune stimulated genotype) responses differently to the

control genotypes. Using a Bonferroni correction the significance level α was reduced to 0.0083 (0.05/6). All analysis was carried out using STATA12.

Zone of inhibition assay

Our treatment line had previously been shown to upregulate the immune response (Gottar *et al.* 2002*)*. However we used the zone of inhition assay to confirm increased immune response in our treated flies. This assay measures antibacterial activity: it is based on the ability of immune proteins to inhibit bacterial growth when placed onto an agar plate seeded with bacteria (*Arthrobacteur globiformis* 125µl of an overnight culture per 50ml of agar). Thirty seven GS1.32>PGRP-Lca flies, 17 fed RU486 and 20 not fed RU486 were used. Each fly was homogenized in 30µl of ringer solution. Five microlitres of the supernatant from the centrifuged solution (1300g for 10 min at 4°C) were pippetted into a hole on the agar plate. This was incubated for 48hrs (30°C). The resultant ZOI were measured as the mean of three diameters.

## Results

Feeding RU486 to GS1.32>PGRP-Lca flies increased their antibacterial activity by 26% ($t = -2.3263$, $df = 29.202$, $p = 0.02715$).

### Immune stimulation effects on memory

Genotype had a significant effect on memory score ($F_{2,109} = 22.46$, $p < 0.0001$). Neither sex, whether RU486 was used, nor odour used had a significant effect on memory score. GS1.32>PGRP-Lca flies, showed a 11.4% decrease in memory scores when fed RU468 relative to those not fed RU468 of the same genotype (interaction between genotype and RU486 was significant $F_{2,109} = 5.76$, $p = 0.0042$). See Figure 1. As feeding RU486 to GS1.32>PGRP-Lca flies leads to an increased immune response, immune stimulation decreases memory scores.

### Immune stimulation effects on sleep

Immune stimulated males (GS1.32>PGRP-Lca fed with RU486) showed a 23% decrease in sleep during the day relative to controls (GS1.32>PGRP-Lca vs GS1.32/+: $F_{1,4607} = 136.29$, $p < 0.00001$, GS1.32>PGRP-Lca vs +/ PGRP-Lca: $F_{1,4535} = 26.87$, $p < 0.00001$) and a 9% decrease at night (GS1.32>PGRP-Lca vs GS1.32/+: $F_{1,4607} = 85.53$, $p < 0.00001$, GS1.32>PGRP-Lca vs +/ PGRP-Lca: $F_{1,4535} = 8.49$, $p = 0.0036$). See Figure 2. There was no corresponding change in mean waking activity during the day ($F_{2,6839} = 0.5$, $p = 0.6044$), or during the night (GS1.32>PGRP-Lca vs GS1.32/+: $F_{1,4607} = 63.34$, $p < 0.00001$, GS1.32>PGRP-Lca vs +/ PGRP-Lca: $F_{1,4535} = 1.96$, N.S.). There was no change in the number of sleep bouts during the day (GS1.32>PGRP-Lca vs GS1.32/+: $F_{1,4607} = 6.42$, $p = 0.0113$, GS1.32>PGRP-Lca vs +/ PGRP-Lca: $F_{1,4535} = 10.43$, $p = 0.0012$) and a small but significant increase (0.5%) at night (GS1.32>PGRP-

193 Lca vs GS1.32/+: $F_{1,4607} = 16.38$, $p < 0.00001$, GS1.32>PGRP-Lca vs +/ PGRP-Lca:

194 $F_{1,4535} = 7.56$, $p = 0.0060$).

## Discussion

Immune stimulated adult flies exhibit reduced levels of sleep both during day and night. Immune stimulated flies have slightly more fragmented sleep at night, as evinced by an increase in the number of sleep bouts. Immune stimulation also leads to a reduction in memory abilities.

The reduction in sleep cannot be explained simply in terms of a generalised increase in activity. Stimulating the immune response had no effect on mean waking activity during the day or night, but immune-stimulated flies slept less than the non-stimulated controls.

Our sleep results agree with a previous study by Shirasu-Hiza showing a similar outcome after gram-positive bacterial infections (Shirasu-Hiza *et al.* 2007). However Kuo (Kuo *et al.* 2010) found that when they infected flies with gram-negative bacteria, the flies slept more. The discrepancies in sleep were explained by Kuo *et al*. as being due to different types of infection. Our work did not use an infection but rather a direct stimulation of the immune response. By upregulating PGRP-Lca we reproduced the immune response associated with gram-negative bacteria. This suggests that if type of infection were the cause of the discrepancies, our results would have just mirrored those of Kuo et al. The discrepencies between these two previous results are difficult to explain as the experiments differed in numerous other methodical aspects, e.g. strength of infection, lighting paradigm, when the phenotype was measured.

Although Imd is one of the canonical immune pathways in insects, over-expression of the Imd pathway can also lead to apoptosis (Georgel *et al.* 2001; Leulier *et al.* 2003). It cannot be excluded that our results could be caused by a side effect: apoptosis of the

219 fat body by the Imd pathway rather than its main effect of immune response. This will
220 be examined in future work.

221 We have shown that immune response decreases sleep and memory in *Drosophila*
222 *melanogaster*. We propose a possible link between all three systems as an interesting
223 area for future research. One of the main hypotheses on sleep function is that sleep
224 periods are favourable for brain plasticity and in the adult brain for learning and
225 memory (Maquet 2001). Like humans, flies with a fragmented sleep show impaired
226 learning compared with flies with consolidated sleep (Seugnet *et al.* 2008). Flies also
227 exhibit decreases in learning after 6 or 12 hours of sleep deprivation (Seugnet *et al.*
228 2006 p. 200). We propose sleep as an intermediate between immunity and memory.
229 We hypothesise that it is not the activation of the immune system *per se* that affects
230 memory in flies, but rather that immune stimulation reduces the length and quality of
231 sleep that in turn, reduces memory ability. However, with our current data, we cannot
232 exclude that in flies the level of immune activation has a direct effect on memory.

233 Our results establishes *Drosophila* as a model for immune-neural interactions. As well
234 as the potential use as a model for mammalian neural-immune links, this work has
235 direct impact on recent concern for insect foragers and the role of multiple stressors in
236 their decline (Gill *et al.* 2012).

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## References

245 Adamo SA, Webster JP (2013) Neural parasitology: how parasites manipulate host
246 behaviour. *The Journal of Experimental Biology*, **216**, 1–2.

247 Ader R, Felten DL, Cohen N (1991) *Psychoneuroimmunology*. Academic Press, San
248 Diego.

249 Alghamdi A, Dalton L, Phillis A, Rosato E, Mallon, E.B. (2008) Immune response
250 impairs learning in free-flying bumble-bees. *Biology Letters*, **4**, 479–481.

251 Alphen B van, Yap MHW, Kirszenblat L, Kottler B, Swinderen B van (2013) A
252 Dynamic Deep Sleep Stage in Drosophila. *The Journal of Neuroscience*, **33**,
253 6917–6927.

254 Aubert A (2007) Invertebrate studies and the evolution of comparative
255 psychoneuroimmunology. *Brain Behavior and Immunity*, **21**, 290–291.

256 Bryant PA, Trinder J, Curtis N (2004) Sick and tired: Does sleep have a vital role in
257 the immune system? *Nature Reviews Immunology*, **4**, 457–467.

258 Cirelli C, Bushey D, Hill S *et al.* (2005) Reduced sleep in Drosophila shaker mutants.
259 *Nature*, **434**, 1087–1092.

260 Demas GE, Adamo SA, French SS (2011) Neuroendocrine-immune crosstalk in
261 vertebrates and invertebrates: implications for host defence. *Functional*
262 *Ecology*, **25**, 29–39.

263 Gegear RJ, Otterstatter MC, Thomson JD (2006) Bumble-bee foragers infected by a
264 gut parasite have an impaired ability to utilize floral information. *Proceedings*
265 *of the Royal Society B-Biological Sciences*, **273**, 1073–1078.

266 Georgel P, Naitza S, Kappler C *et al.* (2001) Drosophila immune deficiency (IMD) is
267 a death domain protein that activates antibacterial defense and can promote
268 apoptosis. *Developmental Cell*, **1**, 503–514.

269 Gill RJ, Ramos-Rodriguez O, Raine NE (2012) Combined pesticide exposure severely
270 affects individual- and colony-level traits in bees. *Nature Genetics*, **491**, 105–
271 108.
272 Gottar M, Gobert V, Michel T *et al.* (2002) The Drosophila immune response against
273 Gram-negative bacteria is mediated by a peptidoglycan recognition protein.
274 *Nature*, **416**, 640–644.
275 Hendricks JC, Finn SM, Panckeri KA *et al.* (2000) Rest in Drosophila is a sleep-like
276 state. *Neuron*, **25**, 129–138.
277 Huber R, Ghilardi MF, Massimini M, Tononi G (2004) Local sleep and learning.
278 *Nature*, **430**, 78–81.
279 Iqbal J, Mueller U (2007) Virus infection causes specific learning deficits in honeybee
280 foragers. *Proceedings of the Royal Society B-Biological Sciences*, **274**, 1517–
281 1521.
282 Isaac RE, Li CX, Leedale AE, Shirras AD (2010) Drosophila male sex peptide
283 inhibits siesta sleep and promotes locomotor activity in the post-mated female.
284 *Proceedings of the Royal Society B-Biological Sciences*, **277**, 65–70.
285 Ishimoto H, Lark A, Kitamoto T (2012) Factors that Differentially Affect Daytime
286 and Nighttime Sleep in Drosophila melanogaster. *Frontiers in Neurology*, **3**,
287 1–5.
288 Kim YC, Lee HG, Han KA (2007) Classical reward conditioning in Drosophila
289 melanogaster. *Genes Brain and Behavior*, **6**, 201–207.
290 Kuo TH, Pike DH, Beizaeipour Z, Williams JA (2010) Sleep triggered by an immune
291 response in Drosophila is regulated by the circadian clock and requires the NF
292 kappa B Relish. *Bmc Neuroscience*, **11**.

293 Lemaitre B, Hoffmann J (2007) The host defense of Drosophila melanogaster. *Annual*
294 *Review of Immunology*, **25**, 697–743.
295 Leulier F, Parquet C, Pili-Floury S *et al.* (2003) The Drosophila immune system
296 detects bacteria through specific peptidoglycan recognition. *Nature*
297 *Immunology*, **4**, 478–484.
298 Mallon EB, Brockmann A, Schmid-Hempel P (2003) Immune response inhibits
299 associative learning in insects. *Proceedings of the Royal Society of London*
300 *Series B-Biological Sciences*, **270**, 2471–2473.
301 Maquet P (2001) The role of sleep in learning and memory. *Science*, **294**, 1048–1052.
302 Mery F, Kawecki TJ (2005) A cost of long-term memory in Drosophila. *Science*, **308**,
303 1148–1148.
304 Moret Y, Schmid-Hempel P (2000) Survival for immunity: The price of immune
305 system activation for bumblebee workers. *Science*, **290**, 1166–1168.
306 Nitz DA, Van Swinderen B, Tononi G, Greenspan RJ (2002) Electrophysiological
307 correlates of rest and activity in Drosophila melanogaster. *Current Biology*,
308 **12**, 1934–1940.
309 Osterwalder T, Yoon KS, White BH, Keshishian H (2001) A conditional tissue-
310 specific transgene expression system using inducible GAL4. *Proceedings of*
311 *the National Academy of Sciences of the United States of America*, **98**, 12596–
312 12601.
313 Richard F-J, Aubert A, Grozinger CM (2008) Modulation of social interactions by
314 immune stimulation in honey bee, Apis mellifera, workers. *BMC Biology*, **6**,
315 50.

316 Riddell CE, Mallon EB (2006) Insect psychoneuroimmunology: Immune response
317 reduces learning in protein starved bumblebees (Bombus terrestris). *Brain,*
318 *Behavior and Immunity*, **20**, 135–138.
319 Roman G, Endo K, Zong L, Davis RL (2001) P (Switch), a system for spatial and
320 temporal control of gene expression in Drosophila melanogaster. *Proceedings*
321 *of the National Academy of Sciences*, **98**, 12602–12607.
322 Seugnet L, Suzuki Y, Shaw PJ (2006) A learning task sensitive to sleep deprivation in
323 Drosophila. *Sleep*, **29**, A367–A368.
324 Seugnet L, Suzuki Y, Vine L, Gottschalk L, Shaw PJ (2008) D1 Receptor Activation
325 in the Mushroom Bodies Rescues Sleep-Loss-Induced Learning Impairments
326 in Drosophila. *Current Biology*, **18**, 1110–1117.
327 Shaw PJ, Cirelli C, Greenspan RJ, Tononi G (2000) Correlates of sleep and waking in
328 Drosophila melanogaster. *Science*, **287**, 1834–1837.
329 Shirasu-Hiza MM, Dionne MS, Pham LN, Ayres JS, Schneider DS (2007)
330 Interactions between circadian rhythm and immunity in Drosophila
331 melanlogaster. *Current Biology*, **17**, R353–R355.
332 Siegel JM (2003) Why we sleep. *Scientific American*, **289**, 92–97.
333 Williams JA, Sathyanarayanan S, Hendricks JC, Sehgal A (2007) Interaction between
334 sleep and the immune response in Drosophila: A role for the NF kappa B
335 relish. *Sleep*, **30**, 389–400.
336

338 Figure Legends
339
340 Figure 1. Memory score for each genotype. Memory score is the proportion of flies
341 that choose the odour they were not trained against. The white boxes represent the
342 mean memory score for the RU486- flies. The grey boxes represent the RU486+ flies.
343 The grey dots are the individual data points.
344
345
346 Figure 2. Sleepbins for each genotype. The black points represent the means of
347 sleepbins for the RU486- flies. The grey points represent the RU486+ flies. The
348 shaded times are night (lights off).

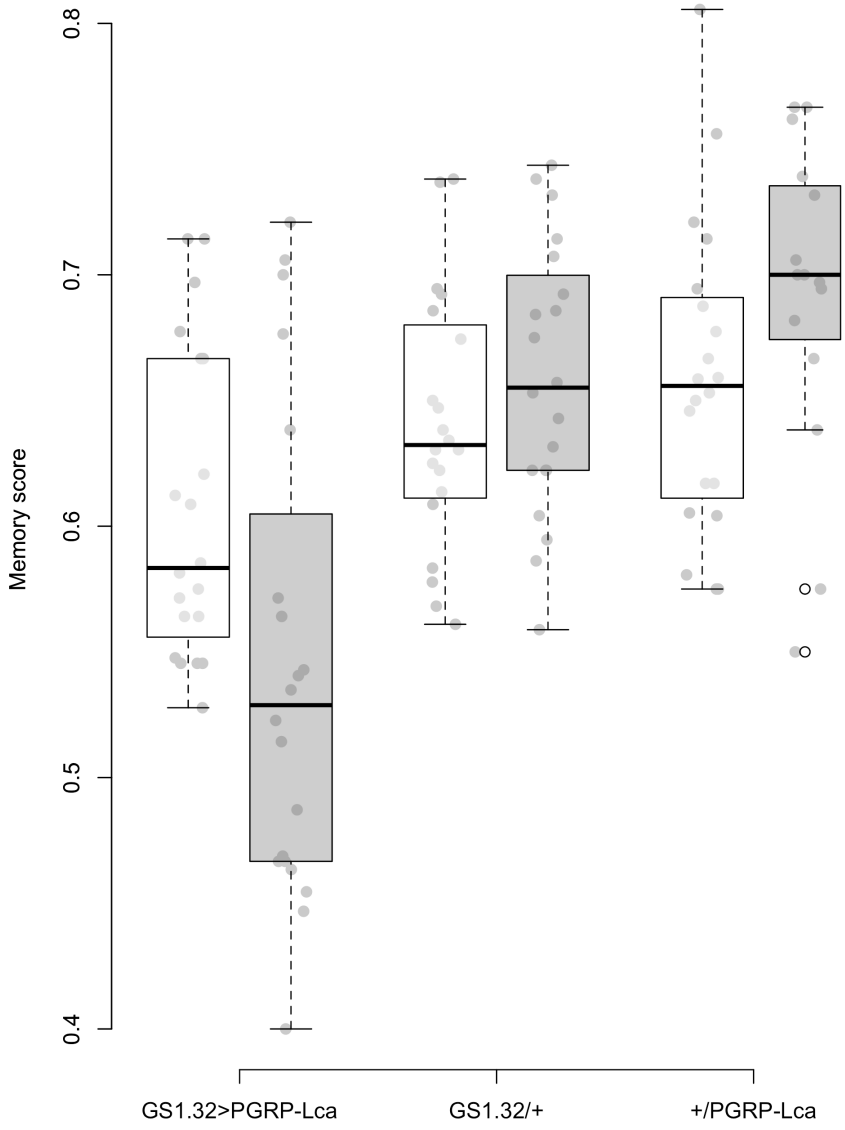


Figure 1

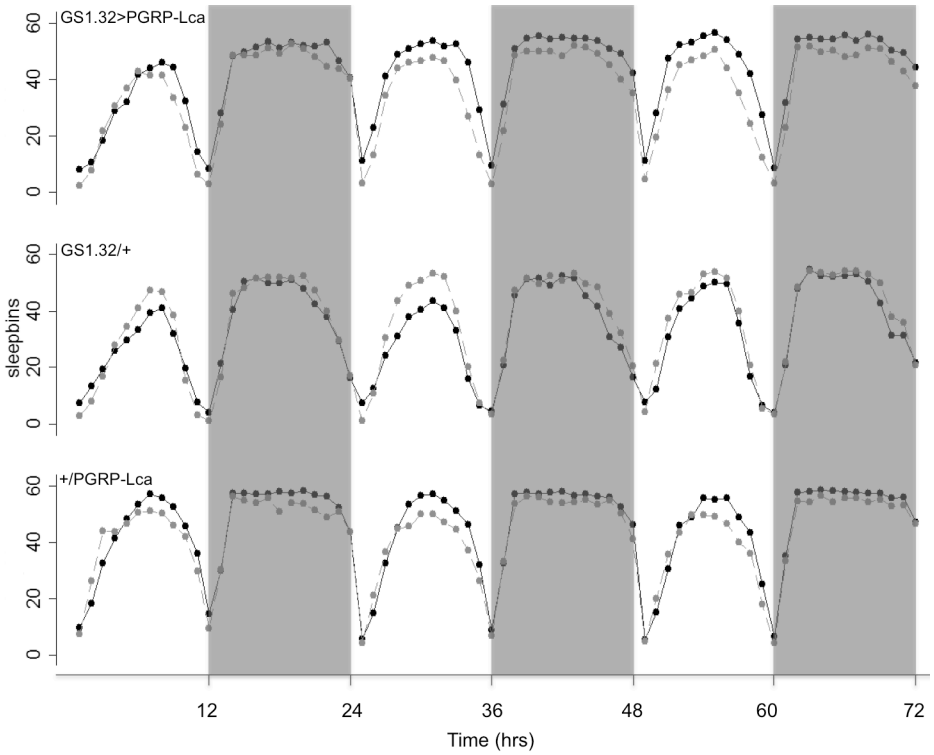


Figure 2