Pheromone binding protein shapes olfactory temporal resolution

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22 **Abstract**

23 **Male moths are capable of orienting towards conspecific females using sex**
 24 **pheromones. Since pheromones are distributed as discontinuous plumes owing to**
 25 **air turbulence, tracking intermittent stimuli with high temporal resolution is**
 26 **suggested to be important for efficient localisation. Here, using a pheromone**
 27 **binding protein (BmPBP1) knockout silkworm, we revealed that the loss of**
 28 **functional pheromone binding protein altered antennal response kinetics resulting**
 29 **in reduced temporal resolution to intermittent pheromone stimuli on the antennae.**
 30 **Behavioural analysis revealed that *BmPBP1*-knockout males exhibited**
 31 **significantly less straight walking, which occurs when detecting pheromone stimuli,**
 32 **especially to high frequency stimuli. Accordingly, *BmPBP1*-knockout males took a**
 33 **significantly longer time to locate pheromone sources and females than did wild-**
 34 **type males. Together, BmPBP1 plays a critical role in determining temporal**
 35 **antennal response kinetics and that an appropriate range of temporal sensory and**
 36 **behavioural resolutions is essential for tracking pheromone plumes for efficient**
 37 **pheromone source localisation in the silkworm.**

38

39 **Introduction**

40 Many animals rely on olfaction to locate potential mating partners or food sources
41 for survival in a natural environment. Odour source localisation is a difficult task for
42 animals because odours are distributed discontinuously owing to turbulent air flow in
43 the natural environment; the stochastic nature of odour distribution further increases the
44 unpredictability of odour arrival¹⁻³. Mechanisms underlying how animals can accurately
45 locate odour sources in such a complex environment have long been a core topic of
46 study in the field of olfactory research.

47 The sex pheromone communication system in moths is one of the best examples
48 of sophisticated odour source localisation in animals, whereby male moths detect and
49 orient toward conspecific females using intermittent sex pheromone information emitted
50 by females. Owing to the simple relationship between sex pheromone chemicals and the
51 localisation behaviour, pheromone source localisation behaviours can be relatively
52 easily induced in the laboratory. As such, this behaviour has been studied as an essential
53 model system to better understand the general mechanisms underlying odour source
54 localisation in animals.

55 Odour source localisation behaviours of male moths consist of two basic
56 behavioural components: an upwind surge and crosswind casting⁴. The surge is a
57 straight upwind flight triggered shortly after exposure to the pheromone filament,
58 whereas crosswind casting is a self-steering counterturning that occurs when a male
59 moth loses contact with the pheromone filaments in the plume. In the silkmoth *Bombyx*
60 *mori*, when exposed to the pheromone, male moths exhibit straight walking at a small
61 turning angle as a reflex behaviour (surge), followed by left and right zigzag turns
62 (casting), with a gradually increasing turn angle when they encounter an air pocket of a
63 pheromone plume⁵⁻⁷. This behavioural sequence is reset in response to every new
64 exposure to a pheromone stimulus. Thus, search-related walking paths are highly
65 dependent on the temporal structure of the pheromone stimuli^{5,8,9}. When the stimulation

66 frequency is low, males walk in complex zigzag paths, whereas a higher frequency
67 results in successive surge behaviours and straighter paths. Therefore, male antennae
68 should possess a sufficient temporal resolution to track intermittent pheromone
69 stimulation. The surge-casting (phasic-tonic) behavioural model for odour source
70 localisation is thought to be a common strategy among moth species underlying their
71 localisation behaviour^{4,10}.

72 The importance of intermittent odour information has also been highlighted in
73 experiments using homogeneous pheromone stimulation that rarely contains fluctuating
74 pheromone filaments in the plume (referred to as a homogeneous pheromone cloud). In
75 homogeneous pheromone clouds, moths cannot sustain an upwind surge and exhibit a
76 casting flight, leading to the inability to localise the pheromone source^{11,12}. Later, to
77 investigate the physiological responses to intermittent stimuli, the characteristics of
78 sensory output to various odour frequencies, including a homogeneous pheromone
79 cloud, were examined in the moths *Grapholita molesta* and *Heliothis virescens*^{10,13}. In
80 response to intermittent stimuli, the electroantennogram (EAG) responses, which
81 monitor the total activity of all olfactory receptor neurons (ORNs) in the antenna, of
82 moths reflected intermittent exposure to pheromone filaments in the plume. However, in
83 a homogeneous pheromone cloud, the responses did not show dynamic changes. These
84 reports conclude that moths naturally receive intermittent pheromone information on
85 their antennae, and this modulates surge-casting localisation behaviour. Therefore,
86 resolving the temporal properties of odour information continuously is thought to be a
87 very important aspect of odour source localisation^{14,15}. However, the temporal sensory
88 and behavioural resolution required for successful odour source localisation and the
89 underlying neural and molecular mechanisms remain to be elucidated.

90 Pheromone binding proteins (PBPs) are small soluble proteins highly enriched in
91 the sensillum lymph space¹⁶. These proteins are reported to contribute to the sensitivity
92 and/or selectivity of antennal responses¹⁷⁻²¹. Also, PBPs are thought to be involved in

the early inactivation of pheromones, following pheromone detection; thus, PBPs are a candidate molecular factor for controlling temporal antennal responses²²⁻²⁵. However, this hypothesis has not been experimentally tested yet. In the silkworm *B. mori*, one of three PBP genes named *BmPBP1*, which is expressed in accessory cells surrounding ORNs and secreted into the sensillum lymph of pheromone sensitive sensilla, is reported to play an important role in pheromone detection²⁶⁻²⁸. In a previous study, we established a *BmPBP1*-knockout silkworm line and showed that the loss of *BmPBP1* affected the sensitivity of antennal responses to pheromone components²¹. In this study, using the *BmPBP1*-knockout silkworm, we revealed that the temporal antennal resolution of *BmPBP1*-knockout male antennae to intermittent pulse trains was significantly reduced. In addition, *BmPBP1*-knockout male moths took significantly longer to locate pheromone sources owing to lowered temporal behavioural resolution, resulting in inefficient source localisation. Based on our results, we will discuss the effects of *BmPBP1* loss and the sensory characteristics that govern the efficiency of odour source localisation behaviour.

108

109 Results

Time constant of antennal responses was altered in *BmPBP1*-knockout male moths. In a previous study, we reported that antennal responses of *BmPBP1*-knockout silkworms to pheromone components were significantly lower than those of wild-type moths, but antennae retained a clear responsiveness to pheromone components (bombykol and bombykal)²¹. Using *BmPBP1*-knockout males, we first tested whether PBPs were also involved in the temporal properties of antennal responses by investigating the response and recovery time constants of EAG responses of *BmPBP1*-knockout male moth antennae to single pulse stimulation with sex pheromone components (Fig. 1a). Comparison of these parameters with the same bombykol dosage (1000 and 10000 ng) revealed that there were significant differences in the kinetic

parameters consisting of latency and recovery time constant at 10000 ng between *BmPBPI*-knockouts and wild-types (Fig. 1b top, 1c). Because the peak EAG amplitudes of *BmPBPI*-knockout male antennae were significantly lower than those of wild-type antennae (Fig. 1b top)²¹, we hypothesised that differences in peak amplitudes could affect the temporal parameters of EAG responses. When we normalised the temporal parameters using peak amplitudes, we found that recovery time constants showed a positive correlation with peak amplitudes, and, as a result, normalised recovery time constants of *BmPBPI*-knockout male antennae were significantly longer than those of wild-type male antennae ($p=0.0014$ for bombykal; $p=0.0017$ for bombykol) (Fig. 1b bottom, 1d). However, the other two parameters did not show any correlation with peak amplitude, and no significant differences were found in the response time constant or latency of antennal responses of *BmPBPI*-knockout and wild-type males (Fig. 1b bottom, 1d). These differences were observed with both bombykol and bombykal (Fig. 1d), suggesting that the loss of BmPBPI delayed the recovery time constant of EAG responses with both bombykol and bombykal.

135

Kinetics of temporal antennal responses to pheromone pulse trains in *BmPBPI*-knockout moths. Because tracking intermittent pheromone pulse trains is important to locate pheromone sources in male moths, we next assessed the effects of loss of BmPBPI on temporal antennal responses to pheromone pulse trains. Particularly, we focused on responses to bombykol because only bombykol triggers the pheromone source localisation behaviour of male silkmoths. We tested three different frequencies of pulse trains, consisting of 0.17 Hz as the low stimulus frequency, 0.83 Hz as the representative of the emission rate of pheromone from females²⁹ and 2 Hz as the frequency that can induce a consecutive surge in male silkmoths⁵ (Fig. 2a). Consistent with single pulse experiments, the recovery time constant of EAG responses became longer in *BmPBPI*-knockout than in wild-type male antennae (Fig. 2a). The time

required to return to baseline levels after the first bombykol stimulation (termination time) was significantly longer in *BmPBPI*-knockout than in wild-type male moths with all tested frequencies (Fig. 2b, 2c). More importantly, the termination times got longer as the frequency increased and EAG responses of *BmPBPI*-knockout never returned to baseline levels during high frequency stimuli in several tested antennae (1 out of 6 tested antennae at 0.83 Hz, 4 out of 12 tested antennae at 2 Hz). However, all EAG responses of wild-type male antennae quickly recovered to baseline levels (n=7 at 0.83 Hz, n=6 at 2 Hz) (Fig. 2c).

To evaluate response recovery after intermittent stimuli, we calculated the valley amplitudes, which were defined as the EAG amplitude where each local minimum amplitude was subtracted from the baseline level amplitude (Fig. 2b, 2d, see also Methods section). The valley amplitudes of *BmPBPI*-knockout males were significantly larger than those of wild-type males when stimuli were delivered at 0.83 and 2 Hz. In comparison, the valley amplitudes of both *BmPBPI*-knockout and wild-type males returned to baseline levels after each stimulus was delivered at 0.17 Hz, and there was no significant difference between the groups (Fig. 2d). These results indicate that delayed recovery time constant in *BmPBPI*-knockout male antennae affects the overall temporal response kinetics of EAG responses to intermittent pheromone stimuli.

Temporal resolution of a single ORN in *BmPBPI*-knockout male moths was lower compared to that in wild-type male moths. To understand the physiological basis of the observed temporal characteristics in EAG experiments, we next performed single sensillum recordings (SSRs) of pheromone sensitive sensilla trichodea (Fig. 3a). We revealed that spontaneous spike activity was not significantly different between *BmPBPI*-knockout and wild-type antennae, suggesting that BmPBPI does not affect spontaneous spike levels (Fig. 3b, 3c). To compare temporal kinetics, we used 10000 ng bombykol as stimuli for *BmPBPI*-knockout male antennae and 1000 ng bombykol as

174 stimuli for wild-type male antennae, which evoked similar spike counts in *BmPBPI*-
 175 knockout male antennae and wild-type male antennae (Fig. 3b, 3c). The spike responses
 176 of *BmPBPI*-knockout male antennae at 0.17 Hz took significantly longer to return to
 177 spontaneous spike levels, while those of wild-type antennae quickly terminated after the
 178 stimulus (Fig. 3d). When antennae were stimulated at 2 Hz, this tendency became
 179 clearer, and the spike responses of all tested ORNs in *BmPBPI*-knockout antennae did
 180 not return to spontaneous spike levels between subsequent stimuli, while wild-type
 181 ORN spike responses quickly returned to spontaneous levels (Fig. 3e, 3f). These results
 182 demonstrate that the loss of BmPBPI delayed response termination of ORNs resulting
 183 in a lower antennal temporal resolution in *BmPBPI*-knockout males.

184

185 **Temporal behavioural resolution of *BmPBPI*-knockout male moths was**
 186 **lower compared to that of wild-type male moths.** To investigate the effects of
 187 delayed termination kinetics on pheromone source localisation behaviours (Fig. 4a), we
 188 first analysed pheromone source localisation behaviours to bombykol using tethered
 189 male moths. Upon exposure to a single pulse of bombykol, each *BmPBPI*-knockout
 190 male moth exhibited full pheromone-induced localisation behaviour, consisting of a
 191 surge behaviour followed by zigzag turns (Fig. S1, Fig. 4b), indicating that the loss of
 192 BmPBPI did not abolish innate behavioural sequences, despite the surge duration of
 193 *BmPBPI*-knockout male moths being significantly longer than that of wild-type male
 194 moths (Fig. 4b).

195 If *BmPBPI*-knockout males perceived intermittent stimuli as continuous signals
 196 owing to delayed response kinetics, we hypothesised that behavioural patterns to pulse
 197 trains should be changed, especially in terms of resetting behaviour sequences. In
 198 response to 0.17 Hz intermittent bombykol stimulation, to which *BmPBPI*-knockout
 199 antennae return to baseline levels, both *BmPBPI*-knockout and wild-type male moths
 200 showed a surge in exposure to each bombykol pulse (Fig. 4c, d), indicating that each

201 pulse induced the resetting of behaviour sequences. However, following 0.83 Hz and 2
 202 Hz bombykol pheromone pulse trains, *BmPBPI*-knockout male moths displayed lower
 203 surge rates—the ratio of surge duration against total locomotion duration (see
 204 Methods)—and higher zigzag turn rates than wild-type male moths (Fig. 4c, d). Further,
 205 *BmPBPI*-knockout male moths exhibited fewer longer surges (defined as a surge lasting
 206 more than 2 seconds) than wild-type male moths (Fig. 4e), indicating that *BmPBPI*-
 207 knockout males failed to exhibit successive surge behaviours in response to intermittent
 208 pheromone stimuli at 2 Hz. Importantly, differences in surge rates were well correlated
 209 with EAG and SSR results; males restarted surge behaviours to each pheromone
 210 stimulus under stimulus conditions that allowed antennal responses to return to baseline
 211 levels, presumably owing to failure to clearly detect the onset of the subsequent
 212 stimulus. Thus, our results indicate that lower temporal antennal resolution reduces
 213 temporal behavioural resolution in *BmPBPI*-knockout males.

214

215 **Wind tunnel experiments revealed behavioural deficits in efficient** 216 **pheromone source localisation in *BmPBPI*-knockout male moths.**

217 To assess whether changes in the behavioural patterns of *BmPBPI*-knockout
 218 males affect odour source localisation, we investigated pheromone source localisation
 219 behaviours to 2 Hz pheromone pulse trains in a wind tunnel under free walking
 220 conditions. We analysed two criteria for efficient pheromone source localisation:
 221 success rate and localisation time. To compensate for differences in EAG sensitivity
 222 (Fig. 1) and to test the effects of pheromone dose on pheromone source localisation, the
 223 behaviours of *BmPBPI*-knockout and wild-type males were tested with 100–10000 ng
 224 and 1–100 ng bombykol, respectively. Both *BmPBPI*-knockout and wild-type males
 225 could localise the pheromone source for all tested doses of bombykol with a similar
 226 success rate (Fig. 5a). However, *BmPBPI*-knockout males took significantly longer to
 227 localise the pheromone source than wild-type males (Fig. 5b). Therefore, though

228 *BmPBPI*-knockout males still possess the ability to localise the pheromone sources, the
229 efficiency of their localisation behaviour in terms of localisation time was significantly
230 reduced in *BmPBPI*-knockout males.

231 The walking traces of *BmPBPI*-knockout males had distinct patterns with more
232 turning and less successive surge behaviour compared with wild-type males (Fig 5c).
233 Indeed, a detailed analysis of the traces revealed that *BmPBPI*-knockout males
234 exhibited significantly shorter surge distances and a larger summed turning angle during
235 localisation than wild-type males (Fig. 5d, e). Exposure to relatively high bombykol
236 concentrations in *BmPBPI*-knockout male moths (KO: 1000–10000 ng) and low
237 bombykol concentrations in wild-type male moths (WT: 1–10 ng) did not alter the
238 characteristics of localisation behaviours (Fig. 5a-e). Lastly, we examined localisation
239 behaviour to female silkmoths and found that behavioural parameters including success
240 rate (Fig. 5f), localization time (Fig. 5g), surge distance (Fig. 5h) and all turn angles
241 (Fig. 5i) showed the same characteristics with those observed to bombykol. Taken
242 together, these results indicate that behavioural deficits caused by the low temporal
243 antennal resolutions of male moths are responsible for the inefficiency of odour source
244 localisation in *BmPBPI*-knockout males.

245

246 Discussion

247 In this study, we demonstrated that the temporal sensory characteristics of
248 antennae are crucial for efficient pheromone source localisation and that *BmPBPI* is
249 involved in shaping the temporal sensory characteristics of antennae to pheromone
250 stimulation in *B. mori*. Since the discovery of PBPs in 1981 in *Antheraea polyphemus*¹⁶,
251 PBPs have been suggested to play important roles in various aspects of pheromone
252 detection in moths. Although previous studies have provided evidence that PBPs can
253 enhance the sensitivity of antennal response to pheromones^{20,21}, no study has provided
254 *in vivo* experimental evidence for the contribution of PBPs for improving temporal

255 sensory resolution, one of the most important aspects in odour source localisation under
256 natural conditions. Our physiological and behavioural analyses using *BmPBPI*-
257 knockout silkmoths present the first evidence supporting the functional role of *BmPBPI*
258 in determining the temporal kinetics of pheromone responses.

259 Odour source localisation utilises the natural intermittency of odour stimulation
260 to promote a surge when a stimulus pulse is encountered. This behavioural strategy for
261 odour source localisation is, at present, widely accepted in moth species, based on the
262 correspondence between physiological antennal responses and surge behaviours during
263 localisation^{10,13}. Therefore, moths require mechanisms that allow for the faithful
264 representation of intermittent odour information at the level of the antennal receptors, as
265 well as a strategy to reflect sensory information in behaviours with sufficient temporal
266 resolution. In this study, we showed that *BmPBPI*-knockout males had a reduced
267 temporal antennal resolution compared with wild-type, resulting in the inefficient
268 localisation to odour sources. Even for the localisation to female silkmoths, similar
269 behavioural deficits, such as a shorter surge distance, longer turning behaviour and
270 longer localisation time, were observed in *BmPBPI*-knockout males. Therefore, these
271 results indicate that moths possess an appropriate range of temporal antennal and
272 behavioural resolutions to efficiently localise odour sources in a dynamically changing
273 environment. Though sensitivity of *BmPBPI*-knockout antennae is lower than that of
274 wild-type antennae, the behavioural deficits are thought to be caused by the alternation
275 of temporal kinetics, but not by the lowered sensitivity, as the behavioural deficits were
276 observed in response to pheromone stimuli that induced similar EAG peak amplitudes
277 in both *BmPBPI*-knockout and wild-type antennae.

278 Our EAG results demonstrated that the recovery time of EAG responses of
279 *BmPBPI*-knockout male antennae was significantly longer than those of wild-type male
280 antennae, which resulted in the lower temporal resolution of EAG responses to pulsed
281 pheromone stimulation. Although EAG is thought to monitor the total activity of all

282 ORNs in the antenna, the actual relationship between EAG responses and the spike
283 activity of individual ORNs is not clear. Thus, it is important to note that consistent
284 temporal characteristics were also observed at the level of single ORN spike responses,
285 showing that EAG response kinetics in *BmPBPI*-knockout antennae reflects changes in
286 temporal spike characteristic of ORNs.

287 Similar temporal alterations in antennal and behavioural responses to odour pulse
288 trains have been previously reported in the oriental fruit moth *Grapholita molesta*^{30,31}.
289 Under cool temperature conditions, antennal responses of *G. molesta* males to
290 pheromones were reduced, and they were unable to keep up with high frequency pulsed
291 pheromone stimuli due to the inability to generate well-contrasted on-off spike
292 representations to each stimulus. This characteristic property of the spike response of
293 ORNs is called “attenuation”³⁰. Under similar cooling conditions, male moths failed to
294 initiate successive surge behaviours and could not locate an odour source³¹. Although
295 the underlying mechanism that affects the antennal response characteristics and
296 behaviours may be different, these studies also indicate the importance of the temporal
297 characteristics of antennal responses for efficient pheromone source localisation. In our
298 study, we revealed that *BmPBPI* is one factor related to temporal antennal resolution
299 and that temporal sensory representations corresponding to odour dynamics play a key
300 role in the quick behavioural reset strategy for efficient localisation.

301 We found that the stimuli frequencies to which EAG responses could recover
302 from peak amplitude to baseline levels corresponded to those where behavioural
303 sequences of male moths were reset. Therefore, after peak neuronal activity, returning
304 to spontaneous baseline activity can be important for tracking odour filaments by
305 continuously resetting behavioural sequences, rather than only peak amplitude detection.
306 Previous studies have reported the temporal antennal resolution of several moth species
307 based on the peak frequency analyses of EAG recordings^{32,33}. According to these
308 reports, antennae of male *B. mori* have a potential temporal resolution to intermittent

309 odour pulses of up to 25 Hz. However, since EAG responses to high frequency
310 pheromone stimulation in those reports do not recover to baseline levels after peak
311 response, there is a possibility that not every odour stimulus is resolved to induce
312 resetting behavioural sequences and initiation of surge behaviour. Thus, the potential
313 temporal resolution of antennae may differ from the antennal temporal resolution for a
314 behavioural reset. In other words, sensory responses may turn into ‘fused’ information
315 for male moth behaviour when tracking an odour plume at a frequency where neural
316 activity does not return to spontaneous baseline activity before a response to a
317 subsequent odour stimulus. In the future, investigation of the behavioural patterns of
318 wild-type animals to high frequency stimuli that induce antennal responses without
319 returning to baseline levels can shed light on antennal response parameters crucial for
320 the reset of behaviour sequences.

321 Previous kinetics model analyses proposed that rapid inactivation of pheromone
322 molecules after they activate pheromone receptors plays a critical role in controlling the
323 kinetics of pheromone responses and that PBPs were one candidate molecular
324 component of pheromone inactivation²³⁻²⁵. Our results showing that the loss of
325 BmPBP1 delayed recovery time constants strongly support the idea that BmPBP1 is
326 involved in the rapid inactivation of pheromone molecules. PBPs have shown to be
327 involved in sensitive detection of pheromones by efficiently solubilising pheromone
328 molecules into the sensillum lymph and protecting them from enzymatic degradation
329 during transportation to the vicinity dendritic membrane of ORNs, where pheromone
330 receptors resides^{21,22}. Therefore, PBPs play multiple functional roles both before and
331 after pheromone detection by pheromone receptors. Further electrophysiological,
332 biochemical and molecular analyses of *BmPBP1*-knockout moths will help reveal the
333 detailed mode of action underlying how PBPs can perform these different functions in
334 the process of pheromone detection.

335 In this study, we reported that BmPBP1 contributes to the temporal control of

336 antennal responses, and its absence lowered the antennal temporal resolution, critically
 337 affecting efficient odour source localisation. Our results could provide a new
 338 methodology for controlling pest insects by modulating odour source localisation
 339 behaviour. Furthermore, strategies to represent spatio-temporal airborne odours in the
 340 sensory system by quickly terminating signals after detection, and to reset sequential
 341 behavioural patterns in response to sensory input, are thought to be equally effective for
 342 the development of artificial odour source searching algorithms.

343

344 **Methods**

345 **Animals and chemicals.** We used *BmPBPI*-knockout moths, which were
 346 generated using transcription activator-like effector nuclease (TALEN)-mediated gene
 347 targeting, as described in a previous study²¹. Larvae were reared on an artificial diet
 348 (Nihon Nosan Kogyo, Yokohama, Japan) at 25°C under a 16:8 h (light/dark)
 349 photoperiod. Following eclosion, one to seven day old male moths were used for the
 350 experiments. Subsequent to all experiments in this study, the genotype of all *BmPBPI*-
 351 knockout males was determined by PCR, as previously described²¹. The purity
 352 (>99.5%) of synthetic bombykol and bombykal was verified by gas chromatography
 353 using previously described conditions^{34,35}, and they were kindly provided by Dr. S.
 354 Matsuyama from the University of Tsukuba, Japan.

355

356 **Electroantennogram (EAG) recordings.** An antenna of a male moth was
 357 excised at the base, and a few antennomeres at the tip were cut off. The antenna was
 358 then mounted on the EAG probe using electrode gel (SPECTRA 360; Parker
 359 Laboratories, Fairfield, NJ, USA). A glass cartridge (inner diameter, 5 mm) was
 360 prepared for stimulation by inserting a piece of filter paper (1.5 × 1.5 cm), and 5 µL of a
 361 pheromone solution in *n*-hexane was administered. A charcoal-purified airstream (1
 362 L/min) was passed through the glass cartridge and directed onto the antenna. The EAG

363 responses were amplified using a custom-made amplifier (Minegishi and Kanzaki,
364 unpublished), band-pass filtered at 0.16 to 300 Hz, and digitised at 1 kHz (USB-6210;
365 National Instruments, Austin, TX, USA). The data were analysed using a custom-
366 written program (MATLAB; Mathworks, Natick, MA, USA). Baseline levels were
367 calculated from 2 sec of data recording before the stimulus. The start time of the
368 response was defined as the time the EAG amplitude exceeded the baseline level by $\pm$
369 2.5 standard deviations. The response time constant was defined as the time of the
370 response phase, from the start of response to 63.2 % of the absolute EAG response
371 amplitude. The recovery time constant was defined as the time of the settling phase,
372 from the peak amplitude to 63.2% of the absolute EAG response amplitude. The valley
373 amplitude was defined as the EAG response amplitude where each local minimum
374 amplitude was subtracted from the baseline level. The termination time was defined as
375 the time from the peak amplitude to the baseline level by $\pm$ 5% standard error of the
376 mean (SEM). The latency was calculated from the start time by subtracting the arrival
377 time of the pheromone on the antenna that was estimated using a method previously
378 described³⁶.

379

380 **Single sensillum recording (SSR).** Single sensillum recordings were performed
381 in a Faraday cage. Moths were fixed on a custom-made acrylic chamber under an
382 Olympus BX50WI (500 $\times$) microscope. The antennae were stabilised with dental wax
383 (GC Corporation, soft plate wax), and the basement segments were fixed with a resin
384 bond (GC Corporation, G-Fix). Spike responses were recorded by a sharpened tungsten
385 wire electrode (diameter 0.5 mm, tip approximately 1 μ m) inserted into the bases of
386 pheromone sensitive long sensilla trichodea on the antennae. As a reference electrode, a
387 silver wire was inserted into the compound eye of the moth. Pheromone was delivered
388 by injecting pheromone pulses into a charcoal-purified and moistened continuous
389 airstream (1 L/min), to reduce any mechanical artefacts. Spike responses were band-

pass filtered at 300 to 3k Hz and amplified (Nihon Kodan, MEZ-8300). All spike responses were digitised at 10 kHz. The spontaneous spike rate was calculated from 2 sec of data recording before the stimulus, with a time bin of 100 ms. We analysed data with a custom MATLAB (Mathworks, MA, Natick, USA) program and Spike2 software (Cambridge Electronic Design Limited, UK).

395

Behavioural experiments using tethered moths. Scales on the dorsal thorax of moths were removed, and a copper wire holder was attached to the thorax with glue. Moths were placed on the top of a 70 mm diameter polystyrene foam ball. The ball was supported by a plastic funnel with an electrical fan attached under the ball. The air flow generated by the electric fan kept the ball floating in the air to reduce friction and enable moths to behave during locomotion. Pulsed pheromone stimulation of 200 ms duration at 0.83 Hz and 2 Hz frequency and stimulation of 1 second at 0.17 Hz frequency was used. Wild-type and *BmPBPI*-knockout males were stimulated with 100 ng and 10000 ng bombykol, respectively. Based on a previous study using tethered moths, the surge behaviour was defined as straight walking with a small angle, at an accumulated angular velocity $\leq 8^\circ$, when the accumulated walking distance ≥ 1 mm, with a forward velocity ≥ 1 mm/sec, using a time bin of 200 ms. The zigzag turn behaviour was defined as turning behaviour with a large angle, at an accumulated angular velocity $\geq 8^\circ/\text{sec}$, with an accumulated forward velocity ≤ 10 mm/sec. These parameters were adjusted to remove trackball noise from oscillations caused by stepping of the moth according to a previous study³⁷. Pheromone source localisation behaviours of moths were recorded with an optical mouse attached behind the polystyrene ball. Surge rate was defined as the ratio of total surge duration to total locomotion duration containing both surge and zigzag turns. We processed data based on these criteria with a custom MATLAB (Mathworks, MA, Natick, USA) program.

416

417 **Wind tunnel experiments.** We performed odour source localisation experiments
 418 in a wind tunnel (1800×900×300 mm L×W×H) at 25–28°C. Pulsed pheromone
 419 stimulation of 200 ms duration at 2 Hz frequency was controlled by an electric valve,
 420 and wind velocity was controlled at 0.5–0.7 m/s. For the odour source localisation
 421 experiments, both *BmPBPI*-knockout and wild-type male moths were used within 1 to 4
 422 days after eclosion. Synthetic bombykol (1, 10, 100, 1000 and 10000 ng) and wild-type
 423 females were used for pheromone stimulation. Moths were placed 50 cm downstream of
 424 a pheromone source. The pheromone source localisation behaviour of male moths was
 425 captured with a digital video camera at a frame rate of 30 Hz. Surge was defined as
 426 straight walking with a small angle, at an angular velocity $\leq 5^\circ/\text{sec}$, with the
 427 accumulated turn angle $\leq 30^\circ/\text{sec}$, with a forward velocity $\geq 1 \text{ mm/sec}$ and a walking
 428 distance $\geq 1 \text{ mm}$, using a bin size of 200 ms based on previous studies³⁸. Behaviours
 429 that did not correspond to these criteria were seen as stopping and switching directions
 430 and were not included in our analyses. The other behaviours (at angular velocity $\geq$
 431 $5^\circ/\text{sec}$, with accumulated turn angle $\geq 30^\circ/\text{sec}$) were classified as zigzag turns. We
 432 processed image data based on these criteria with customised Java and R scripts (R
 433 Development Core Team, 2010).

434
 435 **Statistical analysis.** In our experiments, to assess the statistical differences
 436 between wild-type and *BmPBPI*-knockout moths, we used Student's *t*-test, Fisher's
 437 exact probability test, the Steel–Dwass test and an ANCOVA test. Statistical
 438 significance was calculated using Microsoft Excel 2016, a commercial macroprogram
 439 (Statcel version 4; Seiun-sya, Japan) and custom VBA and MATLAB (Mathworks, MA,
 440 Natick, USA) programs. The error bars shown in the figures represent the SEMs. The
 441 asterisks indicate significant differences between the groups ($*p < 0.05$, $**p < 0.01$).
 442 The different letters indicate significant differences according to the Steel–Dwass test
 443 and Fisher's exact probability test.

444

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563 Y.S. and T.S. analysed the data; and Y.S. and T.S. wrote the paper.

564

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566

567 **Figure legends**

568 **Figure 1. Antennal response kinetics in *BmPBPI*-knockout male moths. (a)**
569 Parameters considered in the antennal response kinetics analysis of EAG recordings. **(b)**
570 Representative EAG responses of *BmPBPI*-knockout (blue) and wild-type (red) male
571 antennae to 10000 ng bombykol stimulation (top), and responses to 10000 ng bombykol
572 stimulation in *BmPBPI*-knockout (blue) and 100 ng bombykol stimulation in wild-type
573 (red) male antennae (bottom). The stimulus was applied for 200 ms, as indicated by the
574 solid line on the trace. **(c)** Kinetic analysis of EAG responses in *BmPBPI*-knockout
575 (blue; n=11) and wild-type (red; n=5) male antennae. Error bars represent $\pm$ SEM. The
576 asterisks indicate significant differences between the groups ($**p < 0.01$), as determined
577 using Student's *t*-test for comparing pairs of data. **(d)** Kinetic analysis of EAG
578 responses of *BmPBPI*-knockout (blue; n=11) and wild-type (red; n=5) antennae

579 normalised by peak EAG amplitude. Broken lines indicate linear regression curves. An
580 ANCOVA test was used to detect significant differences between wild-type and
581 *BmPBPI*-knockout datasets for recovery time, while significant differences between the
582 groups were not detected in the other two parameters (** $p < 0.01$). NS indicates not
583 significant.

584

585 **Figure 2. Analysis of antennal response kinetics to pulsed pheromone stimuli in**
586 ***BmPBPI*-knockout male moths.** (a) Representative EAG responses to pulsed
587 pheromone trains in *BmPBPI*-knockout (blue) and wild-type (red) male antennae at
588 0.17 Hz, 0.83 Hz and 2 Hz. The stimulus was applied for 200 ms, as indicated by the
589 solid line on the trace. The broken line indicates baseline level of each EAG trace. (b)
590 Parameters considered in the antennal response kinetics analysis of EAG recording to
591 pulsed pheromone stimuli. (c) Termination time of EAG responses to pulsed pheromone
592 trains in *BmPBPI*-knockout (blue) and wild-type (red) antennae at 0.17 Hz (KO; n=9,
593 WT; n=9), 0.83 Hz (KO; n=6, WT; n=7) and 2 Hz (KO; n=12, WT; n=6). Error bars
594 represent $\pm$ SEM. The asterisks indicate significant differences between the groups (** p
595 < 0.01), as determined using Student's *t*-test for comparing pairs of data. (d) Valley
596 amplitude of male antennae to pheromone stimuli in *BmPBPI*-knockout (blue) and
597 wild-type (red) at 0.17 Hz (KO; n=9, WT; n=9), 0.83 Hz (KO; n=7, WT; n=7) and 2 Hz
598 (KO; n=16, WT; n=7). The asterisks indicate significant differences between the groups
599 (** $p < 0.01$), as determined using Student's *t*-test for comparing pairs of data. NS
600 indicates not significant.

601

602 **Figure 3. Analysis of antennal temporal resolution to pheromone pulse trains in**
603 ***BmPBPI*-knockout male moths.** (a) Representative spike responses of long sensilla
604 trichodea in *BmPBPI*-knockout (blue) and wild-type (red) male antennae to pheromone
605 pulse stimuli at 0.17 Hz and 2 Hz. Solid lines on the trace represent the timing of

intermittent bombykol stimuli. **(b)** Comparison of spontaneous and elicited spike numbers. Spontaneous spikes were calculated with a time bin of 2 seconds before the first stimulus was presented to *BmPBPI*-knockout (blue; n=9 at 0.17 Hz, n=8 at 2 Hz) and wild-type (red; n=13 at 0.17 Hz, n=11 at 2 Hz) male antennae; spike numbers were calculated with a time bin of 5 seconds from the stimulus at 0.17 Hz and **(c)** with a time bin of 3.5 seconds after the stimulus at 2 Hz. Error bars represent $\pm$ SEM. The asterisks indicate significant differences between the groups ($**p < 0.01$), as determined using Student's *t*-test for comparing pairs of data. **(d)** Comparison of the termination time of spikes between *BmPBPI*-knockout and wild-type at 0.17 Hz. Error bars represent $\pm$ SEM. The asterisks indicate significant differences between the groups ($**p < 0.01$), as determined using Student's *t*-test for comparing pairs of data. **(e)** Spike rate in time bins of 100 ms in *BmPBPI*-knockout (blue; n=9) and wild-type (red; n=13) male antennae at 0.17 Hz and **(f)** those of 100 ms in *BmPBPI*-knockout (blue; n=8) and wild-type (red; n=11) male antennae at 2 Hz. Error bars represent $\pm$ SEM. The asterisks indicate significant differences between the groups ($*p < 0.05$, $**p < 0.01$), as determined using Student's *t*-test for comparing pairs of data. NS indicates not significant.

Figure 4. Behavioural responses of tethered *BmPBPI*-knockout moths to intermittent pheromone pulses. **(a)** Schematic diagram of the pheromone-triggered programmed behaviour and pheromone source localisation strategy of a male silkworm, *B. mori*. **(b)** Analysis of pheromone-triggered programmed behaviour in *BmPBPI*-knockout (blue; n=9) and wild-type (red; n=16) male moths to single pulsed stimulation. Bombykol pulse stimuli were applied for 1 second. Error bars represent $\pm$ SEM. The asterisks indicate significant differences between the groups ($**p < 0.01$), as determined using Student's *t*-test for comparing pairs of data. **(c)** Representative trace of the body angle of tethered *BmPBPI*-knockout (blue) and wild-type male (red) moths to pulsed bombykol stimuli at 0.17 Hz and 2 Hz. **(d)** Surge rate in *BmPBPI*-knockout (blue) and

633 wild-type (red) male moths to bombykol pulse trains at 0.17 Hz (KO; n=9, WT; n=16),
 634 0.83 Hz (KO; n=10, WT; n=7) and 2 Hz (KO; n=9, WT; n=9). Error bars represent $\pm$
 635 SEM. The asterisks indicate significant differences between the groups ($**p < 0.01$), as
 636 determined using Student's *t*-test for comparing pairs of data. (e) Scatter plot of each
 637 surge based on surge duration in *BmPBPI*-knockout (blue; n=9) and wild-type (red;
 638 n=9) male moths. Circles indicate the distribution of individual data. Solid circles
 639 indicate the first surge that was induced by the first bombykol stimuli. NS indicates not
 640 significant.

641

642 **Figure 5. Performance of pheromone source localisation in *BmPBPI*-knockout**
 643 **male moths.** (a) Success rate of pheromone source localisation of *BmPBPI*-knockout
 644 (blue) and wild-type (red) male moths at 2 Hz (WT; n=13 at 1 ng; n=12 at 10 ng; n=18
 645 at 100 ng, KO; n=20 at 100 ng; n=26 at 1000 ng; n=23 at 10000 ng). The different
 646 letters indicate significant differences according to Fisher's exact probability test. (b)
 647 Pheromone source localisation time of *BmPBPI*-knockout (blue) and wild-type (red)
 648 male moths at 2 Hz (KO; n=10 at 100 ng; n=20 at 1000 ng; n=17 at 10000 ng, WT;
 649 n=11 at 1 ng; n=10 at 10 ng; n=15 at 100 ng). Error bars represent $\pm$ SEM. The different
 650 letters indicate significant differences according to the Steel–Dwass test. Black solid
 651 circles indicate the distribution of individual data. (c) Representative trajectory of
 652 *BmPBPI*-knockout (blue) and wild-type male (red) moths to tested bombykol dosage
 653 (WT: 1, 10, 100 ng, KO: 100, 1000, 10000 ng) during pheromone source localisation.
 654 Bombykol pulse stimuli were applied for 200 ms at 2 Hz. Solid lines indicate the
 655 trajectory of the pheromone source localisation behaviour. Broken lines indicate the
 656 estimated boundaries of the pheromone plume. (d) Surge distance of *BmPBPI*-knockout
 657 (blue) and wild-type (red) male moth to bombykol pulse trains at 2 Hz (WT; n=11 at 1
 658 ng; n=10 at 10 ng; n=15 at 100 ng, KO; n=10 at 100 ng; n=20 at 1000 ng; n=17 at
 659 10000 ng). (e) All turn angles of *BmPBPI*-knockout (blue) and wild-type (red) male

660 moths to bombykol pulse trains at 2 Hz (WT; n=11 at 1 ng; n=10 at 10 ng; n=15 at 100
 661 ng, KO; n=10 at 100 ng; n=20 at 1000 ng; n=17 at 10000 ng). **(f-i)** Performance of
 662 pheromone source localisation to a female moth. Success rate (WT; n=10, KO; n=8),
 663 localisation time (WT; n=7, KO; n=5), surge distance (WT; n=7, KO; n=5), and all turn
 664 angles (WT; n=7, KO; n=5) to a female moth. The asterisks indicate significant
 665 differences between the groups (** $p < 0.01$), as determined using Student's *t*-test for
 666 comparing pairs of data. NS indicates not significant.

(a)

(b)
1,426

Bombykal

Figure 2

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Figure 3

Figure 4

Figure 5