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## Collisional mechanism of ligand release by *Bombyx mori* JHBP, a member of the TULIP / Takeout family of lipid transporters.

Stéphane Dupas, Fabrice Neiers, Emma Granon, Erwan Rougeux, Sébastien Dupont, Laurent Beney, François Bousquet, Haq Abdul Shaik, Loïc Briand, Hubert Wojtasek, et al.

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1                                    **Collisional mechanism of ligand release by *Bombyx mori* JHBP,**  
2                                    **a member of the TULIP / Takeout family of lipid transporters.**  
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7 **ABSTRACT:**  
8

9 Juvenile hormones (JHs) regulate important processes in insects, such as postembryonic  
10 development and reproduction. In the hemolymph of Lepidoptera, these lipophilic sesquiterpenic  
11 hormones are transported from their site of synthesis to target tissues by high affinity carriers, the  
12 juvenile hormone binding proteins (JHBPs). Lepidopteran JHBPs belong to a recently uncovered, yet  
13 very ancient family of proteins sharing a common lipid fold (TULIP domain) and involved in shuttling  
14 various lipid ligands. One important, but poorly understood aspect of JHs action, is the mechanism of  
15 hormone transfer to or through the plasma membranes of target cells. Since many membrane-active  
16 peptides and proteins, such as the pore-forming bacterial toxins, are activated by low pH or interaction  
17 with phospholipid membranes, we have examined the effect of these factors on JH binding by JHBPs.  
18 The affinity of *Bombyx mori* and *Manduca sexta* JHBPs for JH III was determined by the DCC assay,  
19 equilibrium dialysis, and isothermal titration calorimetry, and found to be greatly reduced at low pH,  
20 in agreement with previous observations. Loss of binding was accompanied by changes in  
21 fluorescence and near-UV CD spectra, indicating significant changes in protein structure in the  
22 environment of aromatic residues. The apparent dissociation rate constant ( $k_{off}$ ) of the JHBP-JH III  
23 complex was greater at acidic pH, suggesting that low pH favors ligand release by opening of the  
24 binding pocket. The affinity of recombinant *B. mori* JHBP (rBmJHBP) was also decreased in the  
25 presence of anionic phospholipid vesicles. Measurements of steady-state fluorescence anisotropy with  
26 the lipophilic probe TMA-DPH demonstrated that rBmJHBP specifically interacts with anionic  
27 membranes. These results suggest the existence of a collisional mechanism for ligand release that may  
28 be important for delivery of JHs to the target cells, and could be relevant to the function of related  
29 members of this emerging family of lipid-transport proteins.  
30

31 *Keywords:*

32  
33 Juvenile Hormone  
34 JHBP  
35 *Bombyx mori*  
36 *Manduca sexta*  
37 TULIP domain  
38 Takeout-like proteins  
39  
40  
41  
42

43 **1. Introduction**  
44

45 Juvenile hormones (JHs) regulate important processes in insects, such as postembryonic  
46 development and reproduction (Goodman and Cusson, 2012; Jindra et al., 2015). In the hemolymph of  
47 Lepidoptera, JHs are transported from their site of synthesis (the *corpora allata*) to target tissues by  
48 high affinity carriers, the juvenile hormone binding proteins (JHBPs). It is believed that these proteins  
49 protect the hormones from degradation and non-specific adsorption to lipophilic depots (Goodman,  
50 1990; Koeppe and Kovalick, 1986).  
51 For a long time, the details of JHBP-JH interactions have remained elusive. The X-ray structure of  
52 apoJHBP was first determined for the protein from *Galleria mellonella* (Kolodziejczyk et al., 2008).  
53 The structure consists of a long  $\alpha$ -helix surrounded by a twisted, anti-parallel  $\beta$ -sheet with two cavities  
54 forming potential binding sites at the opposite ends of the molecule (Figure S1). It closely resembles  
55 the structure of several other lipid binding proteins, such as the Takeout 1 protein of **the moth**

1 *Epiphyas postvittana* (Hamiaux et al., 2013; Hamiaux et al., 2009), as well as the mammalian  
2 bactericidal/permeability increasing protein (BPI) (Beamer et al., 1997) and the cholesteryl ester  
3 transfer protein (CETP) (Qiu et al., 2007). Phylogenetic analyses have shown that these proteins and  
4 many others, including plant proteins, share one or two copies of an ancestral TULIP lipid binding  
5 motif (Tubular Lipid binding), which allows lipid transport, as well as interaction with phospholipids  
6 at the surface of membranes or lipoproteins (Alva and Lupas, 2016; Wong and Levine, 2017).  
7 Although very ancient and therefore likely playing fundamental functions in lipid trafficking for most  
8 eukaryotes and prokaryotes, the functions of the members of this emerging family are mostly known  
9 from studies in mammals. In invertebrates, the TULIP domain proteins Takeout and Takeout-related  
10 proteins have been involved in various processes (courtship behavior, feeding, aging, JH transport,  
11 perception (Fujikawa et al., 2006; Galikova and Flatt, 2010; Lazareva et al., 2007; Meunier et al.,  
12 2007; Sarov-Blat et al., 2000; Yoshizawa et al., 2011)). Lepidopteran JHBPs appear to constitute a  
13 subclass of the Takeout proteins, characterized by the presence of a conserved disulfide bridge  
14 dividing the large internal hydrophobic cavity into two smaller ligand binding pockets (Hamiaux et al.,  
15 2013; Hamiaux et al., 2009; Kolodziejczyk et al., 2008; Kolodziejczyk et al., 2001). To this day,  
16 JHBPs are the only members with well characterized endogenous ligands.

17 The structure of JHBP from *Bombyx mori* with a bound hormone was solved by both X-ray  
18 diffraction and NMR spectroscopy (Suzuki et al., 2011). This study revealed that ligand uptake and  
19 release are accompanied by substantial conformational changes of the protein, in agreement with  
20 earlier findings on *G. mellonella* JHBP (Krzyzanowska et al., 1998; Wieczorek and Kochman, 1991).  
21 It was shown that the N-terminal helix functions as a gate, which closes upon ligand binding. In the  
22 apo form of the protein in the crystalline state, the gate is open, allowing access of the ligand to the  
23 binding site. In solution, the gate of the apoJHBP is highly mobile and the protein exists in multiple  
24 conformations, including the gate-open conformation, seen in the crystalline state, and the gate-closed  
25 conformation similar to the ligand-bound form. This flexibility of the gate in the apo-JHBP disappears  
26 upon JH-binding. Based on these structural changes a mechanism of JH delivery to target cells has  
27 been proposed ((Suzuki et al., 2011), Figure S1).

28 Release of a hydrophobic ligand from a complex with a binding protein has been previously  
29 demonstrated for a number of proteins. *B. mori* pheromone-binding protein (PBP) (Horst et al., 2001)  
30 binds its ligand only at neutral and basic pH, whereas low pH induces the formation of an additional  
31  $\alpha$ -helix (helix 7), which fills the binding pocket and prevents ligand binding. Analysis by CD and  
32 fluorescence spectroscopy has demonstrated that structural changes, very similar to those evoked by  
33 low pH, were also induced by phospholipid vesicles (Wojtasek and Leal, 1999), and it was therefore  
34 hypothesized that pheromone release could be triggered by phospholipid membranes on the surface of  
35 olfactory neurons, thus providing a mechanism of ligand delivery to target cells.

36 Evidence for pH-induced ligand release has also been shown for several other hydrophobic  
37 ligand binding proteins, including serum retinol binding protein (Ptitsyn et al., 1993) and tear lipocalin  
38 (Gasymov et al., 2004). More generally, acidic pH and interaction with phospholipidic membranes are  
39 two common triggers for membrane-active proteins, such as pore-forming bacterial toxins or viral  
40 fusion proteins (Butko et al., 1994; Harrison, 2015; Jiang and London, 1990; London, 1992;  
41 Menestrina et al., 1989).

42 We have investigated the effects of pH and phospholipid membranes on ligand binding by *B.*  
43 *mori* and *Manduca sexta* JHBPs by several methods. We show that acidic pH substantially reduces  
44 JHBP affinity for JH III, apparently increasing its  $k_{off}$ , which suggests that low pH favors ligand  
45 release, presumably by keeping the protein in the open conformation. We also report that JH III  
46 binding is diminished in the presence of anionic phospholipid liposomes, and that *B. mori* JHBP  
47 significantly interacts with these membranes. Taken together, the results support a collisional model of  
48 ligand release mechanism triggered by specific electrostatic and hydrophobic interactions on the  
49 surface of the plasma membrane of target cells.

## 51 2. Material and methods

### 53 2.1. Purification of native JHBP

1 Hemolymph JHBP from *B. mori* was purified using a modification of a published procedure  
2 (Kurata et al., 1994). The hemolymph was collected from fifth instar day 4 larvae into Falcon tubes on  
3 ice, frozen in liquid nitrogen and lyophilized. The lyophilizate was dissolved in 0.5 mM  
4 phenylthiourea in water before purification, centrifuged for 10 min at 10000 g and dialyzed against 20  
5 mM Tris-HCl, pH 7.5 overnight at 4 °C. The dialyzed sample was applied to a DEAE-Sepharose  
6 (DFF-100, Sigma-Aldrich) column and the proteins were eluted with a stepwise gradient of 50-300  
7 mM NaCl in 20 mM Tris-HCl, pH 7.5. Fractions containing JHBP were pooled, mixed with an equal  
8 volume of 2 M ammonium sulfate and applied to a Phenyl-Sepharose (Fast Flow, high substitution,  
9 GE HealthCare) column. Proteins were eluted with a stepwise gradient of 0.75-0.1 M ammonium  
10 sulfate in 20 mM Tris-HCl, pH 7.5 and then water. Fractions containing JHBP were pooled again and  
11 applied to a hydroxyapatite (Ceramic Hydroxyapatite Type I, BioRad) column. Proteins were eluted  
12 with a stepwise gradient of 10-300 mM sodium phosphate buffer, pH 6.8. Fractions from each  
13 purification step were analyzed by SDS-PAGE and DCC binding assay (Figure S2). This protein  
14 preparation of native *B. mori* JHBP (nBmJHBP) was used for initial binding studies (Figure 1), testing  
15 the effect of ionic strength (Figure S6), and estimation of  $k_{off}$  (Figures 5, S8). Other experiments were  
16 performed with recombinant *B. mori* (rBmJHBP, see below).  
17

## 18 2.2. Expression of recombinant JHBPs

19

20 *B. mori* larvae were reared on mulberry leaves under natural daylight conditions. Fat body was  
21 isolated from fifth instar day 5 larvae, frozen in liquid nitrogen and stored at -70 °C. Total RNA was  
22 isolated with the single phenol-chloroform-isothiocyanate extraction method (Chomczynski and  
23 Sacchi, 1987), and mRNA was isolated with the PolyATtract mRNA Isolation system III (Promega).  
24 The cDNA was synthesized with AMV reverse transcriptase and a primer corresponding to the mRNA  
25 fragment encoding the C-terminus of JHBP. The reverse transcription reaction mixture was used  
26 directly for PCR reactions with Pfu DNA polymerase and primers designed based on the published *B.*  
27 *mori* JHBP cDNA sequence (Vermunt et al., 2001): JHBPBmF (5'-  
28 GCAGAATTCCCATGGGAGATGGAGATGCTCTT) and JHBP-BMX-rev2 (5'-  
29 CGGTGAATTCTTAATTAAGATTTTCG), and the product coding the mature form of the protein  
30 (starting with Asp19 immediately after the signal peptide excision site (Vermunt et al., 2001) was  
31 ligated at the *EcoRI* site of pGEM™-T Easy Vector (Promega). An insert in the right orientation was  
32 excised with *EcoRI* and *NcoI*, and ligated at the *EcoRI* and *NcoI* sites of Pet-22b vector (Novagen,  
33 Merck) in frame with the pelB leader, allowing periplasmic localization. We found 28 nucleotides  
34 differing from the original published sequence (Vermunt et al., 2001), among which 10 yielded amino  
35 acid substitutions. These differences likely stem from the natural allelic diversity amongst *B. mori*  
36 strains (Figure S3). A cDNA kindly provided by W.G. Goodman (University of Wisconsin-Madison),  
37 was used as a template to amplify the sequence encoding *M. sexta* JHBP with primers JHBP-  
38 Manduca-fw (5'-ATATCCATGGGAGATCAAGGGGCACT) and JHBP-Manduca-rev (5'-  
39 CGCTGAATTCAAATAATTAACATCC). The product coding the mature form of the protein  
40 (starting with Asp19 immediately after the signal peptide excision site (Touhara et al., 1993) was  
41 fused in frame with the pelB leader in Pet-22b. As we failed to obtain soluble protein with the pelB  
42 leader system, the sequence coding for pelB was removed by digesting pET-22b (+) with *NdeI* and  
43 *MlsI*, the ends were polished with Klenow, and ligated to yield pET-22b-(minus)-pelB plasmids,  
44 encoding the mature forms with two extra residues (Met1 and Gly2) at the amino-terminus. Shuffle  
45 bacteria (New England Biolabs) were transformed with either *B. mori* or *M. sexta* Pet-22b-(minus)-  
46 pelB JHBP plasmid, grown in the presence of 0.5% glucose and protein expression was induced by  
47 IPTG. Bacterial pellets were lysed for 15 min at 30 °C in a lysis buffer (25 mM Tris-HCl, pH 8.0, 200  
48 mM NaCl, 100 µg/mL lysozyme, and proteases inhibitors), sonicated on ice, and centrifuged for 10  
49 min at 12000 g. Recombinant JHBPs from *M. sexta* (rMsJHBP) and *B. mori* (rBmJHBP) were purified  
50 from bacterial lysates essentially as described above for the native protein from *B. mori* (Figure S2).  
51

## 52 2.3. Ligand Binding Assays

53

54 The DCC (Dextran Coated Charcoal) assay was performed essentially as described previously  
55 (Touhara et al., 1993). Unless indicated otherwise, the continuous buffer system of McIlvaine

1 composed of citrate and phosphate was used to explore the binding for various pH values (McIlvaine,  
2 1921). For typical DCC assays, JHBP (50-300 nM) was diluted in a final volume of 100  $\mu$ L of buffer  
3 containing a JH esterase inhibitor (OTFP: 3-octylthio-1,1,1-trifluoro-2-propanone, a kind gift of B.D.  
4 Hammock (University of California) at a final concentration of 1  $\mu$ M, and about 10000 dpm [ $^3$ H]-JH  
5 III (~ 20 Ci/mmol, Perkin-Elmer). After incubation at room temperature (ca. 20  $^{\circ}$ C) for one hour in a 6  
6 x 50 mm borosilicate glass tube (Kimble-Chase, # 73500-650) precoated with a 10 % polyethylene  
7 glycol solution ((Goodman et al., 1976), Sigma # 95172), 20  $\mu$ L of a DCC slurry (5% (w/v) activated  
8 charcoal (Norit A, ACROS Organics # 134342500), 1 % (w/v) Dextran (Sigma # D4751), 1.5 mM  
9 EDTA) was added and the mixture was gently mixed by vortexing for about 2 s. After a 30 s  
10 incubation, the tubes were centrifuged (ca. 10000 g, 2 min), and the radioactivity of a 50  $\mu$ L aliquot of  
11 the supernatant was measured in a scintillation counter to estimate the amount of bound [ $^3$ H]-JH III. In  
12 these conditions, 10-50 % of [ $^3$ H]-JH III was bound by JHBP. For dialysis assays, 70  $\mu$ L of a buffer  
13 containing about 70000 dpm of [ $^3$ H]-JH III was loaded into each chamber of a disposable dialysis  
14 device with a 10 kDa MW cut-off (Harvard Apparatus, Holliston, MA, U.S.A.). Proteins (1-5  $\mu$ g of  
15 purified JHBP or bovine serum albumin (BSA) used as a control), were added to one chamber of the  
16 device. After overnight incubation at 4  $^{\circ}$ C, an aliquot of each chamber was counted in a scintillation  
17 counter, and the percentage of bound ligand was determined. For experiments involving lipid  
18 membranes, varying amounts (5-600  $\mu$ g) of liposomes were added to the chamber of a dialysis device  
19 with or without JHBP.  
20

#### 21 2.4. *Isothermal titration calorimetry*

22

23 Isothermal titration calorimetry (ITC) experiments were carried out at 293 K and 298 K using  
24 a VP-ITC microcalorimeter (GE Healthcare). *rBm*JHBP was dialyzed against 100 mM sodium  
25 phosphate buffer, pH 7.0, or 100 mM sodium acetate buffer, pH 4.5. Solutions of JH III were prepared  
26 in the same buffer as used for dialysis. The titration sequence included a single 3  $\mu$ L injection  
27 followed by 25 injections of 10  $\mu$ L of ligand solution, with 210 s intervals between injections.  
28 Measurements were performed for 5  $\mu$ M protein with 70  $\mu$ M ligand, or 2.5  $\mu$ M protein with 35  $\mu$ M  
29 ligand. The OriginLab software (GE Healthcare) was used to fit the raw data using a single binding-  
30 site model. For the analysis the data points obtained after the first 3  $\mu$ L injection were discarded. The  
31 experiments were performed 3 times for each ligand and protein concentrations indicated, and the  
32 dissociation constants ( $K_d$ ) were calculated.  
33

#### 34 2.5. *Circular dichroism*

35

36 Near UV CD spectra were recorded between 250 and 320 nm in a JASCO J-815  
37 spectropolarimeter at 20  $^{\circ}$ C, using a 1 cm path length quartz cell (Hellma). *rBm*JHBP (25  $\mu$ M) was  
38 dissolved in McIlvaine buffer at pH 3.5, 4.5, 5.5, 6.5 or 7.5. Data were averaged over 10 accumulated  
39 scans recorded at 1 nm intervals with a 50 nm/min scan speed. The spectra were corrected for buffer  
40 contributions and converted to molar ellipticity in  $\text{deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$ .  
41

#### 42 2.6. *Tryptophan fluorescence spectroscopy*

43

44 The intrinsic fluorescence of *rBm*JHBP was measured with a Cary Eclipse spectrofluorometer  
45 (Varian Instruments) at 20  $^{\circ}$ C, in a 2 mL quartz cuvette with a 1 cm path length equipped with a  
46 magnetic stirrer. The effect of ligand addition was examined using *rBm*JHBP at 10  $\mu$ M final  
47 concentration before and after 15 min equilibration with JH III at various concentrations (1.18, 2.32,  
48 3.42, 6.5, 11.87 and 20.25  $\mu$ M) in 100 mM sodium phosphate buffer, pH 7.0. The proteins were  
49 excited at 293 nm and emission spectra were recorded from 320 to 450 nm with a 10 nm bandwidth  
50 for both emission and excitation. The results were corrected for the dilution due to the addition of  
51 different volumes of JH III solution. For each ligand concentration, two or three spectra were recorded  
52 and averaged.

53 To study pH-dependent changes in fluorescence, a small volume of the concentrated  
54 *rBm*JHBP protein (28  $\mu$ L), was added to a large volume of McIlvaine buffer (972  $\mu$ L) at different pH  
55 values (4.5, 5.5, 6.5, 7.0, 7.5), thus ensuring a minor pH change of the final 10  $\mu$ M protein solution.

1 Fluorescence was recorded as described above. Three independent measurements were performed for  
2 each pH value and the spectra were averaged.

### 3 4 2.7. *Light scattering*

5  
6 The size and homogeneity of rBmJHBP at neutral and acidic pH in McIlvaine buffers were  
7 examined by light scattering using a Zetasizer Nano S apparatus (Malvern, U.K.).

### 8 9 2.8. *Liposome preparation*

10  
11 Solutions of lipids (DMPG (1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol, Sigma #43096),  
12 DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine, Sigma #P-2663), or a 1:1 mixture of both) at a  
13 10 mg/mL concentration in chloroform were evaporated, first by heating at 45 °C under a nitrogen  
14 stream, then for further 2-6 h with a centrifugal evaporator (Speedvac). The lipids were suspended in  
15 water (10 mg/ml), aliquoted and stored at -20 °C. Unilamellar liposomes were obtained by extrusion  
16 of the phospholipid suspension through a 100 nm pore size polycarbonate filter (Liposofast, Avestin),  
17 at 37 °C. The final amount of phospholipid was verified by gas chromatography, and the quality (size  
18 and distribution) of the unilamellar liposomes was verified by light scattering.

### 19 20 2.9. *Determination of fluidity of DMPG liposomes*

21  
22 Membrane fluidity of DMPG liposomes was assessed by the steady-state fluorescence  
23 polarization of 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate (TMA-  
24 DPH) (Sigma Aldrich). The concentration of liposomal preparations was adjusted to 10 mg/mL in  
25 McIlvaine buffer, pH 7.0. Protein solutions (rBmJHBP, heat-denatured rBmJHBP or BSA) were  
26 prepared in 1 mM Tris-HCl, pH 7.0, at a concentration of ca. 40 nM. TMA-DPH was prepared as a 1  
27 mM stock solution in tetrahydrofuran (THF). The liposomal preparations (30 µL) were transferred to a  
28 cuvette containing 2970 µL of buffer (1 mM Tris-HCl, pH 7.5) and labeled with TMA-DPH by  
29 addition of 4 µL of the stock solution. The suspension was stirred for 15 min at 35 °C to allow the  
30 probe to insert in the lipid bilayer. For the study of the effect of the proteins on liposome fluidity, 50  
31 µL of protein solutions were added to the cuvette before labeling with TMA-DPH. Anisotropy  
32 measurements were performed in a range of temperatures between 35 °C and 11 °C, with a Fluorolog-  
33 3 spectrometer (Jobin-Yvon, Horiba Group, USA), using T-format fluorescence polarizers, equipped  
34 with a thermoelectric Peltier junction. The excitation and emission wavelengths were 360 nm (5 nm  
35 bandwidth) and 431 nm (5 nm bandwidth), respectively. Steady-state fluorescence anisotropy ( $r$ ) was  
36 calculated as follows:  $r = (I_{vv} - GI_{vh}) / (I_{vv} + 2GI_{vh})$ , where  $I$  stands for the fluorescence intensity and  
37 the first and second subscripts refer to the setting of the excitation and emission polarizers,  
38 respectively.  $G = I_{hv} / I_{hh}$  is a correction factor for the monochromator's transmission efficiency for  
39 vertically (v) and horizontally (h) polarized light.

### 40 41 2.10. *Statistical analyzes*

42  
43 All analyses (Student's test, curve fittings, determination of pH<sub>50</sub>, determination of the  
44 temperature of phase transition) were performed with Graphpad Prism version 5.0d for Mac OS  
45 X (GraphPad Software, La Jolla, California USA).

## 46 47 3. **Results**

### 48 49 3.1. *Low pH reduces affinity of native and recombinant lepidopteran JHBPs for JH III*

50  
51 A sharp decrease of binding affinity of Lepidopteran JHBP in the low pH range was first  
52 discovered for the *G. mellonella* protein (Ozyhar and Kochman, 1987), and a similar correlation was  
53 later reported for the recombinant *B. mori* JHBP (Suzuki et al., 2011). We have examined the affinity  
54 of the native JHBP from *B. mori*, and recombinant proteins from *B. mori* and *M. sexta* with the  
55 Dextran Coated Charcoal (DCC) assay, and also observed a steep decrease of affinity at low pH in all

1 three cases, with a 50% reduction of binding in the pH range between 4 and 5.5 (Figure 1A). The  
2 native proteins were more sensitive to hydrogen ion concentration ( $pH_{50}$  values for *nBm*JHBP and  
3 *nGm*JHBP (inferred from data in (Ozyhar and Kochman, 1987)) were 5.08 and 5.45, respectively),  
4 than the recombinant proteins from *B. mori* and *M. sexta* ( $pH_{50}$  values were 4.18 and 4.67,  
5 respectively). The cause of the lower sensitivity of the recombinant proteins is unclear. The effect of  
6 low pH on *nBm*JHBP, *rBm*JHBP and *rMs*JHBP was also examined using a dialysis assay (Figure 1B).  
7 The results showed a significant (two- to four-fold,  $p < 0.001$ , Student's t test,  $n = 3$ ) reduction of  
8 binding at pH 4, when compared to pH 7. In addition, the relative sensitivity of the three proteins was  
9 the same as found with the DCC assay (Figure 1B): *nBm*JHBP was the most sensitive, whereas  
10 *rBm*JHBP showed only a *ca.* twofold reduction in affinity at pH 4 in this assay.

11 The dissociation constant ( $K_d$ ) of the recombinant *B. mori* JHBP for JH III was then  
12 determined at pH 4.5 and pH 7.0 by isothermal titration calorimetry (Figure 1C, D). Consistently with  
13 the above data, the  $K_d$  is increased at acidic pH. Interestingly, increasing the temperature to 25 °C had  
14 opposite effects at pH 4.5 and pH 7, as the  $K_d$  was increased at pH 4.5, but lowered at pH 7. This  
15 difference of dependence of the  $K_d$  on temperature may reflect the existence of distinct conformations  
16 of *rBm*JHBP at acidic and neutral pH (Vega et al., 2016). The  $K_d$  measured at 25 °C and pH 7 (37 nM)  
17 is similar to the  $K_d$  for JH II reported for a baculovirus-produced *M. sexta* JHBP (42 nM, (Touhara et  
18 al., 1993)), but much lower than the  $K_d$  for JH III reported earlier for a *B. mori* JHBP expressed in *E.*  
19 *coli* (450 nM, (Vermunt et al., 2001)). The  $K_d$ s reported in this study are, however, higher than those  
20 reported for enantiomerically-pure JH III and native *M. sexta* JHBP which were determined by  
21 equilibrium dialysis (1.86 nM, (Park et al., 1993)). These differences likely result from the varying  
22 conditions used in the different studies, notably the procedures for protein production and/or  
23 purification, the nature and purity of the ligands, and the methods used for binding assays (Park et al.,  
24 1993)). The effect of low pH cannot be accounted for by pH-dependent solubility of the ligand or  
25 ligand breakdown (such as through hydrolysis of the epoxide ring by nucleophilic attack at low pH  
26 (Figure S4)). Moreover, light scattering analysis showed that the apparent size and aggregation of  
27 recombinant *rBm*JHBP were essentially the same at acidic and neutral pH (Figure S5).

28 Since the ionic strength of McIlvaine buffers changes with pH (0.157 M at pH 4, and 0.427 M  
29 at pH 7, (Elving et al., 1956)), we were also concerned that this factor may contribute to changes in  
30 binding. However, binding experiments in the presence of 0-0.6 M NaCl showed that the binding  
31 affinity decreases with increasing ionic strength (Figure S6). Therefore, reduced binding at low pH can  
32 likely be attributed to increased proton concentration, rather than reduced ionic strength.

### 33 3.2. Reduced JH III binding at low pH is reversible

34 We next asked whether this effect of low pH on JHBP affinity could be reversed. When  
35 *nBm*JHBP was first allowed to bind JH at neutral pH and reach equilibrium, adjusting the pH to a  
36 value of 4 was sufficient to reduce dramatically the amount of bound JH. Conversely, after one hour  
37 incubation at pH 4, full binding activity could be recovered by adjusting the pH back to neutrality  
38 (Figure 2).

### 39 3.3. Effect of pH on JHBP structure

40 We next tested the hypothesis that reduced binding at acidic pH could be evoked by a switch  
41 from the closed to the open conformation, as proposed by Suzuki and colleagues (Figure S1) (Suzuki  
42 et al., 2011). Far and near UV circular dichroism (CD) analyses were performed to examine if the  
43 structure of JHBP is different at acidic and neutral pH. For these and further experiments *rBm*JHBP  
44 was used, except as indicated. Little change was observed in far UV CD spectra (Figure S7),  
45 suggesting that the secondary structure of *rBm*JHBP is essentially unchanged at these two pH values,  
46 consistently with the open and closed forms determined by NMR (Suzuki et al., 2011). These data  
47 show in addition that *rBm*JHBP is well structured at both pHs.

48 In contrast, however, near UV CD spectra taken at several pH values showed significant  
49 differences, indicating changes in the tertiary structure of the protein (Figure 3). We also obtained  
50 fluorescence spectra, taking advantage of the fact that *B. mori* JHBP contains a single tryptophan  
51 residue (Trp38) (Vermunt et al., 2001), in the loop forming the gatepost for the gate-forming  $\alpha 1$  helix  
52  
53  
54  
55

1 near the entrance of the ligand binding pocket (Figure 4, Figure S1, (Suzuki et al., 2011)). At neutral  
2 pH, ligand binding induces a change in the environment of Trp38, as evidenced by a significant drop  
3 of fluorescence emission at 293 nm with JH III concentrations ranging from about 1 to 20  $\mu\text{M}$  (Figure  
4 4A). This result shows that the environment of Trp38 changes, and is consistent with the fact that the  
5 nearby  $\alpha 1$  helix, which forms the “gate” of the ligand binding pocket, undergoes a major change of  
6 position upon ligand binding (Figure S1, (Suzuki et al., 2011)). Importantly, lowering the pH also  
7 strongly affected fluorescence intensity (Figure 4B). The fact that a drop in fluorescence emission is  
8 observed in both experiments is not paradoxical, because the fluorescence of tryptophan is known to  
9 be exquisitely sensitive to changes in the local environment (Hellmann and Schneider, 2019; Royer,  
10 2006), and the mere presence (Figure 4A) or absence (Figure 4B) of ligand, may have strong and  
11 hardly predictable effects.

12 Although we cannot exclude that the change in fluorescence seen with decreasing pH is partly  
13 due to the protonation of His206, **which is bound to Trp38** by  $\pi$ - $\pi$  stacking interactions (Suzuki et al.,  
14 2011), this observation, together with UV CD spectra, suggests that at acidic pH, *rBmJHBP* undergoes  
15 a significant change in tertiary structure that may involve the switch of the  $\alpha 1$  helix into the “open”  
16 conformation, and hence trigger ligand release.

### 17 18 3.4. *Binding kinetics*

19  
20 Considering the hypothesis that the reduced binding observed at low pH (Figure 1), or at high  
21 ionic strength (Figure S6), results from the opening of the gate-forming  $\alpha 1$  helix, we expected that in  
22 both conditions the dissociation rate constant ( $k_{\text{off}}$ ) of the *nBmJHBP*-JH III complex would be  
23 increased. To address this point, we attempted to estimate  $k_{\text{off}}$  using a modified DCC assay. As shown  
24 in Figure 5A, the estimated  $k_{\text{off}}$  of the complex was unchanged at high ionic strength, suggesting that  
25 low JH binding in this condition is caused by reduced access of JH III to the binding pocket, rather  
26 than by increased ligand release. In contrast, at acidic pH, the  $k_{\text{off}}$  appeared roughly three times greater  
27 than at neutral pH (Figure 5B). This result suggests that a **high proton concentration** may contribute to  
28 ligand release by *nBmJHBP*. The absolute values for  $k_{\text{off}}$  measured here should be considered as  
29 preliminary and rough estimates (since protein loss was evident for longer incubation times in this  
30 assay (Figure S8)). The  $k_{\text{off}}$  values determined at pH 7 in this study (ca. **0.033 s<sup>-1</sup>**, Figure 5) are similar  
31 to those measured for the native *M. sexta* JHBP-JH III complex by two different approaches (**0.0525 s<sup>-1</sup>**  
32 **and 0.0357 s<sup>-1</sup>**, (Park et al., 1993)), but must be confirmed in future experiments with a dedicated and  
33 accurate method (such as stop-flow spectroscopy). Together with the observation that low pH  
34 provokes a change in fluorescence intensity (Figure 4B), however, these results are consistent with the  
35 hypothesis that low pH contributes to a switch from the “closed” to the “open” conformation of the  $\alpha 1$   
36 helix.

### 37 38 3.5. *Interaction of JHBP with lipid vesicles*

39  
40 It was previously shown that if the dielectric constant of a binding mixture is increased by  
41 adjusting the solution to a final concentration of 30 % ethanol, thereby mimicking the hydrophobic  
42 environment of the cell membranes, JH III was released from JHBP (Suzuki et al., 2011). We have  
43 therefore decided to examine if ligand binding by *rBmJHBP* is modified in the presence of  
44 phospholipid membranes. Unilamellar phospholipid **vesicles** composed of either pure anionic 1,2-  
45 dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG), or a 1:1 mixture of DMPG and 1,2-dimyristoyl-  
46 *sn*-glycero-3-phosphocholine (DMPC, a zwitterionic phospholipid) were prepared, and their influence  
47 on JH binding was assessed by a dialysis assay (Figure 6). JHs are lipophilic hormones, and were  
48 shown earlier to partition into phospholipid membranes (Barber et al., 1981). Therefore, as expected,  
49 DMPG liposomes loaded in one chamber of a dialysis device bound [<sup>3</sup>H]-JH III in a dose-dependent  
50 manner from 5  $\mu\text{g}$  to 200  $\mu\text{g}$  per assay, with a plateau at about 70% bound ligand (Figure 6, white  
51 columns). In this dialysis assay, *rBmJHBP* alone showed robust binding to [<sup>3</sup>H]-JH III at neutral pH  
52 (Figure 6, black column). However, when JHBP was incubated with the ligand in the presence of  
53 DMPG vesicles at an amount equal to or greater than 40  $\mu\text{g}$ , the percentage of bound [<sup>3</sup>H]-JH III was  
54 significantly lower than when *rBmJHBP* was incubated alone (Figure 6, striped columns). This result  
55 therefore suggests that interaction with DMPG liposomes prevents binding of JH III by *rBmJHBP*. The

1 anionic nature of the phospholipid was apparently important for this effect, since even a high (600  $\mu\text{g}$ )  
2 amount of liposomes composed of a 1:1 mixture of the anionic DMPG and zwitterionic DMPC, was  
3 barely sufficient to significantly reduce JH III binding by *rBmJHBP* (Figure 6, grey columns).

4 To examine the effect of anionic phospholipids more closely, the interaction of *rBmJHBP* with  
5 DMPG liposomes was analyzed by a steady-state fluorescence polarization method ((Borenstain and  
6 Barenholz, 1993), Figure 7). The fluorescence anisotropy of the TMA-DPH probe embedded in lipid  
7 membranes is a measure of the motional order within the headgroup region of the phospholipids, as far  
8 down as C<sub>8</sub>-C<sub>10</sub> (Prendergast et al., 1981). DMPG liposomes were incubated first with the hydrophobic  
9 fluorescent probe TMA-DPH alone. The decreasing slope of TMA-DPH fluorescence anisotropy with  
10 temperature was relatively shallow, consistently with earlier observations ((Lamy-Freund and Riske,  
11 2003); Figure 7: liposomes alone). The shape of the temperature-dependent anisotropy curve was  
12 significantly different in the presence of *rBmJHBP* (Figure 7: *rBmJHBP*). Below 20 °C, the motion of  
13 acyl chains was increased, while membrane phospholipids were stabilized between 20 °C and 27 °C.  
14 Importantly, neither BSA nor heat-denatured *rBmJHBP* had an effect on TMA-DPH fluorescence.  
15 These results indicate that *rBmJHBP* strongly interacts with DMPG membranes at neutral pH, and that  
16 this interaction requires a proper folding of the protein.

#### 17 18 **4. Discussion**

19  
20 More than three decades ago, in their study of *G. mellonella* JHBP, Ozyhar and Kochman  
21 reported a steep decrease in its JH binding activity below pH 7, and proposed that this peculiar activity  
22 profile may be shared by JHBPs from other species ((Ozyhar and Kochman, 1987), Figure 1). A  
23 similar pH-sensitivity was later observed for a recombinant *B. mori* JHBP (Suzuki et al., 2011). We  
24 report here that the native JHBP purified from larval *B. mori* hemolymph, as well as recombinant  
25 JHBPs from this species and *M. sexta*, share indeed with their *G. mellonella* homologue a similar  
26 sensitivity profile at low pH (Figure 1).

27 We think that this conserved feature may hint to the existence of a biologically relevant ligand  
28 release mechanism involving the switch of the gate-forming  $\alpha 1$  helix, as proposed by Suzuki and  
29 coworkers (Figure S1, (Suzuki et al., 2011)). This hypothesis is supported by UV fluorescence and  
30 circular dichroism spectrometry, as well as light scattering data, showing that the secondary structure  
31 of *B. mori* JHBP is very similar at acidic and neutral pH, whereas in contrast, significant changes in  
32 tertiary structure are observed, consistently with a conformational switch of the  $\alpha 1$  helix (Figures 3,  
33 S5, S7).

34 Our fluorescence spectrometry data indicate that low pH also strongly influences the  
35 fluorescence intensity of *rBmJHBP* (Figure 4B), which likely reflects a significant change occurring in  
36 the environment of Trp 38, located in the “gatepost” of *B. mori* JHBP (Figure S1). This result would  
37 be expected if the  $\alpha 1$  helix switches to the closed conformation upon ligand binding, and is consistent  
38 with the apparent increase of  $k_{\text{off}}$  of the *nBmJHBP*-JH III complex (Figure 5). In their NMR studies,  
39 Suzuki and colleagues observed that there were only small differences in chemical shifts for most  
40 residues when JHBP was incubated at pH 4 or pH 6, and that the intermolecular NOEs between JH III  
41 and JHBP were conserved in both conditions, indicating that JH III was bound at acidic pH. Although  
42 these results may appear in contradiction with ours, this discrepancy may be explained by the high  
43 (0.6-1 mM) concentrations of JHBP and ligand applied in NMR experiments (Suzuki et al., 2011).  
44 Considering the  $K_d$  values determined in this study (Figure 1D), and the generally accepted maximal  
45 concentration range for juvenile hormones in aqueous solutions (30 to 50  $\mu\text{M}$  (Kramer et al., 1974)),  
46 the law of mass action predicts a high fractional JHBP occupancy (> 90%) at both neutral and acidic  
47 pH at concentrations applied in NMR experiments.

48 The TULIP superfamily of LTPs, to which JHBP belongs, has recently been recognized as  
49 very ancient, since it is present in a wide range of animal, fungal, algal, and plant cells, as well as in  
50 prokaryotic cells (Alva and Lupas, 2016; Wong and Levine, 2017). TULIP domain proteins are  
51 involved in a variety of processes related to lipid shuttling, either between membrane compartments or  
52 lipoprotein particles, such as HDL and LDL. In vertebrates, the plasma phospholipid transport protein  
53 (PLTP) and the cholesteryl-ester transfer protein (CETP), both harboring two TULIP domains, have  
54 well known functions in neutral lipid and phospholipid transfer between lipoprotein particles (Masson  
55 et al., 2009; Tzotzas et al., 2009). Although the details of the molecular mechanisms of docking and

lipid transfer of CETP and PLTP to lipoproteins are still debated, it is now well established that CETP interacts strongly with the outer phospholipid layer of lipoproteins (Lauer et al., 2016; Qiu et al., 2007; Zhang et al., 2012). Its N-terminal end penetrates ca. 50 Å into HDL particles, thus fully crossing the 18-27 Å phospholipid layer. Interestingly, shallow penetration into liposomes has also been observed (Charles and Kane, 2012), and CETP can efficiently load lipids from liposomes and deliver them to lipoproteins (Morton and Greene, 2003; Swenson et al., 1988), or transfer lipids from lipoproteins to liposomes (Morton and Izem, 2014). We report here that JH binding by JHBP is reduced in the presence of DMPG liposomes (Figure 6), and that JHBP strongly interacts with these phospholipid membranes, as judged by its effect on TMA-DPH fluorescence anisotropy in DMPG liposomes (Figure 7). It is therefore tempting to speculate that lepidopteran JHBPs, with their single TULIP domain, could deliver their lipidic hormonal cargo upon docking to plasma membranes, through a mechanism analogous to lipid delivery at the phospholipid surface of lipoproteins by their vertebrate relatives CETP and PLTP. The fact that this effect seems modulated by anionic charge density (since liposomes composed of a mixture of DMPG and DMPC, a zwitterionic phospholipid, had a much smaller effect on JH binding), suggests a primary involvement of electrostatic interactions. This is reminiscent of lipid transfer by CETP and PLTP, which is initiated by the electrostatic interaction of positively charged residues of CETP with negative charges (probably carried by phospholipids) at the lipoprotein surface (Desrumaux et al., 1998; Lagrost, 1994).

The data reported here are also similar to the findings on fatty acid binding proteins (FABPs), a family of small LTPs characterized by an antiparallel  $\beta$ -barrel fold. Fatty-acid binding by Liver FABP (L-FABP), for example, shows a steep decline towards the acidic region, and is strongly inhibited with increased ionic strength, similarly to *BmJHBP* (Stewart et al., 1998). Moreover, L-FABP binds to anionic phospholipid vesicles, and the mode of delivery of fatty acids to target membranes is thought to be collisional, rather than to depend on simple diffusion. It is believed to depend first on electrostatic interactions with anionic phospholipids at the membrane surface, leading to a conformational change inducing ligand release, but involves a subsequent membrane insertion or equivalent hydrophobic interactions (Corsico et al., 1998; Davies et al., 1998, 1999). Since we found evidence for both electrostatic and hydrophobic interactions with phospholipids (Figures 6 and 7), our data also suggest a collisional model of ligand release for *BmJHBP*. It is then conceivable that key interactions stabilizing the  $\alpha 1$  helix in the closed conformation, such as the hydrogen bonds between the  $\alpha 1$  helix and the C terminus described by Suzuki and colleagues (Figure S1, (Suzuki et al., 2011)), may be destabilized as part of the release mechanism.

Further experiments will be required to examine if ligand release actually depends on a locally lower pH, or alternatively, if low pH *in vitro* only mimics a combination of electrostatic forces prevailing in the vicinity of target membranes *in vivo*. For example, ligand release on target membranes enriched in acidic phospholipids could be explained by the creation of a negative surface potential, leading to a local increase in proton concentration.

Collectively, the data presented here support the model of ligand release first envisioned for *G. mellonella* JHBP and *E. postvittana* Takeout 1 (Hamiaux et al., 2009; Kolodziejczyk et al., 2008)1, and described in detail for *B. mori* JHBP by Suzuki and colleagues (Suzuki et al., 2011), whereby JH is released by a movement of the  $\alpha 1$  helix to the open conformation, and suggest that ligand release could be triggered by both electrostatic and hydrophobic interactions with the target membranes. These findings may be of general relevance for other JHBPs and Takeout proteins, since the structure of *G. mellonella* JHBP and *E. postvittana* Takeout 1 proteins are strikingly similar, presenting also an N terminal  $\alpha 1$  helix followed by a loop possibly playing the role of a gatepost (Hamiaux et al., 2009; Kolodziejczyk et al., 2008; Suzuki et al., 2011), and because molecular simulations have suggested that ligand entry and release by *E. postvittana* **Takeout 1** may occur in this region of the protein (Zhang et al., 2016). Ligand release from the other side of the protein may coexist with this mechanism, however, in the case of large ligands fitting in the continuous binding pocket of Takeout proteins (Hamiaux et al., 2009).

## Figure Legends

**Fig. 1.** Ligand binding by lepidopteran JHBPs is reduced at acidic pH.

1 Binding of [<sup>3</sup>H]-JH III by native *B. mori* JHBP (nBmJHBP), recombinant *B. mori* JHBP (rBmJHBP),  
2 and recombinant *M. sexta* JHBP (rMsJHBP) was assayed by the Dextran Coated Charcoal (DCC)  
3 method (A) and dialysis assay (B). To allow comparison, the highest percentage of bound [<sup>3</sup>H]-JH III  
4 observed is set to 100% for each protein. The pH values inducing a 50% reduction in binding (pH<sub>50</sub>)  
5 were determined from the curves in (A) and are respectively equal to 4.18, 4.67, 5.08 and 5.45 for  
6 rBmJHBP, rMsJHBP, nBmJHBP and native *G. mellonella* JHBP (nGmJHBP, data inferred and  
7 redrawn with permission from figure 7 in (Ozyhar and Kochman, 1987)). (C, D) The K<sub>d</sub> values of  
8 rBmJHBP for JH III at two different temperatures were determined by isothermal titration calorimetry  
9 (ITC). (C) Illustration of results of a typical ITC experiment, with 5 μM rBmJHBP and 70 μM JH III,  
10 at pH 7 and 20 °C: raw data (top), and integrated data (bottom). The arrow indicates the slope of the  
11 curve used to determinate K<sub>d</sub>. (D) Summary of K<sub>d</sub> determinations by ITC. \*: p < 0.05, Student's t test,  
12 unpaired, one-tailed, n = 3.

13  
14 **Fig. 2.** Reversibility of the effect of low pH.

15 **Recombinant *B. mori* JHBP (ca. 1.2 · 10<sup>-7</sup> M) was incubated with 2 · 10<sup>4</sup> dpm [<sup>3</sup>H]-JH III for 2 h at pH 7**  
16 **(black column), for 1 h at pH 7, then for 1 h at pH 4 after adjusting the pH (hatched column), or first**  
17 **for 1 h at pH 4, then for 1 h at pH 7 after adjusting the pH (white column). Initial incubations were**  
18 **done in 100 μL diluted McIlvaine buffer (1/10), and pH adjustment was obtained by addition of an**  
19 **equal volume of undiluted buffer at the desired pH. The percentage of bound hormone was determined**  
20 **with the DCC assay. \*\*\*: p < 0.001 Student's t test, unpaired, two-tailed, n = 3 (black and hatched**  
21 **columns) or n = 5 (white column).**

22  
23 **Fig. 3.** Near UV CD spectra of rBmJHBP at different pH values.

24 Near UV CD spectra of rBmJHBP were recorded between 320 and 250 nm. The spectra were recorded  
25 for a 10 μM rBmJHBP solution in McIlvaine buffer with varying pH.

26  
27 **Fig. 4.** Fluorescence emission spectra of rBmJHBP.

28 (A) The spectra of a 10 μM protein solution were recorded after excitation at 295 nm before (solid  
29 line) and after 15 min equilibration with JH III at various concentrations. The measured intrinsic  
30 fluorescence is given in arbitrary units (A.U.) In (B), the spectra were recorded for a 10 μM protein  
31 solution without ligand, in McIlvaine buffer at varying pH.

32  
33 **Fig. 5.** Dissociation kinetics of JH III from JHBP.

34 To estimate the half-life (t<sub>1/2</sub>) of the nBmJHBP-JH III complex and the rate constant of dissociation  
35 (k<sub>off</sub>), nBmJHBP was first incubated in McIlvaine buffer at pH 7, 0 M NaCl with labelled hormone  
36 ([<sup>3</sup>H]-JH III) to achieve equilibrium. At t<sub>0</sub>, an equal volume of DCC slurry was added, allowing buffer  
37 change (0.6 M NaCl (A), or pH 4 (B)), as well as quick and complete removal of unbound hormone  
38 (Fig. S8 (A)). DCC was next eliminated by centrifugation, after incubation for various periods of time.  
39 Since the amount of JHBP left in solution decreased only slowly with time, and was similar in the  
40 different conditions (Fig. S8 (B)), the radioactivity in the supernatant presumably reflects the release  
41 of [<sup>3</sup>H]-JH III by JHBP. (A) Effect of high ionic strength. (B) Effect of pH. n = 3-6 for each time  
42 point. \*\*\* indicates P < 0.0001.

43  
44 **Fig. 6.** Inhibition of binding of JH III to JHBP by model membranes. Binding of [<sup>3</sup>H]-JH III by  
45 rBmJHBP was assayed with a dialysis assay (see Material and Methods for details). The black column  
46 represents [<sup>3</sup>H]-JH III bound to rBmJHBP at equilibrium. Note that DMPG liposomes alone were also  
47 able to bind a significant, but smaller amount of [<sup>3</sup>H]-JH III (white columns). DMPG/DMPC  
48 liposomes bind similar amounts of [<sup>3</sup>H]-JH III (data not shown). When rBmJHBP was incubated  
49 together with 40 μg or higher amounts of anionic liposomes, the total amount of bound [<sup>3</sup>H]-JH III  
50 was significantly reduced (DMPG, striped columns) compared to the JHBP-only control, and was  
51 comparable to the amount bound by DMPG vesicles alone. In contrast, 600 μg of liposomes composed  
52 of a 1:1 ratio of DMPC and DMPG only slightly inhibited binding (DMPG/DMPC liposomes, grey  
53 columns). \*\*\*: p < 0.0001, N=3-9, Student's t test, two-tailed.

54  
55 **Fig. 7.** Steady-state fluorescence anisotropy of TMA-DPH in DMPG liposomes.

1 TMA-DPH was incubated in 1 mM Tris-HCl, pH 7.5 with DMPG liposomes alone, or with DMPG  
2 liposomes and *rBm*JHBP, BSA or heat-denatured *rBm*JHBP. Each curve represents the average of  
3 three replicates. Protein concentration was ~ 4 nM, and DMPG concentration was ~ 145  $\mu$ M, thus the  
4 lipid:protein molar ratio was ~ 1:35000 (lipid:protein mass ratio ~ 1/1000).

## 8 Legends of supplementary figures

9  
10 **Supplementary figure S1.** Crystal structure of *B. mori* holo- (A) and apo-JHBP (B).

11 (A) Juvenile hormone II (JH II, shown as balls and sticks) bound to JHBP (PDB id: 3AOS). The gate-  
12 forming amino-terminal  $\alpha$  helix ( $\alpha$ 1) is shown in red, and the “gatepost” is highlighted in green. W38  
13 is the only tryptophan residue of *B. mori* JHBP, and the main contributor to the JHBP fluorescence  
14 (Fig. 4). The residues in yellow are involved in direct hydrogen bonds that participate in keeping the  
15  $\alpha$ 1 helix in its closed position. They are shown here only as examples of candidate bonds that may be  
16 disturbed at low pH. The closed conformation is further stabilized by polar interactions involving  
17 water molecules, as well as polar and hydrophobic interactions with JH II (Suzuki et al., 2011). (B)  
18 Apo-JHBP (PDB id: 3AOT). The arrow depicts the ca. 70 ° swing of the  $\alpha$ 1 helix allowing JH II  
19 release in the open conformation (Suzuki et al., 2011). The images were done with PyMOL 2.3.1.

20  
21 **Supplementary figure S2.** Purification of native and recombinant JHBPs.

22 (A) Flow-chart for purification. The Coomassie stained gel at the bottom shows purified proteins used  
23 in this study. Purity is estimated to be greater than 95%. Note that both native and recombinant  
24 proteins migrate with apparent molecular weights around 30 kDa instead of the predicted molecular  
25 weight (~ 25 kDa). This electrophoretic behavior has been already noted in previous studies (see for  
26 example (Vermunt et al., 2001)). (B) Representative last purification step of recombinant *B. mori*  
27 JHBP (*rBm*JHBP) on hydroxyapatite (Ceramic Hydroxyapatite Type I, BioRad). Bars represent  
28 concentrations of sodium phosphate used for elution, the dotted line shows [<sup>3</sup>H]-JH III binding capacity  
29 of each fraction. In the Coomassie stained gel at the bottom, stars indicate fractions pooled for  
30 experiments. On the X axis the names of collected fractions are shown. Start: starting material, FT:  
31 Flow Through, W: Wash, MW: Molecular weight markers (kDa).

32  
33 **Supplementary figure S3.** Sequence of the cDNA encoding *B. mori* JHBP used in this study.

34 The first codon (GAT) of the mature protein (after the excision of the putative signal peptide predicted  
35 in (Vermunt et al., 2001) starts at position 7, and the 6 first nucleotides encode 2 residues added in this  
36 study for expression purposes (Met1, Gly2). We found 28 nucleotide differences in our strain (top  
37 sequence), when compared to the sequences published in (Vermunt et al., 2001) (bases in red). Among  
38 these base changes, 13 were synonymous, and the remaining 15 lead to a total of 10 amino acid  
39 substitutions. Three of these amino acid changes (highlighted in yellow) were also found by Vermunt  
40 and colleagues in an independent cDNA referred to as JHBP2. These variants presumably reflect the  
41 allelic variability of the different strains of silkworms used in the two studies.

42  
43 **Supplementary figure S4.** Stability of JH III at various pHs.

44 (A) [<sup>3</sup>H]-JH III (about 10000 dpm) was incubated for one hour at 20 °C in McIlvaine buffer, at various  
45 pH (3 replicates each), and analyzed by TLC. (B) The regions of interest of the silica plate (intact  
46 [<sup>3</sup>H]-JH III and polar metabolites) were scraped off and radioactivity was counted. Student's t-test n =  
47 3 \*: p = 0.0086. Note that although there is a significantly higher degradation of [<sup>3</sup>H]-JH III at pH 4,  
48 it cannot account for the decreased JH binding shown in Fig. 1. (C) Solubilization of [<sup>3</sup>H]-JH III is not  
49 influenced by pH (n = 3).

50  
51 **Supplementary figure S5.** Light scattering analysis of *rBm*JHBP. The size and aggregation of  
52 *rBm*JHBP (in McIlvaine buffer) was assayed with a Zetasizer light scattering device (Malvern, UK).  
53 The apparent radius of *rBm*JHBP was not affected by pH ( $r = 2.56 \pm 0.77$  nm at pH 6.9;  $r = 2.49 \pm$   
54  $0.48$  nm at pH 4.5). Some aggregation was evidenced at pH 4.5 (bottom left), but it represented a  
55 negligible fraction of the total protein. The % volume (right) reflects more accurately the relative

1 abundance in each size category, because intensity depends on light scattering, which is much greater  
2 for a large particle than for a small particle (the intensity of scattering is proportional to the sixth  
3 power of its diameter).

4  
5 **Supplementary figure S6.** Effect of ionic strength on JH III binding by *nBmJHBP*. JH III binding  
6 was measured using the DCC assay, in 10 mM Tris-HCl, pH 7, with NaCl concentrations as indicated.

7  
8 **Supplementary figure S7.** Far-UV CD spectral analysis of *rBmJHBP* at acidic and neutral pH.

9  
10 **Supplementary figure S8.** Control experiments for analysis of ligand release by the DCC method.  
11 (A) Unbound labeled hormone ( $^3\text{H}$ -JH III) is efficiently removed by Dextran Coated Charcoal  
12 (DCC).  $^3\text{H}$ -JH III (about 100000 dpm) was incubated for 30 min at 20 °C, in the same buffers as  
13 those used for analysis of ligand release (Figure 5) and exposed for 20 s at 20 °C to DCC. After  
14 centrifugation, (30 s, ca. 10000 g), the percentage of labelled hormone left in solution was measured by  
15 scintillation counting. Note that this short DCC treatment is sufficient to virtually eliminate all free  
16  $^3\text{H}$ -JH III in the three different conditions. (B) Assessment of *nBmJHBP* removal by DCC. To  
17 confirm that the apparent different half-lives of the complexes shown in Fig. 5 are not merely  
18 reflecting the differential binding of *nBmJHBP* to charcoal in different buffers, *nBmJHBP* was  
19 incubated in the same conditions as those used in Figure. 5. After 20 s (top) or 2 min (bottom)  
20 exposure to DCC, followed by a centrifugation to pellet charcoal (30 s, ca. 10000 g), the amount of  
21 protein remaining in solution was examined by SDS-PAGE and silver staining (Pierce). Note that  
22 there is no or little protein loss after 20 s exposure to DCC. Protein loss occurs after 2 min exposure,  
23 but appears similar in all three conditions. The asterisk indicates a 31 kDa MW marker.

## 24 25 26 27 28 **Acknowledgments**

29  
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32

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