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Elephant Albumin: A Multipurpose Pheromone Shuttle

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Summary

(*Z*)-7-dodecenyl acetate (*Z*7-12:Ac) is present in the urine of female Asian elephants (*Elephas maximus*) approaching ovulation and functions as a female-to-male sex pheromone. Here we show that a significant fraction of the pheromone in the urine is bound to a protein, elephant serum albumin (ESA), and provide evidence for key physiological functions of urinary ESA. Our biochemical and behavioral experiments suggest a three-fold role of ESA in pheromone signaling: (1) transporting *Z*7-12:Ac from serum into urine; (2) extending the presence of the pheromone in the environment without hampering detection; and (3) targeting pheromone delivery to chemosensory organs through localized release of the ligand induced by a pH change. The exploitation of albumin in pheromone transport clearly distinguishes the elephant from other mammals studied, and complements the uniqueness of elephant anatomy, physiology, and behavior.

Introduction

Adult Asian elephant (*Elephas maximus*) females communicate their periovulatory state by excreting a sex pheromone, (*Z*)-7-dodecen-1-yl acetate (*Z*7-12:Ac), in their urine [1]. Quantitative solid-phase microextraction (SPME) showed that the urinary *Z*7-12:Ac concentration varies from nondetectable levels (< 1 nM) during the luteal phase of the estrous cycle to high micromolar levels in the days just prior to ovulation (follicular phase) [2]. Changes in pheromone concentration correlate with oscillations of total urinary protein concentration (from 64.7 ± 1.9 μg/ml in the luteal stage to 97.6 ± 1.4 μg/ml prior to ovulation) and pH (7.7 in the luteal phase, 8.4 prior to ovulation) [2]. Male elephants exhibit a range of responses to the pheromone, beginning with olfactory sniffs followed by check and place responses, in which the male touches the pheromone-containing urine with the muciferous trunk tip [3]. Next, in the flehmen response, the elephant places the mucus-urine mixture on the trunk tip into the paired openings of the vomeronasal ducts in the roof of the mouth [4]. Subsequent to flehmens, penile erections and mounting attempts (even on inanimate objects treated with the pheromone) are frequently observed [1].

The majority of mammalian pheromones have been reported to be secreted in association with proteins of the lipocalin protein family. The mouse pheromones excreted in urine are bound to major urinary proteins (MUPs) [5, 6]. Rat preputial gland secretions contain both the pheromone, dodecyl propionate [7], and members of the lipocalin protein family [8]. In pigs, the male-to-female sex pheromones (androstenone and androstenol) are secreted from the maxillary glands, presumably bound by another lipocalin, pheromaxein [9, 10]. The hamster pheromone may be a small ligand of aphrodisin, a lipocalin present in hamster vaginal discharge [11, 12].

The role of the secretory lipocalins is likely threefold: (1) transporting the pheromone into the secretory solutions [13]; (2) prolonging the period of bioavailability of the pheromone by retarding the rate of its evaporation [14]; and (3) modulating the pheromone activity by affecting the sensory organ responses [15]. In the mouse (*Mus musculus*), arguably the most-studied mammalian olfactory system, the pheromone carrying MUPs are the most abundant proteins in the urine. The MUP-pheromone complexes dissociate slowly [14, 16], thereby extending the period during which the volatile pheromone is bioavailable. Addition of MUPs to the synthetic mouse pheromones alters the spatial pattern of activation in the female accessory olfactory bulb [17]. This probably correlates with the finding that MUPs play roles in individual recognition [18].

In analogy to other mammalian pheromone systems, and because of poor recovery of lipophilic elephant pheromone using organic solvent extraction, we postulated that the elephant pheromone was bound to a urinary protein. The large difference in pH between the female urine (pH 8.4) and male sensory organs (pH 5.5) indicated that pH might be important in pheromone binding and release by the urinary proteins [2]. Since *Z*7-12:Ac also serves as a part of sex pheromone blends in many insect species, techniques used for characterization of insect pheromone binding proteins could be implemented to study this mammalian pheromone system. The in vitro biochemistry of protein-pheromone interactions was evaluated in parallel with the in vivo responses of male elephants to the protein-pheromone complexes. In this manner, we have identified the elephant urinary pheromone binding proteins, revealed the

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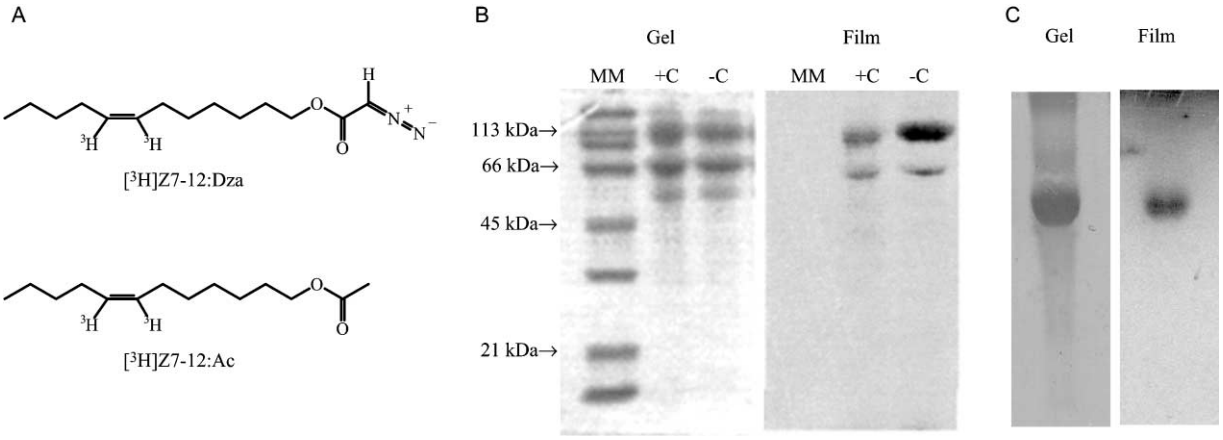

Figure 1. Identification of Urinary Proteins that Bind the Pheromone

(A) The probes used were the tritium labeled elephant pheromone, (*Z*)-7-[^{3}H]-dodecen-1-yl acetate ([^{3}H]*Z*7-12:Ac) and the tritium labeled photoactivatable analog, (*Z*)-7-[^{3}H]-dodecen-1-yl diazoacetate ([^{3}H]*Z*7-12:Dza).

(B) Photoaffinity labeling of female elephant urine. Concentrated follicular phase urine (20 μl) was photolabeled with 0.5 μCi of [^{3}H]*Z*7-12:Dza in presence and absence of nonradioactive *Z*7-12:Ac as a competitor (lanes +C and −C, respectively). The left pane shows the 15% (w/w) polyacrylamide gel of the labeled urinary proteins. Noteworthy is the absence of proteins in the 15–20 kDa range, typical for lipocalins. The right pane shows the autoradiogram of the gel in the left pane. Apparent is labeling of a 130 and a 65 kDa protein (lane −C), which can be competed off with the pheromone (lane +C). A 55 kDa protein band that is not labeled is also visible. The 65 and 130 kDa proteins are the monomer and dimer of albumin, respectively. Lane MM contained molecular weight markers.

(C) Native PAGE/blot of concentrated female urine after incubation with [^{3}H]*Z*7-12:Ac, carried out at elevated pH (8.5). Radioactivity detected by autoradiography on a film (right) corresponds to a strong albumin band on the native PAGE blot (left).

biochemical properties of the pheromone-protein interactions, and extrapolated the most likely physiological and behavioral roles for these interactions. Based on our results, we propose a working model for pheromone perception in elephants, and possibly other mammalian species.

Results

Protein Content of Urine

Female Asian elephant urine contains, on average, between 58 μg/ml (luteal and early follicular phase) and 98 μg/ml (periovulatory period) of protein [2]. Higher urinary protein concentrations coincide with the presence of *Z*7-12:Ac in the urine. The major protein of elephant urine is albumin, as revealed by N-terminal sequencing of proteins separated by electrophoresis. Albumin is present in the urine both as a monomer (66 kDa) and as a dimer (132 kDa). The urine also contains significant amounts of a 55 kDa protein, which was N-terminally blocked. Notably, no significant amounts of protein in the lipocalin molecular mass range were observed in the urine either by MALDI-MS (data not shown) or SDS-PAGE (Figure 1B). Western detection of major urinary proteins (MUPs) in the elephant urine using polyclonal antibodies against the mouse MUPs did not reveal any MUP immunoreactivity (data not shown). No significant differences in protein composition between luteal and follicular phase urine were discernible by SDS-PAGE.

Binding of *Z*7-12:Ac by Urinary Albumin

In order to identify proteins in the urine capable of binding the pheromone, two types of experiments were conducted: (1) photoaffinity labeling with [$^3H_2$]*Z*7-12:Dza, and (2) native PAGE/electroblotting after incubation with [$^3H_2$]*Z*7-12:Ac. Both experiments were performed on concentrated anoestrous female elephant urine.

Photoaffinity labeling (Figure 1B) revealed binding of the photoactivatable analog to two urinary proteins of molecular mass approximately 65 and 130 kDa. Labeling could be displaced by addition of excess nonradioactive pheromone to the mixture prior to irradiation with UV light. Edman sequencing of the purified proteins yielded the N-terminal sequence AYKSEIAHRYKD, 85%–95% identical to N-terminal sequences of known mammalian serum albumins, suggesting that the labeled proteins are the monomeric and dimeric form of an elephant albumin. The molecular mass of the urinary albumin as determined by MALDI-MS (65,918 ± 65 Da) is in good agreement with that of the elephant albumin purified from the serum (65,984 ± 65 Da).

Native PAGE/electroblotting of the concentrated urine, preincubated with the radiolabeled pheromone, did not reveal any binding when performed at pH 6.4 (according to Laemmli) [19]. However, when the electrophoresis was conducted at alkaline pH (8.5), binding of the radioactive pheromone to native ESA was observed (Figure 1C).

Cloning of ESA

The cDNA coding for ESA was PCR amplified from cDNA prepared from female elephant liver tissue. The 5′ primer was designed based upon conserved sequences of known mammalian albumins; the 3′ primer was $(dT)_{18}$. The 2011 bp PCR product was cloned into a pCRII TOPO XL vector (Invitrogen). Six identical clones containing the albumin cDNA were sequenced.

The amplified ESA cDNA encodes a predicted protein of 599 amino acids, including all 586 amino acids of the

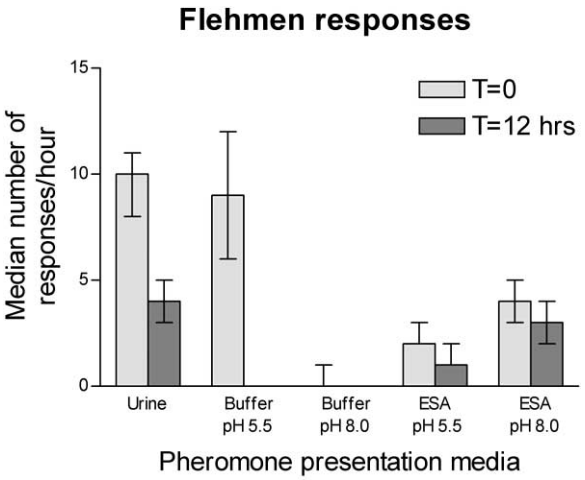

Figure 2. Effect of ESA on the Period of Bioavailability of the Pheromone

Number of flehmen responses to pheromone samples in different media (preovulatory urine, phosphate buffer of pH 5.5 or 8.0, with or without ESA), exhibited per hour to fresh samples (T = 0; light gray) and aged samples (T = 12 hr, dark gray). The data are plotted as median and 25%/75% percentiles. Over the period of 12 hr, the biological activity of the pheromone presented in the acidic and alkaline buffers dropped to zero. The ESA-containing solutions (urine, alkaline ESA solution, and to a lesser extent also the acidic ESA solution) continue to elicit flehmen responses even after 12 hr. Thus, presence of ESA preserves bioactivity of the pheromone samples for 12 hr. The effect is most pronounced when pH is in the alkaline range, similar to the natural pH of elephant urine.

mature protein, all 6 amino acids of the propeptide, and 7 of the probable 18 amino acids of the signal peptide (Figure S1). The predicted protein sequence is highly similar (68%–74% identity, 81%–85% similarity) to known mammalian serum albumins. The predicted molecular mass of the mature protein (65,962 Da) is in good agreement with the molecular mass of the albumin from urine (65,918 ± 65 Da) and serum (65,984 ± 65 Da), as determined by MALDI-MS.

ESA Extends Pheromone Bioavailability

Solutions of the pheromone, *Z*7-12:Ac, at alkaline or acidic pH and with or without ESA, were tested for biological activity immediately after preparation and after 12 hr of exposure to environmental conditions simulating natural habitat. The results are summarized in Figure 2 (flehmen responses − hallmark pheromone responses) and in Figure S2 (olfactory responses − not specific to the pheromone). The freshly made pheromone solution in alkaline buffer elicited only a low number of flehmen responses (median 0, range 0–1, n = 22). In contrast, *Z*7-12:Ac in acidic buffer exhibited a very high biological activity (median 9, range 5–15), comparable to the preovulatory female urine (median 10, range 6–13). The activity of the fresh solutions containing ESA was moderate (2, 1–5 for the acidic solution; 4, 2–5 for the alkaline solution), but significantly higher than the background (0). When the identical solutions that had been aged for 12 hr were used, the pattern of observed biological activity changed. The activity of samples without ESA dropped to 0, while the activity of ESA-containing pheromone solutions persisted (1, 1–3 for the acidic ESA solution; 3, 1–5 for the alkaline solution; 4, 3–6 for the preovulatory urine). At T = 12 hr, the activity of the alkaline ESA solution was statistically significantly higher ($p < 0.0001$ by the Wilcoxon signed rank test) than the activity of the acidic ESA solution, while not significantly different ($p > 0.05$) from that of the preovulatory female urine. In contrast to the flehmen responses, the olfactory responses to the pheromone solutions were similar to each other in numbers and close to background levels for all the different media and both time points, with the exception of the preovulatory urine and the acidic buffer devoid of ESA (both at T = 0), which elicited significantly higher numbers of responses (see Figure S2).

pH-Dependent Pheromone Binding by ESA

The pheromone binding properties of ESA were investigated by photoaffinity labeling (Figure 3A) and a volatile odorant binding assay (VOBA) (Figure 3B), performed at various pH values, in order to mimic the conditions in the urine (alkaline) and sensory organs (acidic). The photoaffinity labeling experiment showed that ESA labeling was pH dependent, with intense labeling at alkaline pH and weaker labeling at acidic pH. The VOBA experiment indicated that on average at pH = 9.0, four pheromone molecules were bound by one molecule of ESA, while at pH = 5.0, less than one pheromone molecule was bound by each ESA molecule. Separation of native proteins preincubated with tritiated *Z*7-12:Ac revealed binding only to ESA, and only when the electrophoresis was conducted at alkaline pH. In contrast, no pheromone binding was detected when the experiment was performed under standard PAGE conditions (pH 6.4, Tris-glycine buffer).

Discussion

Binding of the Pheromone by Elephant Urinary Proteins

We have investigated the protein composition of adult female elephant urine, as well as the pheromone binding properties of the urinary proteins. Since *Z*7-12:Ac is also a common component of insect pheromone blends, we applied two techniques used in insect pheromone research to this mammalian system. Photoaffinity labeling with diazoacetate analogs of insect pheromones has in the past been used to identify the first insect pheromone binding proteins (PBPs) and to find their amino acid residues interacting with the pheromone. While the latter findings are a part of a conflicting body of evidence, the former are universally accepted. In our current research, we have used photoaffinity labeling with [^{3}H]*Z*7-12:Dza, a photoactivatable analog of the pheromone (Figure 1A), to reveal binding of the pheromone by an elephant albumin present in the female urine (Figure 1B). Like photolabeling, autoradiography of nondenaturing electrophoresis gels of protein mixtures preincubated with the radiolabeled pheromone has also been used to identify and characterize insect PBPs. Here we show that autoradiography of elephant urinary proteins preincubated with tritiated *Z*7-12:Ac and separated electrophoreti-

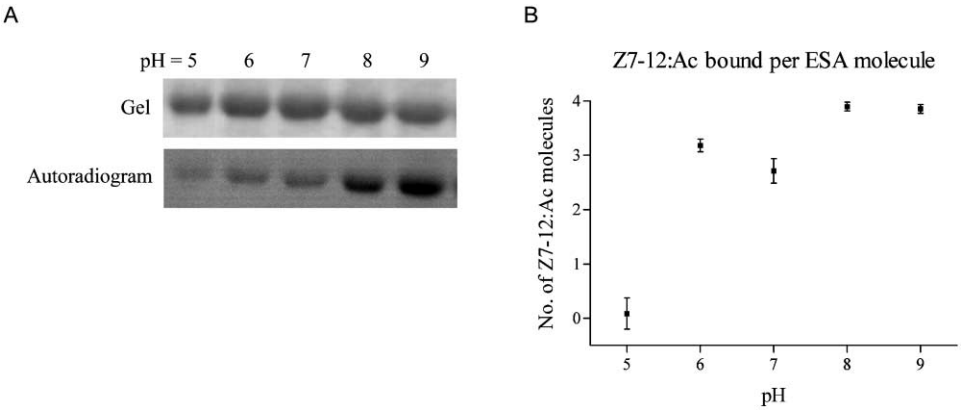

Figure 3. Effect of pH on Pheromone Binding by ESA

(A) Photoaffinity labeling of purified elephant serum albumin at various pH values. ESA (10 μg) in 50 mM phosphate buffer of pH 5.0–9.0 was photolabeled with 0.5 μCi of [^{3}H]*Z*7-12:Dza. Photolabeling is strong at alkaline pH and weak at acidic pH.

(B) Volatile odorant binding assay on purified ESA carried out at pH values indicated. The value axis shows the number of molecules of the pheromone bound per one molecule of ESA. At alkaline pH, up to four molecules of the pheromone are bound to one molecule of ESA under the conditions of the assay. The number of molecules bound decreases gradually from pH 9.0 to 6.0, then drops to less than one molecule at pH 5.0.

cally at alkaline pH also reveals pheromone binding to ESA (Figure 1C).

Several lines of evidence suggest that the elephant urinary albumin is identical to ESA. First, it has long been established that, in mammals, small amounts of serum albumin pass from the bloodstream into the urine under physiological conditions [20]. Second, elephant urinary and serum albumins exhibit identical N-terminal protein sequences. Third, the molecular mass of the serum and urinary albumins (as determined by MALDI-MS) and the predicted molecular mass of the cloned albumin are essentially identical. Fourth, the pheromone present in the preovulatory serum is bound to a protein, as evidenced by a sharp increase in the amount of pheromone detected upon protease treatment [2]. Thus, it is likely that the pheromone is present in blood as an ESA complex. This complex could then cross intact from serum into the urine.

No binding of the pheromone to any putative lipocalins was observed in the urine. Instead, only traces of protein in the lipocalin molecular size range were observed in urine (Figure 1B). Much higher urinary lipocalin concentrations would be expected if lipocalins were to play an important role in elephant pheromone secretion or delivery. The apparent absence of lipocalins from female elephant pheromone urinary proteins distinguishes the elephant pheromone system from all other mammalian pheromone systems studied thus far. Nonetheless, lipocalins are present in the chemosensory organs: the elephant odorant binding protein (OBP) found in male trunk mucus was recently found to act as a pheromone scavenger that may terminate the olfactory response [21].

Effect of pH on Pheromone Binding by ESA

The pH dependence of pheromone binding by ESA is consistent with our previous observation that extraction of the pheromone from urine was facilitated by acidification. It also has important physiological ramifications, due to the large difference in pH between the female urine and the male chemosensory organs [2]. To investigate the effect of pH on *Z*7-12:Ac binding, three experiments were conducted at different pH values: (1) photoaffinity labeling (Figure 3A), (2) VOBA (Figure 3B), and (3) native PAGE with noncovalently bound radiolabeled pheromone (Figure 1C). The photolabeling experiment relies upon two tacit assumptions. First, the binding of the pheromone analog, but not the photolabeling reaction itself, should be affected by pH. Second, the binding profile of the pheromone must be similar to that of the photoactivatable analog. Thus, although the photolabeling experiment showed a strong effect of pH on ESA labeling, additional evidence for the pH dependence of pheromone binding by the ESA was required.

In the VOBA experiment (Figure 3B), true equilibrium binding of the pheromone is observed. However, in VOBA, it is not possible to control the concentration of the ligand in solution, since the concentration depends on the solubility of the ligand. Since the solubility of *Z*7-12:Ac in PBS buffer shows weak pH dependence (data not shown), the free pheromone concentration in active samples of acidic pH was higher (ca. 15 μM) than in neutral or alkaline samples (11 μM). Samples at pH 6.0, in which the increased pheromone solubility resulted in higher free pheromone concentration therefore showed disproportionately high *Z*7-12:Ac binding. Since the dissociation constants of the complex between ESA and *Z*7-12:Ac are unknown, it was not possible to accurately quantify the extent of this distortion. Nevertheless, the VOBA showed that *Z*7-12:Ac binding to ESA at equilibrium is higher at alkaline pH and lower at acidic pH. Native PAGE of the ESA-[^{3}H]*Z*7-12:Ac complex carried out at alkaline pH (8.4) and acidic pH (6.4) indicated that the affinity of ESA for the pheromone is higher, or the dissociation rate of the complex is slower, at alkaline pH. Together, the three experiments provide convincing evidence that the binding of the pheromone by ESA is stronger at alkaline pH than at acidic pH. The biochemical evidence for the effect of pH on pheromone binding

by ESA is further enhanced by our behavioral results showing significantly higher ($p < 0.0001$) retention of the pheromone by ESA at alkaline pH than at acidic pH (Figure 2).

The change in affinity of the ESA for the pheromone probably occurs through a conformational change in the ESA molecule. Mammalian serum albumins are known to adopt several pH-dependent conformations [22]. At neutral or slightly acidic pH, albumins exist in an N (neutral) form; when the pH is raised, a reversible transition to a B (basic) conformation occurs. At acidic pH, the A (acidic) conformation is prevalent. The A-B transition is thought to affect the overall shape of the albumin molecule, rather than just the protonation state of acidic and basic amino acid side chains. It is also known that the pH-induced conformational changes are accompanied by changes in ligand binding affinities [23, 24]. It has been hypothesized that these conformational changes in serum albumins are of physiological importance [25]. While serum albumins from several mammalian species have been crystallized (in many different crystal forms, with and without ligands), all known structures are of the A or N conformations. Since it is likely to be the B conformation of ESA that is responsible for binding of the pheromone, the current serum albumin structures do not allow us to make justifiable predictions about the molecular basis of the pH effect on binding of the pheromone by ESA.

ESA and Transport of the Pheromone into Urine

Interestingly, there are indications that binding of the pheromone by ESA may facilitate transport of the albumin into the urine. The total urinary protein concentration oscillates even when compared to urinary creatinine concentration [2]. Urinary creatinine concentration is commonly used as a measure to assess constant kidney function and serves as a reference point for dilution. Therefore, while the kidney function remains constant, the total urinary protein concentration oscillates in synchrony with the urinary pheromone concentration. This paradox could be explained if retention of ESA by kidneys was affected by binding of the pheromone by the albumin. That is, when heavily liganded by the pheromone, ESA would be more likely to pass into the urine, either through increased permeation of the glomerular filter or through reduced reabsorption by the proximal tubules. Taking into account the abundance of the ESA in the urine and the existence of multiple fatty acid binding sites in mammalian albumins [26], we estimate that approximately 25% of the pheromone is bound to the urinary ESA during the follicular phase of the estrous cycle. Thus, even though the ESA-mediated pheromone transport into urine is not likely to be the only mechanism present, it can account for a significant amount of the pheromone being transported.

In the murine pheromone system, the urinary pheromones [13] have been proposed to be conveyed into the urine by lipocalins, major urinary proteins (MUPs). As in the elephant, the fraction of a mouse urinary pheromone (6-hydroxy-6-methyl-3-heptanone) bound by MUPs in the mouse urine has been estimated to be around 25% [27]. Thus, the transport and bioavailability roles of ESA in the elephant, and MUPs in the mouse, may be similar. However, MUPs are absent from the urine of many mammals, including the Asian elephant, while the presence of albumin in urine is common in mammals, including rodents [28]. Therefore, the use of albumin to transport mammalian pheromones into urine may be more prevalent than transportation by lipocalins. Albumin may perhaps even complement MUPs in animals that do produce MUP-rich urine.

ESA and the Period of Pheromone Bioavailability

Nondenaturing PAGE demonstrated that the pheromone remained bound to ESA for 1 hr or more. Therefore, we tested the hypothesis that ESA extends the period of pheromonal activity of the excreted urine by complexing the pheromone and thereby reducing its evaporation, as had been shown for the mouse MUPs [14]. The hypothesis was tested with behavioral experiments in which male elephants were presented with pheromone solutions at different pH values and in the presence or absence of ESA. A solution of the pheromone in alkaline buffer exhibited only limited biological activity, attributable to low water solubility of the pheromone at high pH and its tendency to adsorb onto surfaces. Adsorbed pheromone could be recovered by addition of ESA or a small amount of acetone (data not shown) to increase the amount of the dissolved pheromone. The solubility of the pheromone at low pH was about 100 times higher than at high pH (data not shown). Solubility did not pose a problem in behavioral experiments carried out at acidic pH, and the *Z*7-12:Ac solution in acidic buffer elicited high numbers of responses. Only the solutions containing ESA (preovulatory urine, alkaline ESA solution, and to a lesser extent also the acidic ESA solution) exhibited physiological activity after a period of 12 hr. Our results are consistent with the previous finding that areas sprayed with estrous urine can elicit pheromone responses for periods of up to 24 hr [1]. We therefore postulate that the presence of ESA in the urine extends the period of biological activity of the pheromone-containing urine.

Retarding pheromone evaporation is well suited to the elephant pheromone system. Due to the limited cohabitation of both sexes in Asian elephant society, it is important that the chemical signal produced by the female persists in the environment long enough (hours) to be encountered by a male. However, prolonged periods of presence (days) of the signal would be counterproductive, as the information carried by the signal (oncoming ovulation) would become temporally inaccurate. This temporal restriction on the elephant pheromone contrasts with the mouse urinary compounds that function as priming pheromones (puberty onset acceleration, pregnancy suppression, territory marking) [5, 29], in which prolonged presence of the pheromone is beneficial to the signaling individuals. Dominant male mouse urine induced territorial avoidance by subordinate males for up to 48 hr [30], and correspondingly, the complexes of mouse pheromones with MUPs endured extensive extractions and protein purification procedures for days [6, 16]. In contrast, the elephant pheromone is bound by ESA in a complex that dissociates in 2–3 hr under

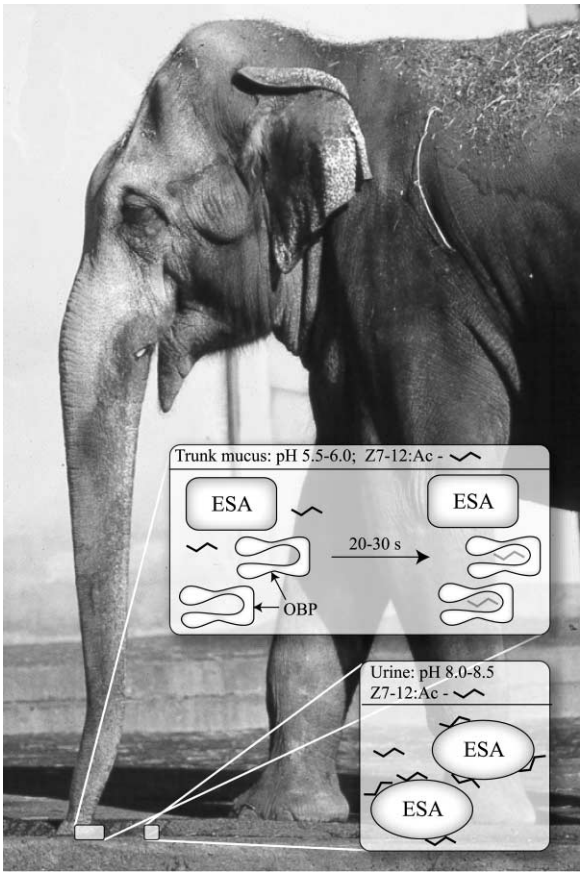

Figure 4. Role of ESA in Pheromone Detection

*Z*7-12:Ac is present, bound to ESA in the alkaline female urine. When the urine comes in contact with the acidic environment of the trunk mucus, ESA releases the pheromone, which becomes available for detection by receptor cells. Pheromone present in the trunk or chemosensory organs for extended periods of time becomes bound and effectively sequestered by an odorant binding protein (OBP).

the conditions of nondenaturing electrophoresis, even though our results demonstrate that it can persist for more than 12 hr in nature. Thus, the comparatively weaker binding of the elephant pheromone by ESA is consistent with the constraints imposed by the organization of the elephant society.

Binding of *Z*7-12:Ac by ESA and Recognition of the Pheromone

The pH of female elephant urine around the time of ovulation rises to 8.4, while the pH in the chemosensory organs is in the acidic range (pH 5.5) [2]. We have now also shown that binding of the pheromone by ESA is affected by pH. During pheromonal responses, the pheromone-containing urine is in direct contact with the acidic environment of the trunk mucus or the vomeronasal organ. Our results suggest that the acidic pH of the trunk mucus or the sensory organs causes the pheromone to dissociate from ESA, creating a pulse of free pheromone (Figure 4). Both the increased free pheromone concentration and the temporal pattern of the pheromone concentration may be important for recognizing the pheromone and finding its source (urine left behind by a female). Urinary ESA thus acts as a fixative that facilitates recognition of the pheromone and localization of its source.

The elephant urinary proteins are unlikely to mediate recognition of individual females, as the protein composition of female elephant urine does not appear to bear a "signature" of the excreting female. Thus, the male elephant recognizes the ovulating female through inducing urination by the females in his vicinity by touching the female's urogenital area with the tip of his trunk and sampling the urine for the presence of the pheromone. In contrast, in mice, recognizing the identity of the signaling individual from the signal alone can be important for the recipient, e.g., in territorial marking or pregnancy-block induction by an unfamiliar male [31]. Therefore, in mice the identity of the signaling individual is embedded into the pheromonal signal by a pattern of MUPs characteristic of the individual, which is present in the urine [18].

Significance

We report an emerging story of pheromone transport and recognition in the Asian elephant, an endangered species of which only a few thousand individuals remain. Our experiments have revealed that serum albumin, a ubiquitous vertebrate protein, plays an unexpected and multifaceted role in excretion, maintenance in the environment, and detection of (*Z*)-7-dodecenyl acetate, the female-to-male elephant sex pheromone. Based on results of biochemical and behavioral experiments, we propose a three-fold function of the pheromone binding by ESA. First, ESA serves as a transport vehicle of the pheromone from serum to urine. Second, binding by ESA extends the period for which the pheromone remains available for detection in the environment, without hampering detection. Third, dissociation of the ESA/pheromone complex induced by low pH in the vicinity of chemosensory organs enhances detection of the pheromone by producing a pulse of the volatile pheromone as the male elephant performs the characteristic "flehmen" behavior. Release of the pheromone from ESA presents the first step in a sequence of biochemical events during which the elephant pheromone is delivered to the chemosensory organs, detected by receptor proteins (unpublished data), and sequestered (by odorant binding proteins [21]). Use of albumin in urinary pheromone transport by the Asian elephant, although distinct from other mammalian pheromone systems studied thus far, may not be limited to elephants. A strong correlation of the biochemical properties of ESA with pheromone-induced behaviors fits the particular lifestyle requirements of Asian elephants. This accord suggests the possibility that distinct requirements for pheromone transport may be met by distinct proteins in different mammalian species, or perhaps even within a species. Apart from the field of study of mammalian pheromones, our results demonstrating the physiological importance of pH dependence of ligand binding by ESA, and suggesting a role of ESA in transporting the pheromone into urine, are relevant to the study of drug delivery and clearance through the kidneys.

Experimental Procedures

Radiolabeled Probes

Synthesis of the radioactive probes (Figure 1A) was performed at the National Tritium Labeling Facility, Lawrence Berkeley National Laboratory, Berkeley, CA and The University of Utah, as described [21].

Photoaffinity Labeling

In a typical reaction, 0.5 μCi of [^{3}H]*Z*7-12:Dza (0.88 nmol) dissolved in 0.5 μl of ethanol was added to 25 μl of protein solution. Anoestrous female elephant urine concentrated 1000× by ultrafiltration through a YM3 membrane (Millipore, Bedford, MA) was used as one solution, and compared with a solution of purified ESA. In competition experiments, a 1000-fold excess of *Z*7-12:Ac was added to the protein mixture prior to addition of the photoaffinity probe. Following a 5 min incubation at room temperature, the mixture was irradiated (254 nm, 1 min) using a Rayonet UV reactor (four 8 W lamps). The samples were analyzed by SDS-PAGE on a 7.5% (w/w) polyacrylamide gel, stained with Coomassie blue staining, impregnated with En^{3}Hance (NEN Life Sciences, Boston, MA), miniaturized with 50% (w/w) PEG 8000 [32], and dried. The dried gel was exposed to a sheet of a BioMax MS film (Kodak, Rochester, NY) for 4–7 days at −80°C.

Nondenaturing Electrophoresis/Electroblotting after Incubation with the Tritium-Labeled Pheromone

Electrophoresis and electroblotting of protein mixtures after incubation with the tritiated pheromone was performed as described [21], with modifications. Typically, 1 μl of a methanolic solution containing 1 μCi of [^{3}H]*Z*7-12:Ac was added to 25 μl of ESA-containing solution (either concentrated anoestrous female elephant urine or purified ESA). The mixture was incubated at room temperature for 5–10 min prior to separation by PAGE under nondenaturing conditions on a 7.5% (w/w) polyacrylamide gel using a pH 8.4 gel buffer. The proteins were electrotransferred onto a poly(vinylidene) difluoride (PVDF) membrane. All electrophoretic procedures were conducted at 4°C. Radioactivity present on the blot was detected by autoradiography (BioMax MS film, BioMax TranScreen LE intensifying screen, both by Kodak). An exposure time of 2 weeks was used in experiments carried out at pH 6.4; 1 week exposure was sufficient when the experiment was performed at pH 8.5. After processing the film, proteins present on the PVDF membrane were visualized by Coomassie blue staining.

Cloning of cDNA Encoding ESA

Total RNA was isolated from 20 mg of female Asian elephant liver tissue using the guanidinium thiocyanate method. Messenger RNA was purified using a PolyATract kit (Promega, Madison, WI), and reverse transcribed using the AMV reverse transcriptase (Promega) and $(dT)_{18}$ as a primer. A band at 2100 bp was PCR amplified using a primer based on the conserved sequences of known mammalian serum albumins (CTCTTCAGCTCTGCTTATTCC, corresponding to the peptide sequence LFSSAYS, which is highly conserved even at the DNA level) and $(dT)_{18}$, and a Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA). The PCR product was cloned into a pCR2.1 TOPO XL plasmid (Invitrogen). Six identical clones were sequenced in full, along both strands, in four overlapping sequencing reactions.

Purification of the ESA for Binding Studies

The protein solution (serum or concentrated urine, 1 ml) was transferred into 20 mM Tris-HCl buffer, pH 7.4, by gel filtration on a PD-10 column (AP Biotech, Piscataway, NJ). The desalted protein solution (2.5 ml) was loaded onto a 30 ml Affi-gel Blue Gel (Bio-Rad) column, and eluted with a 0–1.5 M gradient of NaCl in 20 mM Tris-HCl buffer pH 7.4. ESA-containing fractions were pooled, then desalted and concentrated by ultrafiltration using a YM30 membrane (Millipore). The crude ESA was further purified by ion exchange chromatography on a Poros HQ-10 column (Perseptive Biosystems, Framingham, MA). The protein was eluted with a 0–0.5 M gradient of NaCl in 20 mM Tris-HCl, pH 7.4. Fractions containing pure ESA (>95% by SDS-PAGE) were pooled, desalted, and then concentrated by ultrafiltration using a YM30 membrane (Millipore). Traces of bound ligands were removed by acidification to pH 4.0 with 0.1 M HCl, followed by treatment with activated carbon. The activated carbon was removed by filtration through glass wool, and the protein solution was neutralized with 0.1 M NaOH.

Volatile Odorant Binding Assay

The assay was performed as described [33], with some modifications [21]. Purified ESA (50 μl of 300 μg/ml) was incubated overnight at 25°C in a closed chamber containing 2 μl of *Z*7-12:Ac. Aliquots (25 μl) were injected under a layer of heptane (100 μl), and an aqueous solution of Proteinase K (Invitrogen, 10 μl of 1 mg/ml) was added. The mixture was incubated at 37°C overnight. The amount of pheromone in the heptane extract was determined by quantitative gas chromatography using a Hewlett Packard 5890 Series II gas chromatograph equipped with an HP-5 column (30 min × 0.32 mm × 0.25 μm film). The system was precalibrated with triplicate serial dilutions of *Z*7-12:Ac in heptane, in the range of concentrations 373 nM–382 μM. The molar response was linear throughout in this concentration range ($R^2 = 0.9992$). ESA concentration in volatile odorant binding assay (VOBA) samples was calculated from UV absorption at 210 and 280 nm using the modified Edelhoch method [34]. The UV spectra were collected on a computerized Hewlett-Packard model HP8453 spectrophotometer and analyzed using UV-Visible Chemstation software, version A.02.05. Elephant odorant binding protein, known to bind *Z*7-12:Ac [21], was used as a positive control, and buffers free of protein were used as a negative control. The amount of bound pheromone was determined by subtracting the amount of pheromone that dissolved in the protein-free buffer from the amount of pheromone detected in the ESA-containing sample.

Pheromone Bioavailability Assay

A behavioral assay developed to evaluate the biological activity of samples presented to elephants [35] was used in a modified form. Briefly, a 100 μM solution of *Z*7-12:Ac in 20 mM phosphate buffer of either pH 8.0 or 5.5, with or without ESA (40 μg/ml), was placed at a random site within a ground level concrete area prior to the entry of the male elephant. Control solution, lacking the pheromone component, were placed at least 20 ft away from the pheromone sample. Each pheromone solution was tested thrice with each of seven male elephants available. Behaviors were recorded using a video camera and all occurrences of standard chemosensory behaviors were noted. The assay was performed as a double-blind study. Assays were conducted with both freshly mixed samples and samples that had been exposed to the environment for 12 hr in open trays, thus mimicking the aging of the estrous female urine in the natural environment.

Use Of Animals

All animal experiments were carried out in accordance with the US and local regulations. Female elephant urine was obtained from elephants at Riddle's Elephant Sanctuary (RES), Greenbrier, AR, and at Washington Park Zoo, Portland, OR. Approval from the respective IACUC committees was current. Female elephant serum was obtained from elephants at RES and at the Ringling Center for Elephant Conservation, Polk City, FL. Liquid samples were collected by L.E.L.R. (Oregon Graduate Institute), with assistance from staff of the elephant facilities, from living, nonanesthetized animals using noninvasive techniques. Elephant tissue samples were provided by L.E.L.R. from necropsy of euthanized elephants at Washington Park Zoo and Six Flags Marine World.

Supplemental Data

Supplemental Data, consisting of two figures, are available at http://www.chembiol.com/cgi/content/full/11/8/1093/DC1.

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and Dr. H. Lin. Mass spectra were measured on a MALDI/TOF mass spectrometer Perseptive Voyager-DESTR (PE/Applied Biosystems) by Dr. V. Nanayakkara at the University of Utah Mass Spectrometry Facility directed by Dr. J.A. McCloskey. Synthesis of [^{3}H]*Z*7-12:Ac was accomplished with assistance from Dr. H. Morimoto at the National Tritium Labeling Facility in Berkeley, CA. The Ringling Center for Elephant Conservation, Riddle's Elephant Sanctuary, and Oregon Zoo generously shared their elephants and provided environment and resources for fluid collections and bioassays. Financial support was provided by the National Institutes of Health (Grant RO1-DC03320 to L.E.L.R.) and by the University of Utah (Graduate Research Fellowship to J.L.).

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