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LETTER TO THE EDITOR

An Approach to Search for Putative Pheromones in Birds via Chemical Analysis—A Reply to Mardon J, Saunders SM, and Bonadonna F

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Our work on the positive identification of male pheromones of budgerigars (*Melopsittacus undulates*) was recently published as a peer-reviewed paper in *Chemical Senses* journal, where the combination of chemical and behavioral assays "robustly demonstrates that a blend of 3 long-chain alkanols synergistically acts as a male pheromone in budgerigars" (Zhang et al. 2010). Such a claim was questioned by Mardon et al. on the basis of 4 important issues, which I now address.

Selective use of 23 glandular compounds was determined by the chemical knowledge of pheromones in animals

Growing evidence suggests that pheromones (specially referring to volatile pheromones unless otherwise noted) have a large convergence across the animal taxa such as moths, bark beetles, flies, spiders, birds, rodents, and elephants (Rasmussen et al. 1996; Rasmussen and Greenwood 2003; Wyatt 2003, 2005; Burger et al. 2004; Howard and Blomquist 2005; Zhang, Rao, et al. 2007; Zhang, Zhao, et al. 2007; Zhang, Liu, et al. 2008; Zhang, Sun, et al. 2008; Xiao et al. 2009, 2010; Whittaker et al. 2010). Therefore, the chemistry and methodologies for chemiosignals are supposed to be interlinked from arthropods to mammals. The knowledge of the chemical properties (e.g., volatility) of pheromonal components from various animals will certainly help us to choose the advisable fraction of potential pheromone components from whole chromatogram of gas chromatography (GC). The bioassay evidence for the late-eluted ester waxes to be active pheromones has not been reported yet, and thus, the wax esters were often screened out from possible pheromone components in birds and rodents (Wyatt 2003; Zhang, Rao, et al. 2007, Zhang, Zhao, et al. 2007, Zhang, Liu, et al. 2008; Zhang, Sun, et al. 2008; Zhang et al. 2009, 2010, Supplementary material). However, the early-eluted fraction logically selected by us included many components such as alkanols and pentanoates showing structural similarities with many known pheromone components in animals (Wyatt 2003; Zhang et al. 2010). In agreement, several other papers on avian putative chemosignals have also focused on the uropygial gland–secreted volatiles regardless of the wax esters (Burger et al. 2004; Bonadonna et al. 2007; Soini et al. 2007; Whittaker et al. 2010).

Quantitative comparison of the relative abundances of scent volatiles proves a potent way to screen for putative pheromones

Because animals normally have little control over the absolute amount for each compound in a mixture once a scent is deposited in the environment or painted on their body surfaces, the relative abundances or ratios of scent volatiles are suggested to be more dependable to code for specific olfactory information (Singer et al. 1997; Sun and Müller-Schwarze 1998). Although the real ratio calculated by using absolute amount of each compound may be better, it is often unmanageable work to exactly quantify each of numerous GC-eluting volatiles by comparing their GC areas in the samples with standard curve of GC areas versus concentrations of authentic analogues. Alternatively, such a relative abundance or ratio can be reflected by the percent GC peak area and provide an efficient way to screen for numbered putative pheromone components from numerous scent compounds. Quantitative comparison of the relative abundance of each scent volatile between sexes led to our discoveries of some putative pheromone components and consequent definitive pheromones validated by further bioassay in Brandt‘s voles (*Lasiopodomys brandtii*), mice (*Mus musculus*), rats (*Rattus norvegicus*), and golden hamsters (*Mesocricetus auratus*) (Zhang, Zhao, et al. 2007; Zhang, Liu, et al. 2008; Zhang, Sun, et al. 2008; Liu et al. 2010). Our recent identification of female and male pheromones of a spider species (*Pholcus beijingensis*) recurred to the approach (Xiao et al. 2009, 2010). Indeed, Singer et al. (1997) has demonstrated that the major histocompatibility complex (MHC)–determined urine-borne chemosignals are composed of a mixture of volatile carboxylic acids covarying in relative concentrations with MHC types 10 years ago. Such a way may be universal to decipher how a large numbers of sex-shared scent volatiles to compose sex pheromones in addition to a small quantity of sex-unique compounds, for example, with male pheromone components of mice, namely, *E*-b-farnesene, *E*,*E*-a-farnesene, *R*,*R*-3,4-dehydro-exo-brevicomin, and 6-hydroxy-6-methyl-3-heptanone (Singer et al. 1997; Novotny et al. 1999; Wyatt 2003; Zhang, Liu, et al. 2008; Zhang, Sun, et al. 2008; Zhang JX, unpublished data).

In the budgerigar, the gladular octadecanol, nonadecanol, and eicosanol exhibited higher relative abundance in males and were regarded as male putative pheromone components accordingly (Zhang et al. 2010). The next effort was thus focused on measuring their amounts in male budgerigars. Because several pentanoates predominated and characterized the glandular secretion of females, 3 alkanols must have a lower relative abundance regardless their absolute amounts in the budgerigar, exactly as you mentioned that the higher relative contribution of alkanols in males exclusively resulted from the presence of additional highly abundant compounds, that is, pentanoates, in the secretions of females (Zhang et al. 2010). These sexual characteristics have been statistically tested as shown in Table 1, where the huge inter-individual variation might be the basis on which individual chemical information is formed (Zhang et al. 2010). Figure 3 only presented a nonstatistically tested representative GC profile for each sex (Zhang et al. 2010). The female-biased pentanoates might be female putative pheromones and were not listed in Table 3 because of our direct interests in male pheromones (Zhang et al. 2010).

Twelve-h social separation might arouse females to court unfamiliar males in monogamous birds

Our pilot experiments revealed that the females living for 12 h in female group showed a stable preference for male odor to female odor, but the females always living in a sex-mixed group did not show a preference in Y maze (data not shown). The separation-induced female olfactory preference for males over females might synthesize female avoidance to female odor and attraction to male odor in Y maze. In mice, females whether caged singly in female group or in mixed-sex group show a consistent olfactory preference for male odor to female odor, suggesting that social habituation or familiarity does not completely eliminate the innate sexual attraction in polygamous rodents. In monogamous birds, the olfactory memory shaped by a female budgerigar on a courting male in the mixed-sex group might confuse the sex preference by the female in the face of unfamiliar male and female individuals for a while; however, the female might diminish or lose the memory after 12-h separation, resulting in an arousal of re-courtship. However, no matter what reason such an olfactory preference of females is, it could be used as an experimental model to explore the chemical communication of budgerigars with reason. Such an olfactory preference of females was substantiated by our data to be mediated by male and female uropygial gland secretion and its male-biased alkanols in the budgerigar (Zhang et al. 2010).

The pheromonal identity of the alkanol blend was confirmed by its behavioral effects comparable to glandular secretion

Our statement “to mimic the lower quantities found in females, we created a 4-fold reduced-dose alkanol blend of 8 μg” was inexact. Fortunately, the involved behavioral tests were only staged validation, which may indicate that the alkanol blend showed a dosage-dependent effect on female budgerigars (Zhang et al. 2010). Finally, we compared behavioral effects of the 3 alkanol blend at male dose with that of whole female glandular secretion at female dose to decisively confirm the identity of male pheromone components of 3 alkanols (Zhang et al. 2010). We have seen more recently that the 3 alkanols differently activated cFos expression of male and female brain regions (data not shown).

In addition, we failed to observe the olfactory sex preference of males by means of the same methods as used for females (data not shown); we may need to develop a new experimental model for further exploring male olfactory responses and female pheromones. Although previous work was mainly focused on female-biased chemosignals in birds, mate choice is more important in females with higher reproductive investment than in males, and female birds often assess male quality through audition, vision, and possible olfaction, and thus, all physical and chemical signals emitted by males should be useful for male choice by females in birds. Males of some birds (e.g., the budgerigar) without sexually dimorphic vocalization and plumage coloration might emit stronger chemical signals for compensatory attractiveness to females as some songbirds have sweeter songs and brighter plumages than female birds do.

The starting temperature of 70 °C for the gas chromatograph was optimal for the samples from budgerigars

We believed that we did not loose any low molecular weight volatiles due to the starting temperature of 70 °C of the GC program, which was determined after some pilot experiments with a starting temperature of 40 °C, at which nothing was eluted on the early chromatogram fraction. It is common sense that both GC conditions and sample preparation should be optimized for different samples to organize the running time efficiently. We even regulated the starting temperature to 100 °C for the volatiles of mouse preputial gland but 50 °C for mouse urine volatiles (Zhang, Rao, et al. 2007; Zhang, Liu, et al. 2008).

If my explanation for our study on the budgerigar is acceptable, other work on Bengalese finches (*Lonchura striata*) will be intelligible (Zhang et al. 2009).

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