BRIEF COMMUNICATION

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SUPPLEMENTARY MATERIALS

Expanded Methods:	1
Figures (Expanded Methods):	4
Figure (Whole-Brain Activations):	1
Table (Whole-Brain Activations):	1
Caption (Whole-Brain Activations):	1

Second-Hand Stress: Neurobiological Evidence for a Human Alarm Pheromone

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Alarm pheromones are airborne chemical signals, released by an individual into the environment, which transmit warning of danger to conspecifics via olfaction. Using fMRI, we provide the first neurobiological evidence for a human alarm pheromone. Individuals showed activation of the amygdala in response to sweat produced by others during emotional stress, with exercise sweat as a control; behavioral data suggest facilitated evaluation of ambiguous threat.

The existence of alarm pheromones is well-established in mammals, with animals exposed to odors secreted by acutely stressed conspecifics expressing neurobiological and behavioral changes that are indistinguishable from their reactions to predators[1]. In recent years, a significant body of research has explored the role of human reproductive pheromones, which appear to exist and exert influence on humans in many of the same contexts in which they exist for non-human mammals [2]. This strong conservation across species is biologically suggestive, and predicts that human alarm pheromones may also exist and assume functional importance.

To date, only five studies worldwide have published reports on human alarm pheromones. Two studies[3, 4] found that individuals were able to identify, solely by smelling sweat collected on axillary pads, whether the sweat donor had been watching a frightening versus benign film. Using a similar collection paradigm with frightening and benign films, another study[5] found that subjects, when smelling the stress, but not neutral, sweat showed improved accuracy in completing a word-association task. Two studies collected sweat from individuals preparing to take a difficult examination with exercise sweat as the control. In one study, females exposed to the stress odor were less likely to judge a face as positive when primed with a positive face[6], while in the other, auditory stimuli provoked an increased startle response[7] when subjects breathed sweat collected during the stress condition.

We set out to determine whether inhaling the sweat of people who were emotionally stressed produced, in a group of unrelated individuals, neurobiological effects associated with emotional arousal. The chief excitatory area associated with emotion is the amygdala[8], which has been reliably activated in human neuroimaging studies that induce emotional arousal as well as animal studies using rat alarm pheromones[9].

To obtain human sweat stimuli, we first collected axillary samples obtained from 144 individuals participating in a stress condition (first-time tandem skydive) and a control condition (running on a treadmill for the same duration of time at the same time of day). Sweat donors jumped from 4km (13,000 ft.), with one full minute of free-fall at a vertical speed of 193km/hr and four minutes under the parachute. Because the tandem-master controlled the descent, the skydiving condition produced an emotional but not physical stressor for our sweat donors, while the exercise condition produced a physical but not emotional stressor. Significant increases in both subject cortisol-levels (repeated-measures ANOVA, *pre–post Stress vs. Exercise*: F=39.87, p=0.000, N=40) and state-anxiety (paired t-test: t=10.02, p=0.000, N=40), confirmed that the paradigm was successful at inducing emotional stress. The sweat collection and storage protocols

were designed to prevent bacterial growth, which gives otherwise odorless sweat its characteristic aversive odor.

Axillary samples, once extracted and pooled for each condition, were then used as olfactory stimuli for five experiments. Two fMRI experiments assessed amygdala activation as well as possible gender interactions that could indicate confounds due to reproductive pheromones. Since the amygdala is also known to play a role in general olfactory processing, for the next two experiments we used a double-blind forced-choice discrimination task, as well as Likert scales, to determine whether there were odor differences (either intensity, valence, or qualitative) between the test and control samples that could confound the neurobiological results. Finally, we tested the behavioral implications of the amygdala activation, to investigate how stress sweat affects threat-perception using psychometric curves generated by subjects' responses to morphed neutral-to-threatening faces.

Subjects for all experiments were screened for anosmia prior to testing. For the fMRI and behavioral experiments, odor presentation was controlled with synchronized nasal inhalation; for the odor discrimination experiments, individuals were asked to sniff the sample. To control for potential reproductive pheromone confounds, we included only heterosexual subjects. Sweat collection and storage protocols, GC-MS validation of the sweat extraction methods, evaluation of trial-specific respiratory parameters demonstrating compliance with synchronized breathing protocols, as well as other experimental parameters, are described in greater detail in the Online Supplementary Materials.

fMRI Experiments: In the first experiment, we presented sweat from 40 male donors to 16 subjects (50% female) while their brains were scanned using fMRI. In a second (replication) experiment, using different subjects and scanners, we presented sweat from an additional 40 donors (50% female) to a different group of 16 subjects (50% female) undergoing fMRI, increasing power by doubling the number of stimulus presentations. Because we hypothesized that putative alarm pheromones would modulate activity in brain structures related to fear, our analyses focused on the amygdala; all values were corrected for multiple-comparisons using small-volume correction (SVC). For both experiments, these revealed significant activation of the left amygdala (*1st Exp:* t=4.80/Z=3.68, $p_{(svc)}$=0.02 [MNI *x, y, z*=−16, −8, −16], N=16; *2nd Exp:* t=6.19/Z=4.30, $p_{(svc)}$=0.000, [MNI *x, y, z*=−21, −3, −15], N=16; *Figure 1*) in response to the stress sweat as compared to the exercise sweat. For both experiments, activity was concentrated most strongly in the superficial, or corticoid, amygdala (*1st Exp:* t=4.80/Z=3.68, $p_{(svc)}$=0.008, N=16; *2nd Exp:* t=6.19/Z=4.30, $p_{(svc)}$=0.000, N=16)—a region known to have substantial olfactory inputs in primates; homologous structures in other mammals have been implicated in pheromonal processing[10]. Because activation patterns were equivalent for same-sex and opposite-sex donor-detector pairs (repeated-measures ANOVA: *1st Exp*: F=1.76, p=0.21, N=16; *2nd Exp*: *Donor Sex*: F=0.21, p=0.65; *Detector Sex:* F=1.31, p=0.27; *Donor Sex*Detector Sex*: F=0.004, p=0.952, N=16), our findings suggest that reproductive pheromones were unrelated to the effect[11].

Odor Perception Experiments: While odor intensity and valence can cause differential amygdala responses[12, 13], subjects rated both odors, using Likert scales ranging

from one ("undetectable"/"pleasant") to ten ("very strong"/"unpleasant") as mild (*Stress*: μ=2.6, s.d.=2.3, *Exercise*: μ=2.6, s.d.=2.3; Wilcoxon sign-ranks test: Z=1.11, p=0.28, N=26) and neutral (*Stress*: μ=4.5, s.d.=1.1, *Exercise*: μ =4.8, s.d.=0.8; Wilcoxon sign-ranks test: Z=1.56, p=0.12, N=26). To investigate whether the stress and exercise sweat odors were qualitatively distinct, we conducted a double-blind forced-choice odor discrimination experiment, in which 16 subjects (50% female) identified whether 16 odor-pairs (50% different), randomly presented, were identical or different; subject ratings were not significantly different than chance (one-sample t-test: t=0.64, p=0.53, N=16). The data suggest that the test and control odors were indistinguishable, and therefore rule out non-specific olfactory processing as a likely explanation for amygdala activation in response to the Stress vs. Exercise contrast.

Threat-Perception Experiment: Since data from our previous experiments suggested that the observed amygdala activation reflected emotional rather than olfactory processing, we tested whether breathing stress sweat vs. exercise sweat from 64 donors (50% female) behaviorally affected perception of ambiguous threat. Psychometric curves were generated from a forced-choice design in which 14 subjects (36% female) indicated via response-button whether briefly-presented (200ms) male faces, morphed between neutral and angry expressions, were "more neutral" or "more threatening." For each subject, stress and exercise conditions produced psychometric curves, each composed of nine points ranging from neutral (10%) to angry (90%), with each point the average of 14 face presentations. Threat-levels were presented randomly, with experimental conditions counter-balanced for order. Values for slope, σ, were calculated for each curve using sigmoidal fitting. These showed sharpened discrimination (mean 43% increase) between neutral versus angry faces in response to the stress sweat (*Stress:* σ=0.192, s.d.=0.101; *Exercise:* σ=0.134, s.d.=0.066; repeated-measures ANOVA: F=8.30, p=0.01, N=14, *Figure 2*). No differences between conditions were observed for inflection-points (F=1.35, p=0.27, N=14), suggesting that the effect was specific to increasing accuracy in evaluation of ambiguous threat, rather than attribution of threat to neutral stimuli.

Our findings indicate that there may be a hidden biological component to human social dynamics, in which emotional stress is communicated via chemosensory cues. Sweat collected during an acute emotional stressor, and subsequently presented to an unrelated group of individuals, produced significant brain activation in regions responsible for emotional processing without conscious perception of distinct odor; behavioral data, our own as well as those from previous studies, suggest the emotional processing may be specific to enhancing vigilance and sharpening threat-discrimination.

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Figure 1: Breathing stress-derived sweat modulates amygdala, the primary brain region associated with emotional arousal. The activation map *(a)* reflects the STRESS−EXERCISE contrast, and was produced using height threshold $t = 3.7$, $p<0.001$ (uncorrected) and extent threshold $k = 5$ voxels. Whole-brain random-effects analyses revealed that the strongest activation in response to this contrast was the left amygdala (see Supplementary Online Materials Figure 3 and Table 1 for whole-brain activations); there were no significant de-activations. The MNI coordinates of the maximally activated voxel, located in the left amygdala, are [-21, -3, -15]. Corresponding time-courses for this voxel *(b)* are shown for STRESS−REST and EXERCISE−REST contrasts.

Figure 2: Psychometric curves generated by a forced-choice assessment of ambiguous threat show sharpened discrimination between threat and non-threat while breathing stress-derived sweat. For each subject, data for each condition (STRESS, EXERCISE) were fitted with the sigmoid function, where p_0 and $p_0+\Delta p$ define upper and lower asymptotes, A_0 is the inflection point, and σ defines slope. Significant differences between conditions were seen for slope, with individuals under the STRESS condition more closely approximating ideal discrimination, shown by the dotted line.

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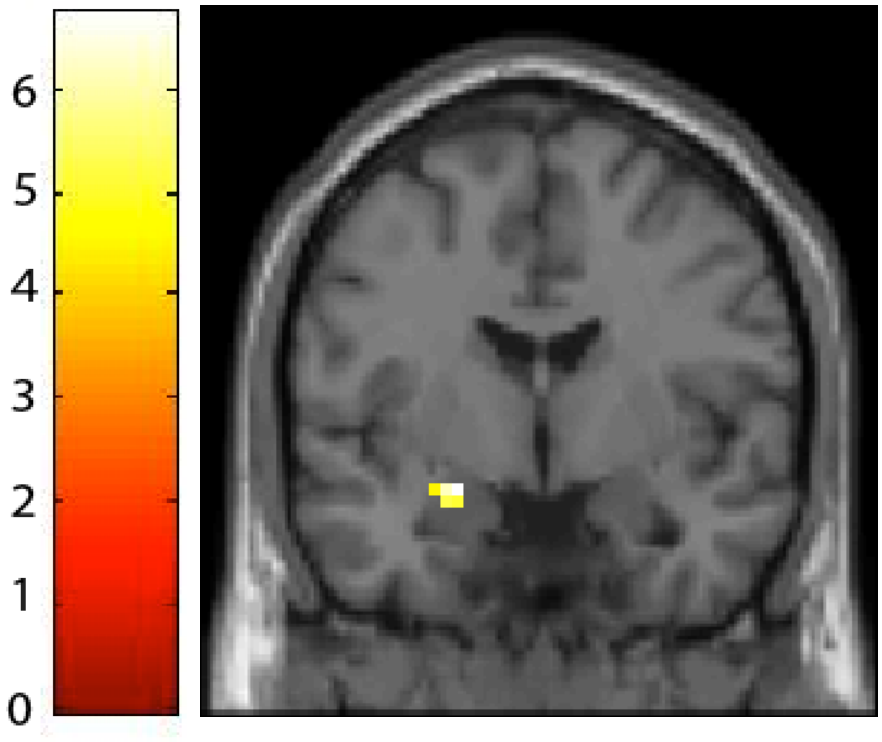
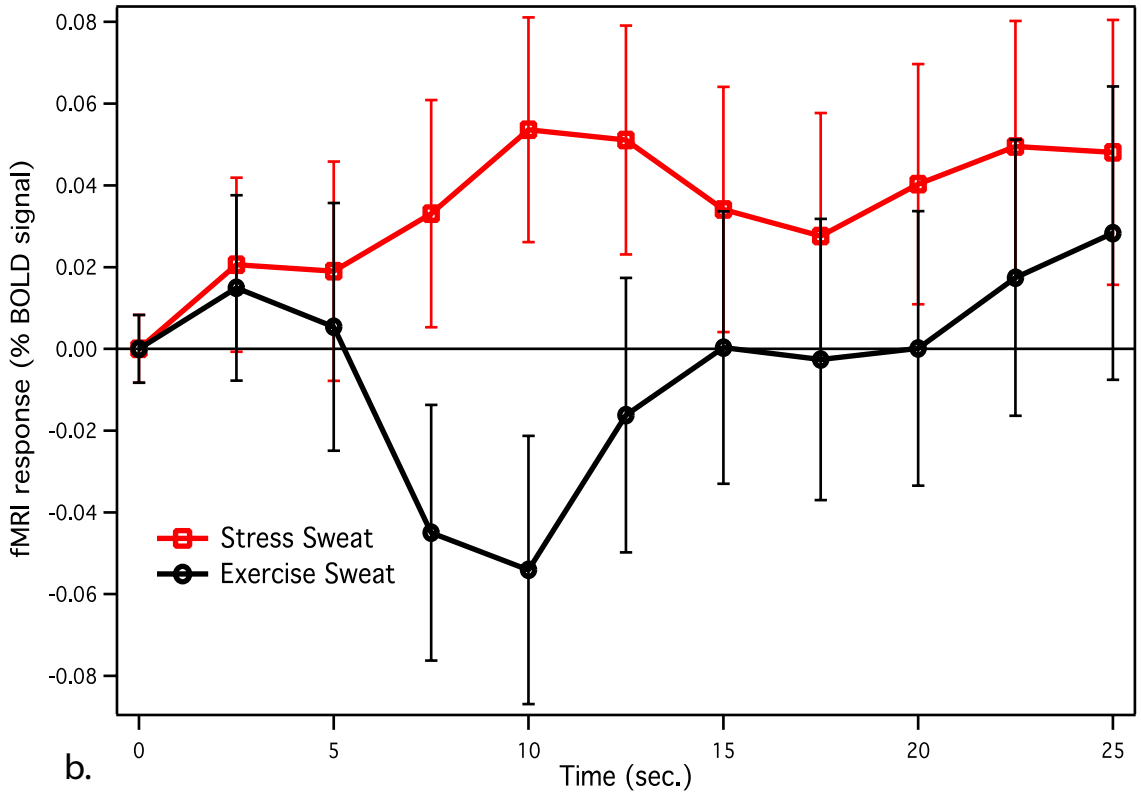


a.



b.

Figure 1 (Mujica-Parodi)

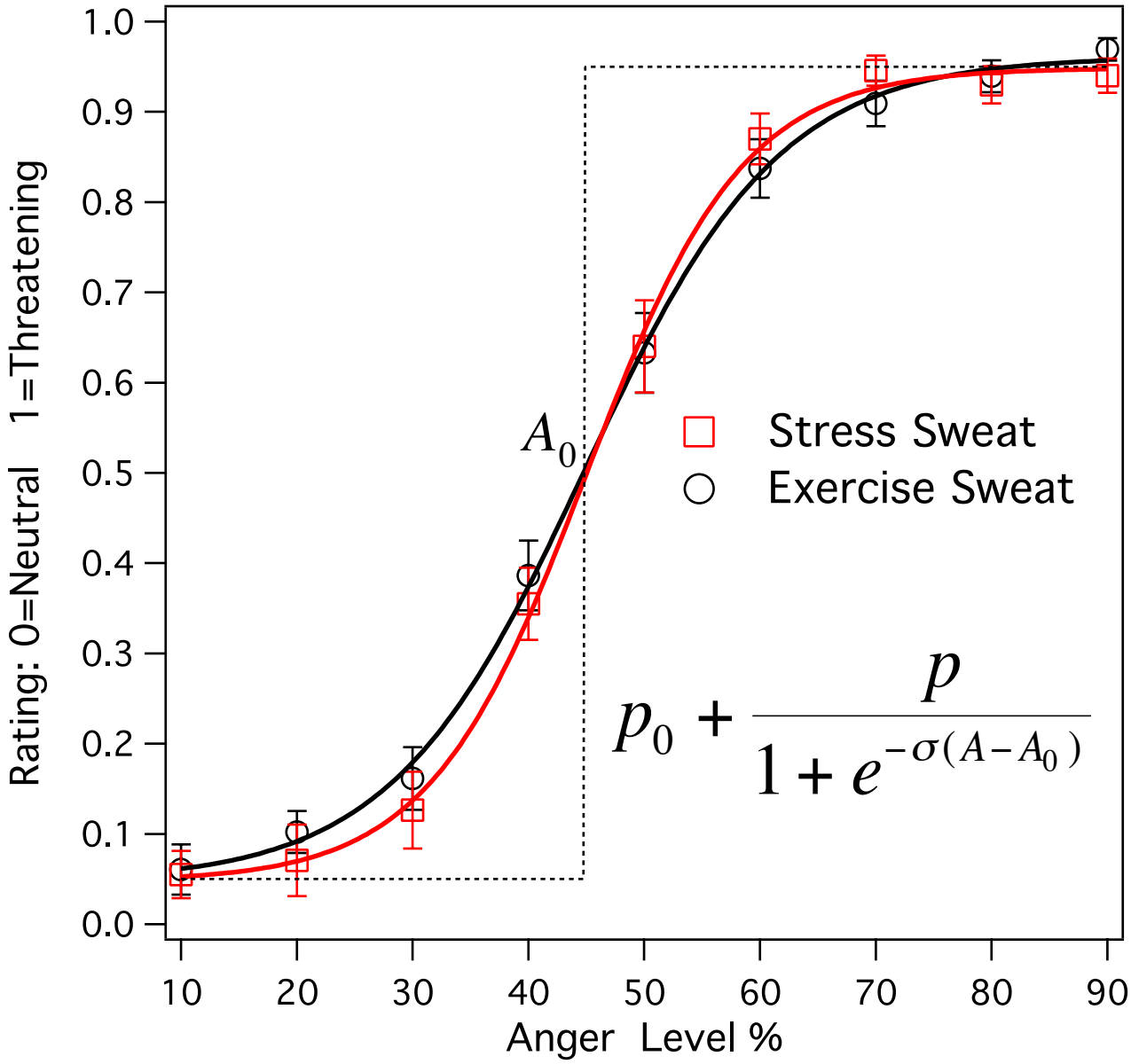


Figure 2 (Mujica-Parodi)