In order to obtain a strong resetting effect, KaiC must have sufficiently high sensitivity to ADP, at the upper end of the range of effective affinities we estimated experimentally (fig. S5). We used this model to predict a full phase-response curve based on this competitive inhibition mechanism and compared it to our experimental data and to measurements of the phase shift induced by darkness in vivo (Fig. 4C) (*19*). The agreement between these data and the model indicates that varying the relative nucleotide concentrations in the reconstituted oscillator approximates the response of the circadian clock in living cyanobacteria to changes in illumination, and an increase in the amount of ADP appears to alter the phase of the oscillator through inhibition of KaiC's kinase activity.

In the model, the ATP/ADP ratio describes a family of limit cycles that differ in the amplitude of the phosphorylation rhythm. Indeed, KaiABC reactions in buffers with tonically lowered ATP/ADP ratios continue to oscillate with a circadian period, but KaiC cycles through distinct patterns of phosphorylated states (fig. S6). If the ATP/ADP ratio is lowered abruptly, phosphorylation is inhibited, and the system must adjust to a new limit cycle. If this transition produces amounts of Ser^{431}-phosphorylated KaiC sufficient to block KaiA activity, a large phase shift can result.

We have described a simple entrainment mechanism for a circadian clock in which enzymatic activity is directly tied to the availability of biochemical energy in the cell. In this view, no signaling pathway that specifically targets the oscillator is required to couple the clock to the environment, and the core oscillator proteins interact directly with metabolites, as has been reported for KaiA in vitro (*27*). Although factors outside the Kai proteins have been implicated in light-driven input to the cyanobacterial circadian system, including LpdA and the histidine kinase CikA (*18*, *20*), strains lacking these proteins can be effectively entrained with repeated light-dark cycles, supporting the hypothesis that there exist basic synchronization mechanisms intrinsic to the KaiABC core oscillator itself. Because unexpected darkness will unavoidably lead to changes in the production and consumption of ATP in an obligate phototroph, KaiC's sensitivity to ADP represents a robust intrinsic mechanism for maintaining synchrony with the environment.

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Suppression of Avian Influenza Transmission in Genetically Modified Chickens

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Infection of chickens with avian influenza virus poses a global threat to both poultry production and human health that is not adequately controlled by vaccination or by biosecurity measures. A novel alternative strategy is to develop chickens that are genetically resistant to infection. We generated transgenic chickens expressing a short-hairpin RNA designed to function as a decoy that inhibits and blocks influenza virus polymerase and hence interferes with virus propagation. Susceptibility to primary challenge with highly pathogenic avian influenza virus and onward transmission dynamics were determined. Although the transgenic birds succumbed to the initial experimental challenge, onward transmission to both transgenic and nontransgenic birds was prevented.

The diversity of avian influenza viruses (AIVs) and their propensity for interspecies transmission make them a global threat to animal and public health communities. Cross-species transmission of influenza viruses may occur directly or be facilitated by intermediate host species that amplify and diversify virus populations, notably domestic chickens, ducks, and pigs (*1*). Although control of AIV infection in its wild aquatic bird reservoir is impractical, control of AIV in domesticated hosts is possible (*2*). The diversity of viral antigenic subtypes and their potential for evolutionary shift and drift are a challenge, particularly because current vaccines do not generally achieve sterile immunity even against antigenically well-matched viruses (*3*). One potential route to control AIVs in commercial poultry is to use genetic modification to introduce novel genes that confer resistance to infection (*4*, *5*). Here we evaluate transgenic expression of an RNA hairpin molecule capable of inhibiting influenza viral polymerase activity (*6*).

An RNA expression cassette (Fig. 1A) was designed to use a chicken U6 promoter (*7*) to express the short hairpin RNA molecule, decoy 5 (D5, Fig. 1B) (*8*). This decoy contains the conserved 3'- and 5'-terminal sequences of influenza virus genome segments that encompass the complementary RNA (cRNA) binding site for

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influenza A virus polymerase (9, 10) and has the potential to interfere with virus replication and packaging. A structurally similar decoy molecule (D7, Fig. 1B), containing specific mutations that prevent binding by the viral polymerase, provided the negative control. We confirmed the efficacy and specificity of the D5 expression cassette by expressing it in DF-1 chick embryo fibroblast (CEF) cells with a lentiviral vector plasmid (fig. S1A), cotransfected with an AIV-based minireplicon system (8, 11) (Fig. 1C), in which activity of influenza polymerase is proportional to the amount of the reporter protein produced (12). The D5 lentivector was then used to generate transgenic chickens (8) carrying the D5 cassette inserted at a single location on chromosome 2 (fig. S1, B and C). One transgenic cockerel was crossed with stock hens, and the resulting ~1:1 transgenic (TG-D5) and nontransgenic (non-TG) progeny were used in the challenge studies described below. Expression of the green fluorescent protein (GFP) reporter gene, present in the integrated vector, was detected in all transgenic birds. Decoy RNA expression was below the limit of detection of Northern blot analysis, consistent with our experience in transiently transfected cells, in which we have found this RNA to be unstable in the absence of the viral polymerase. The minireplicon AIV polymerase assay, performed with CEFs isolated from TG-D5 and non-TG embryos, showed that expression of the reporter in the TG-D5 CEFs was on average 0.24 times that from the non-TG embryos ($P = 0.004$; 95% confidence interval 0.10 to 0.56), indicating that the D5 cassette in the transgenic CEFs was effective in this assay (Fig. 1C).

The susceptibility of the TG-D5 birds to highly pathogenic avian influenza (HPAI) infection was evaluated in two in vivo studies. In study 1, the susceptibility to direct infection and onward transmission in 3-week-old transgenic or nontransgenic birds was compared (8). Ten TG-D5 and 10 non-TG chickens were directly infected with a high dose [10^5 EID_{50} (50% egg infectious dose)] of H5N1 HPAI (A/turkey/Turkey/1/2005). One day post-infection (dpi), these challenged birds were housed with 10 uninfected birds (the "in-contact" group) of the same transgenic status, and the health of the birds was monitored over the next 11 days (fig. S2). All the directly infected birds died between 2 and 4 dpi, with negligible difference between the time to death for TG-D5 and the non-TG birds [$P = 0.50$; Bonferroni adjusted Mann-Whitney test (8)]. However, clear and statistically significant differences in mortality and time to death were apparent for the in-contact groups ($P = 0.017$), where 7 out of 10 non-TG in-contact birds died by the fifth day of the study, with a mean time to death (excluding survivors) of 3.3 (SD = 0.5) days after exposure. Only 2 out of 10 of the TG-D5 in-contact group died (on day 5 and 5.5 after exposure), whereas the others remained healthy for the duration of the study. These results suggest that susceptibility to a high dose of H5N1 HPAI virus challenge

was similar for the TG-D5 and non-TG birds, but that there were clear differences in transmission and/or susceptibility after contact exposure.

Study 2 was designed to investigate whether the results obtained in study 1 reflected reduced levels of virus shedding from the transgenic challenge group and/or reduced susceptibility to infection via contact exposure to these infected birds. Ten TG-D5 and 10 non-TG sibling birds were directly infected as before, but with a dose of virus one-tenth that used in the earlier study (10^4 EID_{50}). Each of these groups was then split into two groups of five, and each group of five was housed with 12 TG-D5 or 12 non-TG birds (i.e., four groups of 17 birds housed together from dpi = 0; Fig. 2 and fig. S3), plus two unexposed control birds. Direct infection of the non-TG birds was again 100% lethal, with peak mortality at 2 dpi and the remaining birds succumbing to infection between days 4 and 6, consistent with this being caused by subsequent rounds of infection (Fig. 2, groups 1 and 2, "non-TG challenged"). In 6 out of 10 birds, mortality resulted directly from infection, and 4 out of 10 birds were euthanized on welfare grounds because of their severe clinical presentation. The infected TG-D5 birds also showed peak clinical signs at 2 dpi, when 1 out of 10 died directly from the infection and five were euthanized on welfare grounds (Fig. 2, groups 3 and 4, "TG-D5 challenged"). Four out of 10 of the birds survived to at least day 7. One of these (#4458) was euthanized on clinical grounds, with one healthy bird (#4446) for comparison. The sick bird (#4458) did not have viral antigen in any of its tissues and was not shedding detectable levels of viral RNA. The two remaining birds were healthy for the 10-day duration of the study. The pattern of mortality in the directly challenged birds is consistent with the TG-D5 birds having reduced transmission capacity that affected the successive waves of mortality apparent in the non-TG challenged birds. Although these data taken alone were not statistically significant, this hypothesis is supported by the analysis of the in-contact birds described later.

Direct infection of non-TG birds resulted in viral shedding from the cloaca and oropharynx and efficient transmission to the in-contact birds with 100% mortality, irrespective of whether these were TG-D5 or non-TG (Fig. 2 and fig. S3, groups 1 and 2, "in-contact"). However, the transmission dynamics to the TG-D5 birds were noticeably prolonged (Fig. 2, group 2, "in-contact"). This is an interesting observation but the effect was of marginal statistical significance ($P = 0.06$ before and $P = 0.18$ after adjustment; Mann-Whitney), if we accept that the power of the study to detect this difference is likely to be compromised at this level of aggregation. In marked contrast, contact with the directly infected TG-D5 birds did not result in clinical disease in either the TG-D5 or non-TG in-contact birds, because no in-contact birds died from influenza (Fig. 2, group 3 and 4 "in contact") and none of the birds sero-converted (as determined by enzyme-linked

immunosorbent assay and hemagglutination inhibition assay performed with day 10 sera). Of the 24 in-contact birds, 14 remained healthy until the end of the study, 9 healthy birds were killed for postmortem examination (8) during the course of the study, and one bird (#5033) became sick
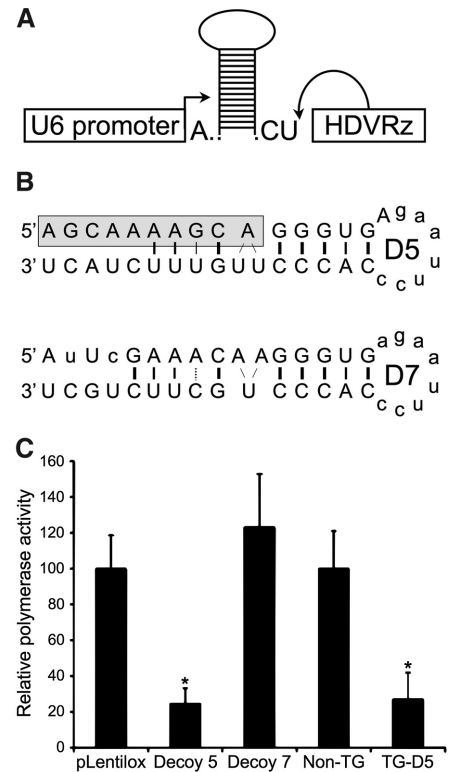

Fig. 1. Inhibition of influenza virus polymerase by decoy RNA hairpins. (**A**) The decoy hairpin RNA expression cassette comprised a chicken U6 RNA promoter, the decoy sequence, and hepatitis delta virus antigenomic ribozyme (12). (**B**) Structure of the RNA decoys: Decoy 5 (D5) comprises the conserved 5′- and 3′-terminal nucleotides from segment 8 cRNA with the polymerase primary binding site in the shaded box. Decoy 7 (D7) corresponds to the viral RNA sequence with mutations at position 2 and 4 of the 5′ end known to ablate polymerase binding. Lowercase letters indicate non–influenza-related sequences. (**C**) Polymerase activity in DF-1 and CEF cells determined by minireplicon-based luciferase expression assay. DF1 cells were transfected with plasmids expressing the 3P/NP genes (derived from A/turkey/England/50-92 H5N1) and chicken polymerase I–driven luciferase minireplicon. These were cotransfected with pLentilox, pL-D5, or pL-D7 expressing no decoy, decoy 5, or decoy 7, respectively, as indicated. Data are means ± SD from three replicate transfections. Similarly, the activity of the same minireplicon system was determined by transfection into CEFs prepared from decoy 5 transgenic (TG-D5, $n = 8$) or nontransgenic (non-TG, $n = 2$) embryos. Luciferase levels are plotted relative to the empty vector (pLentilox) and non-TG controls as appropriate. Polymerase activity in decoy 5 versus vector control transfected cells and non-TG versus TG-D5 CEFs were significantly different ($P = 0.02$ and $P = 0.004$, respectively).

from an apparently unrelated cause. Comparing the time to death for the in-contact birds between the non-TG and TG-D5 challenge birds (i.e., groups 1 and 2 versus 3 and 4) showed that these differences were highly statistically significant ($P = 0.0004$; Mann-Whitney test), indicating that the transgenic chickens have altered infection transmission dynamics for H5N1 HPA1 virus.

Figure 2 also shows the quantity of viral RNA [$\log_{10}$ RNA copies (8)] detected in buccal swabs sampled daily (parallel data for cloacal swabs are shown in fig. S3). The shedding patterns were typical of those observed for HPAI virus infection and transmission in poultry, with shedding from the oropharynx often preceding that from the cloaca (13). High levels of viral RNA were detected in 10 out of 10 buccal samples and 9 out of 10 cloacal samples from the directly infected non-TG challenge groups (Fig. 2 and fig. S3, groups 1 and 2). Shedding occurred in phases, with 60% of the birds shedding by 2 dpi, in parallel with the waves of mortality. The in-contact groups housed with the non-TG challenge birds showed an initial low level of viral RNA coinci-

dent with the peak shedding from the challenged birds (we interpret this as evidence of initial exposure at this time), followed by successive waves of shedding similar to that seen for the non-TG challenge birds but occurring ~2 days later. Similar levels of virus RNA were detectable in the buccal and cloacal swab samples from both the non-TG and TG-D5 in-contact groups (fig. S3).

In the directly infected TG-D5 birds (Fig. 2 and fig. S3, groups 3 and 4), viral RNA was detected by 2 dpi in 5 out of 10 buccal samples but in only 2 out of 10 cloacal samples (or maximally 4 out of 10, including the two for which H5 real-time reverse transcriptase–polymerase chain reaction (RRT-PCR) data are unavailable but that were positive in the immunohistochemical analysis). Half of the birds succumbed to the initial direct infection, but the virus shed by these birds was insufficient or not able to infect the remaining in-contact birds. Again, low levels of viral RNA were detected in the buccal samples from many birds in the in-contact groups at the time of peak shedding from the challenged birds. This low level of infection or exposure failed to establish a

productive, systemic infection, as shown by the lack or very low level of viral RNA shed from the cloacae of these birds and by the absence of mortality.

Histopathology and immunohistochemistry of all major tissues and organs (table S1) revealed viral antigen and characteristic histopathological changes associated with AIV infection in 7 out of 13 directly infected (TG-D5 and non-TG) birds, consistent with their virus shedding status. The histopathological changes included multifocal necrosis in the spleen, bursa, thymus, brain, and pancreas, and interstitial pneumonia and perivascular and alveolar edema in the lung. There were no marked differences in the severity, nature, and distribution of histopathological changes or expression of viral antigen between the TG-D5 ($n = 5$) and non-TG ($n = 2$) directly infected birds examined. None of the birds (non-TG or TG-D5) that were in contact with the directly infected TG-D5 birds were positive for viral antigen or showed any typical histopathological changes (16 out of 16 birds tested on dpi 2, 8, or 10). Lymphoid hyperplasia was observed in the spleen, thymus, and bursa for most of these birds at later time

Fig. 2 data — Groups 1 and 2

Group		Bird #	0	1	2	3	4	5	6	7	8	9	10	IHC
Group 1	Non-TG challenged	5019	-	3.2	6.3									nt
		5020	-	3.8	5.2									nt
		5021	-	4.9	5.5									nt
		5018	-	-	4.0	*								nt
		5022	-	-	-	-	2.0	7.2						nt
	Non-TG in Contact	5023	-	-	2.3	2.9	6.0							nt
		5024	-	-	1.5	4.0	5.8							nt
		5026	-	-	2.3	0.9	5.8							nt
		5027	-	-	2.0	-	6.0							nt
		5028	-	-	2.9	-	6.3							nt
		5030	-	-	2.0	3.5	5.5							nt
		5035	-	-	2.9	*	5.5							nt
		5025	-	-	2.3	*	2.9	6.0						nt
		5029	-	-	1.2	-	3.8	6.9						nt
		5032	-	-	2.9	-	4.9	6.6						nt
		5036	-	-	-	-	4.6	6.6						nt
		5031	-	-	2.9	-	2.3	1.5	1.2	5.8				nt
Group 2	Non-TG challenged	5013	-	-	3.5									-
		5014	-	-	5.2									+
		5016	-	-	5.8									+
		5017	-	-	-	3.5	5.5							nt
		5012	-	-	-	-	2.9	-	*					nt
	TG-D5 in Contact	4463	-	-	3.2	-	6.9							nt
		4464	-	-	3.2	-	4.9							nt
		4471	-	-	-	2.9	5.8							nt
		4465	-	-	1.8	-	4.6	-	-					nt
		4467	-	-	-	-	2.3	-	*					nt
		4469	-	-	1.5	-	1.2	4.0	*					nt
		4470	-	-	2.3	-	2.3	2.0	*					nt
		4474	-	-	-	-	-	-	*					nt
		4462	-	-	2.9	-	5.5	1.5	5.2	6.6				nt
		4466	-	-	1.5	-	2.0	1.5	4.6	5.8				nt
		4468	-	-	-	-	-	-	-	3.5				nt
		4475	-	-	1.2	-	2.3	-	5.8	-				nt

Fig. 2 data — Groups 3 and 4

Group		Bird #	0	1	2	3	4	5	6	7	8	9	10	IHC
Group 3	TG-D5 challenged	4457	-	4.0	6.6									+
		4459	-	4.0	*									+
		4461	-	4.9	*									+
		4458	-	-	-	-	-	-	-	-				-
		4460	-	-	*	-	-	-	-	-	*	-		-
	Non-TG in Contact	5001	-	-	*									-
		5002	-	-	*									-
		5005	-	-	*	-	-	-	-	-				-
		5010	-	-	-	-	-	2.0	-	-				-
		5033	-	-	-	-	-	-	-					-
		5003	-	-	*	-	-	-	-	-	-	-	-	-
		5004	-	-	1.2	*	-	-	-	-	-	-	-	nt
		5006	-	-	1.8	-	-	-	-	-	-	-	-	-
		5007	-	-	*	-	-	-	2.9	-	-	-	-	nt
		5009	-	-	*	-	-	-	-	-	-	-	-	-
		5011	-	-	1.8	-	-	-	-	-	-	-	-	nt
		5034	-	-	*	-	-	*	-	-	-	-	-	nt
Group 4	TG-D5 challenged	4442	-	-	-									-
		4443	-	4.9	4.3									+
		4444	-	2.3	*									+
		4446	-	-	-	-	-	-	*	1.5				-
		4445	-	-	-	-	*	*	2.0	-	-	-		-
	TG-D5 in Contact	4454	-	-	-									-
		4456	-	-	-	-								-
		4448	-	-	0.9	-	-	-	-	2.0				-
		4450	-	-	-	-	-	-	2.0	-				-
		4472	-	-	-	-	-	4.0	-	-				-
		4447	-	-	-	-	-	-	-	1.2	-	-	-	nt
		4449	-	-	-	-	-	-	-	2.6	-	-	-	-
		4451	-	-	-	-	*	-	-	-	-	-	-	nt
		4452	-	-	1.8	-	-	-	-	-	-	-	-	nt
		4453	-	-	-	-	-	-	-	-	-	-	-	nt
		4455	-	-	3.2	-	*	*	-	2.3	-	-	-	-
		4473	-	-	1.2	-	-	-	-	1.5	-	-	-	-

Fig. 2. Mortality and virus shedding data for challenge and in-contact groups in study 2. Groups of five TG-D5 or non-TG "challenged" birds infected with influenza virus A/turkey/Turkey/1/05 (H5N1) on day 0 were housed with groups of 12 TG-D5 or non-TG "in-contact birds." groups 1 and 2: Non-TG challenged birds; groups 3 and 4: TG-D5 challenged birds; Groups 1 and 3: Non-TG in-contact birds; and groups 2 and 4: TG-D5 in-contact birds. Survival is indicated by the length of the green bar. Terminal block color indicates day and cause of death (black, found dead; magenta, moribund; orange, healthy birds killed for immunohistological studies). Numbers for each day are the $\log_{10}$ of the number of viral RNA copies present in buccal swab samples (data for cloacal swabs are shown in fig. S3). Sample negative by RT-PCR (–), sample unavailable (*). The IHC column summarizes the immunohistochemistry data shown in table S1. Virus antigen detected (+); no virus antigen detected (–); not tested (nt).

points, but was not seen at earlier time points or in the two unexposed control birds. This may be indicative of antigen or viral exposure or an abortive infection.

These data show that the TG-D5 chickens did not efficiently transmit infection to birds housed with them, but the specific mechanism underlying this effect is not known. Polymerase decoys may disrupt replication by direct binding to polymerase or indirectly by influencing the level of expression of the recently discovered, putative regulatory small viral RNA molecules (14, 15) (which may also have a role in innate immunity). Although decoy 5 suppressed polymerase activity in cell culture, this did not translate into a quantitative reduction in virus shedding from infected birds (Fig. 2) (nor have we found any effect in ovo or in fibroblast cell culture). Polymerase-RNA interactions may be involved in the virus packaging process, but after passage through TG-D5 chick embryo fibroblasts in cell culture, we have not found any effect on the genome:plaque-forming unit ratio of the virus to support the hypothesis that the decoy induced the formation of defective virus particles. The standard intravenous pathogenicity index of the virus shed from one of the TG-D5 chickens (#4457, dpi = 2) was determined after a single passage in embryonated hens' eggs and found to be unaltered, indicating that passage through TG-D5 chickens does not rapidly select for a stable genetic change that reduces the virulence of the shed virus.

Our goal was a proof-of-principle demonstration that genetic modification can be used to prevent avian influenza infection in chickens. The TG-D5 birds exhibited a marked absence of onward transmission of infection, even to unprotected (nontransgenic) chickens housed in direct contact with them. This property could have a major impact on susceptibility and propagation of infection at the flock level and supports the concept of genetic modification for controlling AIV infection in poultry. Our strategy offers substantial potential benefits over vaccination. Although conventional AIV vaccines can achieve strain-specific clinical resistance to primary challenge, sterile immunity is not achieved (3). Such vaccination can allow the cryptic circulation of virus in flocks, facilitating antigenic drift and posing a risk to unvaccinated birds and humans that come into contact with them. In contrast, onward transmission and circulation at the flock level are absent in the TG-D5 chickens. The decoy 5 RNA corresponds to an absolutely conserved sequence that is essential for the regulation of viral transcription, replication, and packaging of all subtypes of influenza A virus, offering pan-subtype A protection, whereas vaccination offers no protection against unmatched viral strains. Unlike proposed microRNA-based strategies (4, 5), the development of resistant virus is intrinsically unlikely, requiring mutations in the polymerase and the promoter of all eight genome segments simultaneously, a statistically highly improbable event.

The control of avian influenza by genetic modification brings obvious health benefits to consumers and producers, as well as welfare and productivity benefits to the birds. Nevertheless, it is important to assess any genetic modification for potential hazards. Here, the transgene encodes an innocuous decoy RNA, expressed at steady-state levels that are barely detectable by conventional methods and unlikely to present a risk to consumers, birds, or the wider environment. There are no apparent ill-effects on uninfected transgenic birds, which are phenotypically normal and show no significant deviation from the expected Mendelian frequency or differences in hatch weights (fig. S4 and table S2). The transgene is not expected to alter susceptibility to other pathogens, although this has yet to be confirmed. Transgenes can be introduced into multiple founder lines as discrete traits without affecting other genetic properties of the lines. This will facilitate the permanent introduction of novel disease-resistance traits into the mass population of production birds via conventional breeding techniques, with little impact on genetic diversity or valuable production traits. Our approach is technically applicable to other domestic species that are hosts of influenza A, such as pigs, ducks, quail, and turkeys. Further development of transgenic disease resistance in poultry and other farm animals will undoubtedly stimulate debate about the application of this technology in food production.

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Human Tears Contain a Chemosignal

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Emotional tearing is a poorly understood behavior that is considered uniquely human. In mice, tears serve as a chemosignal. We therefore hypothesized that human tears may similarly serve a chemosignaling function. We found that merely sniffing negative-emotion–related odorless tears obtained from women donors induced reductions in sexual appeal attributed by men to pictures of women's faces. Moreover, after sniffing such tears, men experienced reduced self-rated sexual arousal, reduced physiological measures of arousal, and reduced levels of testosterone. Finally, functional magnetic resonance imaging revealed that sniffing women's tears selectively reduced activity in brain substrates of sexual arousal in men.

Charles Darwin suggested that expressive behaviors initially served emotion-relevant functions, before evolving to serve as emotion-signals alone (1, 2). Thus, the behavior of emotional tearing, considered uniquely human

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(3), is a paradox: Whereas tears clearly serve as an emotional signal (4), tears were not related to any emotionally relevant function. Despite psychological theories on the meaning of tears (5, 6) and biological theories describing tears as an adaptation related to their eye-protective nature (3) or a mechanism for expelling toxic substances (7), the functional significance of emotional tears remains unknown (8).

Tears are drops of liquid produced by the lacrimal, accessory lacrimal, and Meibomian glands, which contain proteins, enzymes, lipids, metabo-