monitor fast neuronal activity in single cells in live flies and mice. The authors used a microbial rhodopsin protein called Ace as the basis for their voltage sensor. Microbial rhodopsins are light-sensitive ion channels, and were initially adopted in neuroscience for their ability to generate electrical currents and so to modulate neuronal activity[1]. More recently, these proteins have been used to monitor electrical currents because they fluoresce in a voltage-dependent manner[5]. They are fast and sensitive sensors, but their use in live organisms[6] has been hampered by the fact that they fluoresce only weakly.

The researchers bypassed this obstacle by fusing Ace with the fluorescent protein mNeonGreen. In this configuration, blue–green light excites mNeonGreen, which emits green–yellow fluorescence. A portion of this fluorescence is absorbed in a voltage-dependent manner by Ace, causing mNeonGreen-emitted fluorescence to decrease as the membrane voltage rises and neuronal activity increases, and to increase as the membrane voltage falls (Fig. 1). *In vitro*, the Ace–mNeon fusion protein acts six times faster and can resolve closely spaced, repeating action potentials much more accurately than similar protein fusions[7].

To assess the capabilities of their tool *in vivo*, Gong and colleagues compared it with GECIs in live mice and flies. Measurements taken using Ace–mNeon during a visual task corroborated previous measurements taken with GECIs. In mice, Ace–mNeon flawlessly reported single action potentials in neurons at the surface of the brain's cortex region, 20 times faster than is possible using GECIs. This is an impressive achievement, because intact mammalian tissue is opaque and can be naturally fluorescent — both of which are factors that can mask the signal from fluorescent proteins.

In flies, Ace–mNeon recorded more than 18,000 action potentials with perfect accuracy, and detected odour-evoked subthreshold and fast voltage changes that a GECI failed to pick up. Furthermore, the authors used the protein to track voltage propagation from one side of a cell to the other with submillisecond precision. Such precision tracking was previously unachievable in live flies.

Although the sensor's performance is impressive, major challenges remain before it can replace GECIs *in vivo*. First, the authors used conventional fluorescence microscopy for *in vivo* imaging. The effectiveness of this type of imaging for sensor detection relies on sparse expression of Ace–mNeon, limiting the number of cells that can be imaged concurrently. Second, for maximum impact, a fast sensor requires fast imaging, but imaging speed and field of view are inversely correlated in current imaging techniques, so rapid imaging limits the ability to simultaneously investigate many cells. The combination of fluorescence microscopy and limited field of view meant that Gong *et al.* could study only a handful of cells at a time. A third challenge is that, although mNeonGreen is three times more stable to light than other rhodopsin-paired fluorescent proteins, extended continuous imaging sessions still 'bleach' the protein, decreasing its fluorescence. This limitation could be bypassed by using multiple short exposures, or by spacing measurements widely enough for protein turnover to replace the photobleached sensors.

The benefits of using GEVIs such as Ace–mNeon to image activity in live animals are undeniable. Nonetheless, better hardware is required to realize the full potential of these voltage reporters. Until that is available, calcium sensors will remain the gold standard for studying densely labelled cell populations simultaneously over extended imaging sessions, especially in deep brain areas. The development of technologies such as microendoscopy[8] and fibre photometry[9] has enabled calcium imaging of subcortical brain regions, and fine-tuning these techniques for use with GEVIs is an exciting possibility for the future. Overall, Gong and colleagues' study highlights the power of microbial rhodopsins, especially when paired with strongly fluorescent proteins, and the need for continued development of these tools hand-in-hand with microscopy techniques. ■

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1. Emiliani, V., Cohen, A. E., Deisseroth, K. & Hausser, M. *J. Neurosci.* **35,** 13917–13926 (2015).
2. Gong, Y. *et al. Science* **350,** 1361–1366 (2015).
3. Chen, T.-W. *et al. Nature* **499,** 295–300 (2013).
4. Knöpfel, T., Gallero-Salas, Y. & Song, C. *Curr. Opin. Chem. Biol.* **27,** 75–83 (2015).
5. Kralj, J. M., Douglass, A. D., Hochbaum, D. R., Maclaurin, D. & Cohen, A. E. *Nature Meth.* **9,** 90–95 (2012).
6. Flytzanis, N. C. *et al. Nature Commun.* **5,** 4894 (2014).
7. Gong, Y., Wagner, M. J., Li, J. Z. & Schnitzer, M. J. *Nature Commun.* **5,** 3674 (2014).
8. Lecoq, J. *et al. Nature Neurosci.* **17,** 1825–1829 (2014).
9. Lerner, T. N. *et al. Cell* **162,** 635–647 (2015).

EVOLUTION

A lizard that generates heat

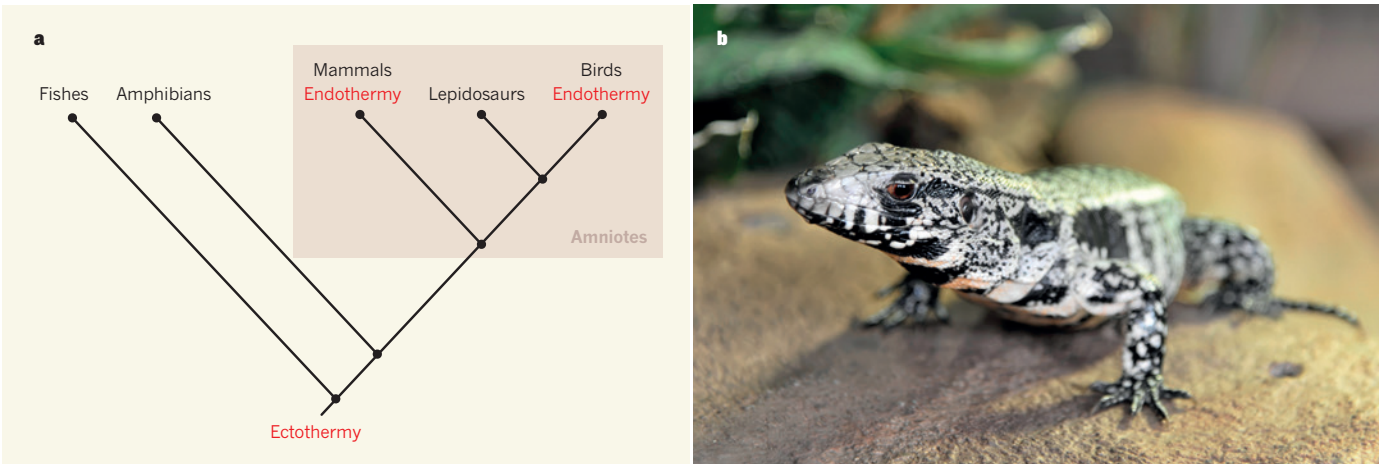
Birds and mammals generate heat to regulate body temperature, but most non-avian reptiles cannot. The discovery of endothermy during the reproductive period of a tegu lizard sheds light on the evolution of this characteristic.

COLLEEN G. FARMER

The avian and mammalian lineages diverged 320 million years ago, and since that time both lineages have converged on a radically different approach to life from that of their common ancestor. Birds and mammals are endotherms, meaning they use internal heat to regulate their body temperature; their ancestor, and many extant animals such as amphibians and non-avian reptiles, are ectotherms that rely on external heat sources (Fig. 1). Understanding the convergent evolution of endothermy in birds and mammals is a central question in evolutionary physiology, because thermal biology is linked to fundamental traits such as body size, food requirements and aspects of reproduction. Writing in *Science Advances*, Tattersall *et al.*[1] report the remarkable discovery that a lizard species uses endothermy during its reproductive period. Their finding supports the idea[2,3] that the ability to exert control over temperature during reproduction was the common selective agent that drove the evolution of endothermy in birds and mammals.

The transition from aquatic to terrestrial habitats presented animals with new challenges to reproduction; chief among these was the fact that eggs laid on land are at risk of desiccation and are subject to greater fluctuations in temperature than eggs laid in water. One lineage of animals — the amniotes — evolved eggs containing a series of fluid-filled membranes, which reduced the risk of desiccation (Fig. 1). Many amniotes further evolved an ability to exert control over temperature during reproduction. For example, viviparity (giving birth to live young rather than laying eggs) allows females to control developmental temperature by gaining heat through basking, and has evolved independently more than 100 times in lizards and snakes[4].

Tattersall *et al.* studied black and white tegu lizards (*Salvator merianae*), which inhabit tropical, subtropical and temperate climates throughout the plains east of the Andes Mountains. During autumn and winter, the lizards hibernate in burrows, after which their reproductive phase begins. Males undergo a surge in



IMAGEBROKER/EGON BÖMSCH/FLPA

Figure 1 | Thermal strategies during reproduction. **a**, Most fishes and aquatic amphibians are ectotherms, which rely on external sources of heat. These animals lay many small eggs in water, exerting no parental control of temperature during reproduction. That strategy changed with the evolution of amniotes — animals whose eggs have fluid-filled membranes that allow development on land. Mammals and birds have both evolved endothermy, meaning they generate body heat, which they can use to incubate eggs (monotremes and birds) or retained embryos (marsupials and placentals). By contrast, lepidosaurs, which include lizards and snakes, are ectotherms that mostly exert reproductive temperature control through egg incubation after basking or by retaining embryos through to live birth. **b**, Tattersall *et al.*[1] report that black and white tegu lizards (*Salvator merianae*), which are ectotherms during most life stages, use endothermy during their reproductive period.

testosterone and gonadal growth, emerge from their burrows and establish territories, but initially forgo foraging. After the reproductive period, they reduce activity, feed heavily and gain weight[5,6]. When females end hibernation, they mate and deposit yolk in their eggs, which entails a heavy energy investment — clutch mass is typically about 40% of body mass[7,8]. Clutches are laid in nests made of various materials, including moist grass, small sticks and other litter[6], which probably improves the insulating properties of the nest.

After laying, the females remain with the eggs for up to around 75 days[6] with little or no foraging activity. Female attendance greatly influences nest temperature; one study found attended nests to be 5 °C warmer than a control nest where females were barred from brooding[9]. Because these lizards are capital breeders — that is, their reproduction is decoupled temporally from food acquisition and assimilation — changes in body temperature during their reproductive period cannot be explained by an increase in metabolism associated with feeding.

Tattersall *et al.* investigated the relationship between reproduction and thermoregulation in sexually mature tegu lizards reared in a captive colony. The body temperatures of both male and female lizards were equal to their burrow temperatures during most of the hibernation period, except for between days 160 and 180, during which the researchers recorded an increase in body temperature above burrow temperature. This is the period in which the lizards rouse from hibernation and begin the reproductive period. The lizards supplemented their endogenous heat production by basking to gain heat during the day, retreating to their burrows at night. Remarkably, body temperature remained elevated throughout the night, whereas during the non-reproductive season, body temperature equilibrated with the temperature of the burrow.

With these observations, Tattersall *et al.* have established that, during the reproductive period and when insulated by a burrow, these relatively small (around 2-kilogram) lizards can generate heat that raises their body temperature by up to 10 °C above ambient, and that this thermogenesis is not related to feeding or activity. Furthermore, the observations refute conventional wisdom that small animals lacking body insulation, such as hair and feathers, cannot significantly increase their body temperature.

The authors also placed reproductive-phase, fasting lizards in a temperature-controlled chamber for 8 days, and found that they maintained body temperatures that were greater than ambient. Disturbing the lizards caused their body temperatures to decline, possibly owing to increased heat dissipation as a result of elevated peripheral blood flow. This observation may explain why endothermy has been missed by other researchers, who have measured body temperature in disturbed animals rather than quiet, undisturbed animals.

Tattersall and colleagues' work not only provides the first evidence of endothermy in a lizard, but also complements previous findings of endothermy during reproduction in pythons[10]. Like the tegu lizards, diamond pythons (*Morelia spilota*) construct insulated nests and achieve body temperatures of up to 13 °C above ambient when brooding[11]. We now know that reproductive endothermy is not an oddity of one clade of snakes. Indeed, there is increasing evidence that many species of bird and mammal improve their capacities for thermogenesis and endothermy during reproduction (reviewed in refs 2, 3).

The selective drivers for the evolution of endothermy are debated. However, convergent evolution is one of the strongest lines of evidence for the adaptive significance of a trait. Thus, this discovery in lizards corroborates the idea that the initial selective benefits of the evolution of endothermy in birds and mammals were reproductive[2,3]. Studies of pythons have shown that the thermal regime during incubation affects the incubation period as well as the characteristics of the hatchlings (such as initial growth rates, escape behaviour and willingness to feed[12]), providing several potential bases on which reproductive endothermy may provide an evolutionary advantage.

Intriguing questions remain. How do these lizards generate body heat, and do so only at certain times? Precisely how does thermogenesis facilitate the lizards' reproduction — might it expand the geographical range over which this species can reproduce, or alter the time window for reproduction? Are tegus and pythons alone, or are there other reproductively endothermic non-avian reptiles? Reproductive endothermy may yet be discovered in other species if they are studied using methods that do not disturb them during the reproductive period, when insulating nests reduce rates of heat dissipation and metabolism is increased by the synthetic demands of reproduction. ■

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1. Tattersall, G. J. *et al. Sci. Adv.* **2,** e1500951 (2016).
2. Farmer, C. G. *Am. Nat.* **155,** 326–334 (2000).
3. Farmer, C. G. *Am. Nat.* **162,** 826–840 (2003).
4. Shine, R. *Annu. Rev. Ecol. Evol. Syst.* **36,** 23–46 (2005).
5. Winck, G. R. & Cechin, S. Z. *J. Nat. Hist.* **42,** 239–247 (2008).
6. Fitzgerald, L. A., Chani, J. M. & Donadío, O. E. in *Neotropical Wildlife Use and Conservation* (eds Robinson, J. & Redford, K. H.) 303–316

(Univ. Chicago Press, 1991).
7. Manes, M. E., Noriega, T., Casal, C. & Apichela, S. *Cuad. Herpetol.* **21,** 21–29 (2007).
8. Lopes, H. R. & Abe, A. S. in *Manejo y Conservación de Fauna Silvestre en América Latina* (eds Fang, T. G., Montenegro, O. L. & Bodmer, R. E.) 259–272 (Inst. Ecol., La Paz, 1999).
9. Manes, M. E., Ibanez, M. A. & Manlla, A. *Rev. Argentina Prod. Anim.* **23,** 119–126 (2003).
10. Hutchison, V. H., Dowling, H. G. & Vinegar, A. *Science* **11,** 694–695 (1966).
11. Slip, D. J. & Shine, R. *J. Zool.* **216,** 367–378 (1988).
12. Shine, R., Madsen, T. R. L., Elphick, M. J. & Harlow, P. S. *Ecology* **78,** 1713–1721 (1997).

INFECTION BIOLOGY

Small RNA with a large impact

A simultaneous comparison of the RNA molecules expressed by *Salmonella* bacteria and human cells during infection reveals how a bacterial small RNA alters the transcript profiles of both the bacteria and the host cells. SEE ARTICLE P.496

MATTHIAS P. MACHNER & GISELA STORZ

What happens when bacteria encounter or enter host cells? How does each of the species respond, the bacteria to survive in their new environment and the host cells to either tolerate non-harmful bacteria or defend against pathogenic ones? To answer these questions, it is imperative to understand how gene transcription in both cells changes during the encounter. Over the years, approaches applied to this problem have ranged from *in vivo* gene-expression technology[1] to sequencing the full complement of bacterial or host-cell transcripts[2,3] (the transcriptome). However, such analyses have largely focused on messenger RNAs and have profiled either the bacteria or the host, not both at once. In this issue, Westermann *et al.*[4] (page 496) go beyond the individual organisms by using dual RNA-seq, an approach that simultaneously profiles bacterial and host transcriptomes throughout the course of an infection.

The RNA-seq method takes advantage of the ever-increasing depth of sequencing (the number of reads for a particular sample) now possible. Westermann *et al.* first assessed whether the dual RNA-seq approach accurately reflected known gene regulation in human HeLa cells and in the bacterium *Salmonella enterica* serovar Typhimurium (hereafter *Salmonella*), a common cause of food poisoning, during infection. The authors' data confirmed that, as previously reported[5], transcription of invasion-related genes in the genomic region known as *Salmonella* pathogenicity island 1 (SPI-1) was reduced after bacterial internalization, whereas transcription of SPI-2 genes, which promote intracellular survival, increased.

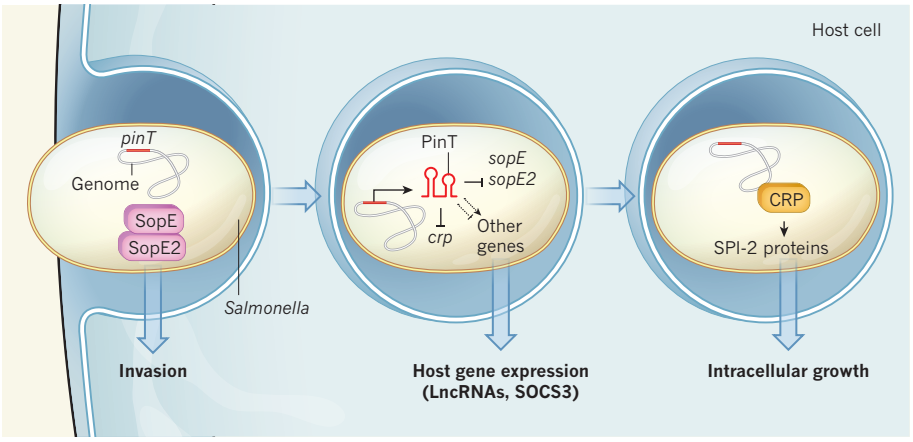

Figure 1 | PinT orchestrates gene expression in *Salmonella* and its host cells. When *Salmonella* bacteria invade cells, expression of the genes *sopE* and *sopE2* facilitates bacterial invasion. After the bacteria are internalized, the levels of the transcripts from these genes fall. By simultaneously monitoring the RNA molecules present in both *Salmonella* and host cells over the course of an infection, Westermann *et al.*[4] found that a small regulatory RNA expressed in *Salmonella*, which they name PinT, induces this repression by base-pairing with the *sopE* and *sopE2* messenger RNAs. PinT also base-pairs with the mRNA that encodes CRP, a protein that activates transcription of genes encoding SPI-2 proteins. This repression is reduced later in infection, allowing the SPI-2 proteins to regulate the bacterium's intracellular growth. The authors also observed differences in host-cell transcripts when the cells were infected with *Salmonella* mutants lacking PinT, including altered levels of long non-coding RNAs (lncRNAs) and the mRNA for SOCS3. This suggests that PinT targets other bacterial genes that influence host-cell gene expression.

Having validated the sensitivity of the approach, Westermann *et al.* focused on mRNAs and regulatory RNAs whose expression changed during the course of the 24-hour infection. In bacteria, small regulatory RNAs (sRNAs) that base-pair with target mRNAs to modulate the mRNA's stability or translation are integral to a wide range of stress responses, including the response to host cells[6]. Thus, the authors were intrigued by an 80-nucleotide sRNA, which they denoted PinT, whose expression was highly induced during infection, and which was activated by the bacterial PhoP/Q system, known to be crucial for *Salmonella* survival in the intracellular environment.

A striking finding of the dual RNA-seq analysis was that tens of bacterial and hundreds of host-cell transcripts were affected merely by the presence or absence of PinT (Fig. 1). On the bacterial side, overproduction of PinT led to reduced levels of the mRNAs encoding SopE and SopE2, two SPI-1 effector proteins that mediate host-cell invasion by *Salmonella*. These mRNAs were elevated in strains lacking the *pinT* gene. By mutating the *pinT*, *sopE* and *sopE2* sequences, the authors revealed that the inhibitory effect of PinT occurred through direct base-pairing with the mRNAs. Dual RNA-seq also revealed a role for PinT in repressing SPI-2 genes later in infection. However, control of these genes was indirect and occurred through PinT base-pairing with the mRNA that encodes the cyclic AMP receptor protein (CRP), an activator of transcription of SPI-2 genes. These data indicate that PinT, on bacterial internalization, controls the temporal expression of both SPI-1 effectors and SPI-2 virulence genes, thus facilitating the bacterium's transition from an invasive state to a state of intracellular replication.

Westermann *et al.* then compared the transcriptomes of the host HeLa cells challenged with either wild-type *Salmonella* or a strain lacking PinT. They discovered numerous changes in cells infected with the PinT-lacking mutant, including altered levels of many long non-coding RNAs (lncRNAs), hyperactivation of mitochondrial genes, increased abundance of mRNAs for proteins involved in innate immune pathways (such as the interleukin-8 mRNA) and accelerated activation of SOCS3, a protein that regulates the inflammatory JAK–STAT signalling pathway. The last finding is of particular interest, because properly balanced JAK–STAT signalling is essential for