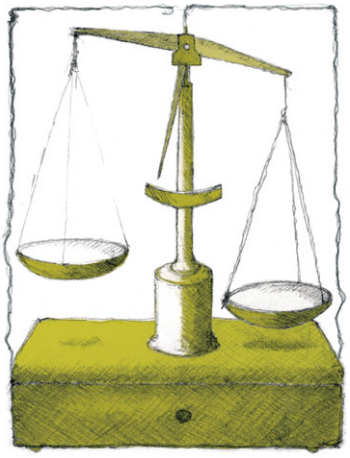
Keeping track of the literature isn't easy, so Outside JEB is a monthly feature that reports the most exciting developments in experimental biology. Short articles that have been selected and written by a team of active research scientists highlight the papers that JEB readers can't afford to miss.

Outside JEB

BRAINLESS LEARNING



LEARNING WITHOUT A BRAIN

You don't need your brain to walk. You don't even need it to catch yourself after you stumble. And now, it appears, you may not even need it to learn some new skills. A recent report suggests that the spinal cord itself, without the brain at all, is able to adapt to a new environment – and possibly even anticipate how its environment has changed.

Chad Heng and Ray de Leon of California State University in Los Angeles studied neonatal rats whose spinal cords were completely severed at the mid-thoracic level, cutting off connections between the rats' brains and hind limbs. Despite the spinal cord damage, many of the rats spontaneously recovered their ability to walk. This didn't surprise the researchers: it's well-known that the spinal cord contains the neural networks that drive regular walking, as well as reflex pathways that allow it to respond to simple perturbations like tripping or stepping in a hole.

But the researchers wanted to see whether the spinal cord can adapt to more complex effects – in essence, whether it can learn. So they built a system to alter the forces on one hind leg. They connected a small robotic arm to one ankle and programmed it to resist the leg's forward motion with a backward force proportional to its velocity – a 'viscous' perturbation, similar to dragging the leg through a vat of honey.

When they turned on the viscous force, it threw the rats' stepping off. At first, they didn't quite manage to put the leg attached to the arm (the 'perturbed' leg) on the ground before they tried to lift up the other one, so that the support phases of the two limbs didn't overlap at all. By the second step after the force came on, the support phases started to overlap slightly, and by the sixth step, the rats had recovered normal overlapping support between the two limbs. They still had some problems – steps on the perturbed side were slow and short – but, overall, they were able to produce an effective response to a rather unnatural, complicated stimulus.

How was the spinal cord adapting to the stimulus? The changes might have been some sort of complex reflex – a 'feedback' strategy, which involves comparing the leg's position with an internal model of where it should be. Or they might have involved learning – a 'feed-forward' strategy, which would mean that the spinal cord was altering that internal model of the leg's motion.

The fact that it took several steps for the rats to adapt suggests a feed-forward strategy. But if learning really occurred, the spinal cord should overcompensate once the viscous force was stopped, taking several steps to relearn the normal force regime. Unfortunately, here the researchers' data are mixed. Four of their 10 rats overcompensated when the researchers turned the force off, overlapping the support phases of the two limbs much longer than normal. The other six rats, though, didn't step at all when the force went off – they tripped – which made it impossible to determine whether they were overcompensating or not. So there weren't enough data for a statistically significant effect, but the trend suggests learning.

Regardless of the mechanism for the adaptation, whether it's a complex feedback pattern or feed-forward learning, Heng and de Leon have shown that the spinal cord is even more complicated than we thought. More than just transmitting information from the brain to the body, more than providing reflex patterns, the spinal cord may cooperate with the brain in learning new environments to produce effective behaviours.

10.1242/jeb.001164

Heng, C. and de Leon, R. D. (2007). The rodent lumbar spinal cord learns to correct errors in hindlimb coordination caused by viscous force perturbations during stepping. *J. Neurosci.* **27**, 8558-8562.

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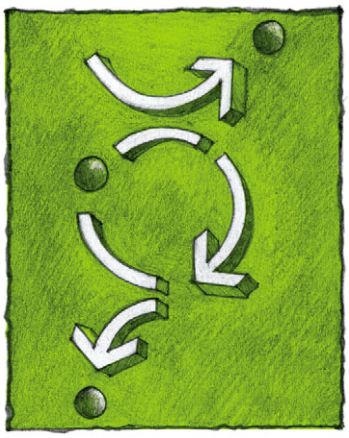
BACTERIAL SIGNALS



FLIES TUNE INTO BACTERIAL SIGNALS

In many species of fly, females are attracted to lay eggs where other females have previously laid. The activity of large numbers of larvae makes the food more edible, often by increasing the rate of substrate decay, thereby aiding larval growth and survival. In the housefly, *Musca domestica*, however, the carnivorous habits of the larvae means that newly hatched larvae may turn into food for older maggots. Kevin Lam and co-workers from Simon Fraser University investigated their hypothesis that to avoid this danger, female flies should be attracted to lay near to freshly deposited eggs and avoid older egg masses.

They first tested whether the presence of eggs of different ages affected survival rate. They found that significantly fewer flies hatched from a mixture of fresh and 24-h-old eggs compared with a single-age control. This supported the idea that mixed-age larval masses represent a significant cost for houseflies. The authors then measured female egg-laying preferences, finding that female flies preferred to lay on food containing freshly laid eggs over egg-free food, but preferred egg-free food over food containing 24-h-old eggs. This showed that the females were using a signal to discriminate between the two egg ages.

To test the hypothesis that this signal was chemical, the authors washed eggs of various ages, treated food with the resultant solution and observed the egg-laying responses of female flies. The results were identical to the real-life situation: the females preferred the fresh egg solution and avoided the 24 h egg solution. When presented with clean food and food containing a solution from 8 h or 16 h eggs, the females showed no significant choice, suggesting that the chemical cue was gradually accumulating or changing over time.

The authors hypothesized that the source of this cue might be microorganisms on the eggs. They therefore filtered the solution from washed 24 h eggs to remove any potential microorganisms and found that females did not avoid laying eggs on food treated with this solution. Having identified microorganisms as the source of the signal that alters oviposition behaviour, the authors next demonstrated that the flies' attraction to microorganisms associated with fresh eggs shows a dose–response curve and that no significant effect was induced by microorganisms isolated from food that had no eggs on it.

The final part of the team's study involved a shift from chemical ecology to microbiology. They isolated 19 different strains of bacteria from fresh egg-washes and discovered that a single Gram-negative oxidase-positive strain, *Klebsiella oxytoca*, strongly inhibited oviposition on its own. This led them to hypothesize that changes in the amount of *K. oxytoca* on eggs might form the basis of the signal used by females. To test this idea, they first showed that the number of *K. oxytoca* found on eggs increased by 1.46×10^8 between 0 and 24 h. To see whether this increase could alter behaviour, they added this amount of *K. oxytoca* and of 14 other bacterial strains to fresh eggs. Only the addition of *K. oxytoca* increased female avoidance behaviour, demonstrating that female flies use the level of this bacterium as a measure of egg age.

Chemical communication in insects often involves direct measures of age, for example through changes in cuticular hydrocarbons. This investigation shows that insects can also take advantage of the presence of other organisms – in this case, bacteria – to detect evolutionarily significant age changes. This intriguing and rigorous study provides researchers studying chemical communication with a stimulating question: how many other examples of inter-kingdom cues are there?

10.1242/jeb.001198

Lam, K., Babor, D., Duthie B., Babor, E.-M., Moore, M. and Gries, G. (2007). Proliferating bacterial symbionts on house fly eggs affect oviposition behaviour of adult flies. *Anim. Behav.* **74**, 81-92.

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NEURON SURVIVAL

MORE NEUROGLOBIN MAKES GOOD SENSE

Most vertebrate brains are exquisitely sensitive to low oxygen levels and respond to this hypoxia with a variety of protective mechanisms to increase anaerobic metabolism and oxygen delivery, minimizing the pathological effects of deficient energy supplies. From the initial reports of their existence several years ago, the heme proteins neuroglobin and cytoglobin have been the subjects of much study: able to bind oxygen reversibly, these proteins could act as neuronal and tissue 'myoglobins', yet their true roles are unclear. Possible functions include serving as an intracellular oxygen carrier or oxygen sensor, or involvement in the metabolism of nitric oxide or reactive oxygen species (ROS) generated when hypoxic cells are reoxygenated. Extensive ROS generation damages cells by oxidizing lipids, proteins and DNA. Thus, compounds that decrease ROS aid in cell survival, and it has been shown that the over-expression of neuroglobin or cytoglobin promotes cell survival after hypoxia or ischemia.

Sylvia Dewilde and her colleagues at the University of Antwerp investigated the function of these heme proteins by either over-expressing or under-expressing (using antisense treatment) cytoglobin and neuroglobin in human neural cells in cell culture and exposing the cells to either oxygen deprivation (anoxia), or oxygen and glucose deprivation. They then compared cell survival and production of the ROS hydrogen peroxide (H_2O_2), as well as mRNA and protein levels in the cell groups.

The unmanipulated cells easily tolerated as much as 32 h anoxia, though when deprived of glucose and oxygen, cell death began to increase after 12 h and only 46% of cells survived 32 h, showing that neuronal cells are far more vulnerable to

cell death when both oxygen and glucose are absent. To investigate how an excess of heme proteins affected cell survival, the team over-expressed neuroglobin or cytoglobin in some cells, finding that they did not alter cell survival in normoxic or anoxic conditions, when viability was already high. However, survival significantly increased in these cells after glucose and oxygen deprivation, indicating a protective role for the proteins.

Because ROS production is thought to be a key event in neuronal cell death after glucose deprivation and reoxygenation, and neuroglobin and cytoglobin are potential ROS scavengers, the group then looked at H_2O_2 levels. H_2O_2 did not increase in anoxic or glucose deprivation conditions in unmanipulated cells. Neuroglobin and cytoglobin overexpression both decreased H_2O_2 release compared to normoxic cells, while under-expressing the heme proteins increased H_2O_2 levels, demonstrating that both neuroglobin and cytoglobin expression are correlated with oxidative stress, which may then result in cell death.

Although both the heme proteins decreased ROS release and increased cell survival, experiments looking at their normal expression suggested that neuroglobin and cytoglobin may have different functions, or at least different mechanisms of regulation. Neuroglobin mRNA and protein are upregulated under glucose deprivation and remain high upon reoxygenation. By contrast, cytoglobin is upregulated in anoxia and returns to basal levels upon reoxygenation. These increases are apparently not sufficient *in vivo* for neuroprotection, as alterations in ROS production and cell survival were seen only after heme protein over-expression. If antisense treatment for neuroglobin or cytoglobin increases ROS and cell death, then it is clear that upregulating these heme proteins makes good sense!

10.1242/jeb.001172

Fordel, E., Thijs, L., Martinet, W., Schrijvers, D., Moens, L. and Dewilde, S. (2007). Anoxia or oxygen and glucose deprivation in SH-SY5Y cells: a step closer to the unraveling of neuroglobin and cytoglobin functions. *Gene* **398**, 114-122.

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HUNGRY FOR AIR

Anyone who has undertaken strenuous exercise, tried to hold their breath at the bottom of a swimming pool or climbed high mountain peaks will stress the importance of getting enough oxygen. This is also true for insects, although perhaps they admit their cravings for air somewhat less openly. Regardless, biologists have long appreciated the fact that insects have remarkable tolerance to low oxygen levels. But how is it possible for a fruit-fly to survive a lack of oxygen for hours when humans can barely endure several minutes? This is especially mystifying when considering flies' relatively high rates of resting energy consumption associated with their flight ability.

Jacob Feala, at the University of California, and colleagues from the Burnham Institute for Medical Research, San Diego, explored the energy pathways that *Drosophila* use during several hours of oxygen deprivation, or hypoxia, in order to better understand any potential metabolic advantage that these flies may have. First, the team employed nuclear magnetic resonance (NMR) spectroscopy to describe the changes in metabolic pathway end-products during hypoxia. The key by-products of energy metabolism found during these experiments were lactate, alanine and acetate. Obtaining high lactate concentrations was not particularly surprising, since this is the most common by-product of ATP production during oxygen shortage in mammals. However, discovering alanine and acetate was a little more unusual.

But the end-products of biochemical reactions do not fully explain what happens during hypoxia, especially if one is interested in knowing which pathways are employed. So, using the NMR results, and armed with the knowledge that most biochemical energy reactions in insects start with glycogen, trehalose and proline breakdown, the team subsequently built a model of all the potential ATP-producing pathways that might yield these mystery metabolites. Specifically, Feala's group included several pathways that produce alanine and acetate in order to find optimal production pathways for each of the biochemical compounds recorded during the NMR trials. To provide a simplified analogy, this process is like trying to work out the route that someone might have travelled on a large, complex railway system while the only information available is the starting station and end destination. Finally, using the refined information-based model, the team explored hypoxia adaptation by computer simulations of different energy and oxygen conditions.

Many hours of number-crunching revealed that the ability of the flies to produce these three biochemical end-products – alanine, acetate and lactate – could help them survive low oxygen by improving ATP production and the efficiency of glucose consumption, and also reducing proton production, which can lead to damaging pH fluctuations. It seems, therefore, that having more options for fermentation of an energy source contributes to the hypoxia tolerance of these flies. So do you want to hold your breath underwater for several hours? Well, Feala and co-workers have shown that all you need to do is take a deep breath and re-direct your anaerobic biochemical pathways to produce lactate, alanine and acetate instead of only lactate as you typically do when you run out of air.

10.1242/jeb.001206

Feala, J. D., Coquin, L., McCulloch, A. D. and Paternostro, G. (2007). Flexibility in energy metabolism supports hypoxia tolerance in *Drosophila* flight muscle: metabolomic and computational systems analysis. *Mol. Syst. Biol.* **3**, 99.

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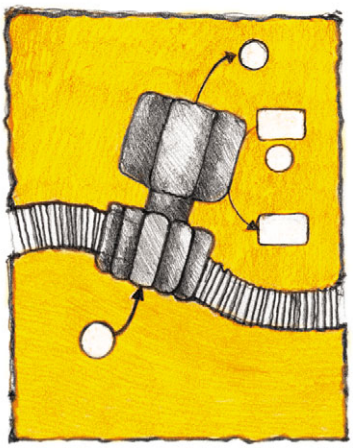
INTERNAL CLOCKS



TIMELESS CLOCK

We don't see it, we don't hear it, yet our internal clock ticks and controls our everyday life more than we realise. Only when we live against our internal clock do we become aware of its arcane rhythm, for instance when we suffer jet lag after a long-distance flight. What makes the internal clock so important is that it orchestrates vital physiological functions, such as body temperature or blood pressure.

Internal clocks are synchronized by external rhythms. Nature is affected by two of them, the daily sun cycle and the annual cycle of the seasons. Correspondingly, two types of internal clocks exist in animals and plants: a circadian and a photoperiodic clock; regulating daily and seasonal activities, respectively. The circadian clock is synchronized by light–dark cycles, the photoperiodic clock by day length. It is an old debate whether the fundamental mechanisms underlying the circadian clock also account for the photoperiodic clock. In two studies published recently, a British and Italian team of scientists led by Charalambos Kyriacou and Rodolfo Costa showed that both clocks are based on distinct genetic programs, suggesting that the mechanisms underlying both clocks are different.

Remarkable progress has been made in elucidating the mechanism of the circadian clock, particularly in *Drosophila melanogaster*. A big breakthrough was the discovery of circadian clock genes, such as *timeless* (*tim*), whose gene products oscillate in a daily rhythm, or *cryptochrome* (*cry*), which encodes a photoreceptor, CRY, required for the entrainment of circadian rhythms to light–dark cycles by influencing, among others, oscillations in TIM protein levels. By contrast, the molecular basis of the photoperiodic clock is largely unknown. To shed light on it, the team investigated the photoperiodic timing of diapause, the suspension of insects' development as an adaptation to changing seasons. They did this for the first time in the 'clock model' *Drosophila* and found that diapause in European populations occurs more frequently at shorter day lengths and at northern latitudes.

When the scientists analyzed the *tim* gene in different *Drosophila* populations, they found two natural alleles, *s-tim*, which encodes the shorter, ancestral variant of the TIM protein (S-TIM), and *ls-tim*, which encodes a longer, newly derived variant (L-TIM) of the TIM protein. It was previously known that CRY synchronizes the circadian clock by binding to TIM in a light-dependent manner and thereby promoting its degradation. But does the newly discovered L-TIM exhibit the same binding properties as S-TIM? Performing interaction assays in yeast, the team showed that L-TIM binds less tightly to the CRY photoreceptor. As a consequence of altered binding properties, photosensitivity of the circadian clock declines due to increased L-TIM stability. Thus, fly populations with different TIM alleles set their internal clock with different accuracies.

However, does the discovered allelic variation also affect the photoperiodic timer? To answer this question, the team carefully analyzed the allele frequencies in natural populations. They found that the alleles have spread unevenly throughout Europe. The ancestral *s-tim* is more prevalent in the north, with the younger *ls-tim* more in southern Europe. Analysis of homozygous flies showed that *ls-tim* flies generally undergo diapause more frequently than *s-tim* flies, suggesting that *ls-tim* confers an advantage by making the flies more adaptable to the changing European seasons. However, the team found no hint of an interaction between the *tim* genotype and the photoperiod in the induction of diapause, implying that the circadian and the photoperiodic clock use different mechanisms.

The clock gene *timeless* hence appears to have two functions in *Drosophila.* In addition to its key role as an oscillator of the circadian clock, it directly affects initiation of diapause without biasing the photoperiodic clock. Although the team of Kyriacou and Costa provide important clues on *timeless* function, the molecular mechanisms underlying the photoperiodic clock are still elusive.

10.1242/jeb.001180

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