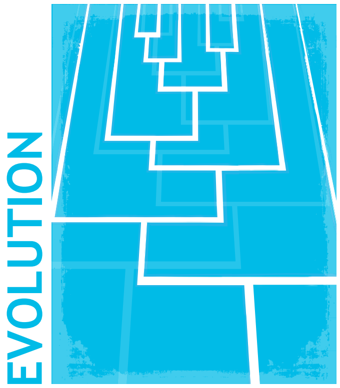
© 2017. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2017) 220, 735-737

OUTSIDE JEB

Haemoglobin's low affinity for predictability



There's more than one way to crack an egg, shear a sheep or do pretty well anything else, but what about the evolution of physiological traits? If the trait in question is a protein's function, then shouldn't biophysical constraints on protein structure demand that the independent evolution of the same function arise from the same amino acid substitution? Chandrasekhar Natarajan, from the University of Nebraska, USA, and his team were sufficiently intrigued by this question to devise a truly impressive comparative study to get to the bottom of it, and used haemoglobin, and its affinity for oxygen, as their representative protein.

The team started by clustering 56 avian species into 28 closely related species pairs, each pair comprising a low- and high-altitude native relative. After isolating each bird's haemoglobin, the team measured their affinities for oxygen and found that the high-altitude inhabitants consistently had higher affinity haemoglobins than their low-altitude counterparts, the evolutionary result of adaptation to a consistently more hypoxic environment. The question then became whether the evolved increases in haemoglobin–oxygen affinities were caused by amino acid substitutions at the same sites.

They set about answering this by sequencing each species' haemoglobins and searching for common amino acid substitutions that might be responsible for the higher affinities that evolved independently in multiple high-altitude species. When assessing their results across the entire family tree of birds, they found no consistency in the amino acid substitutions that they identified in the high-affinity haemoglobins, suggesting that there are multiple ways to make a high-affinity haemoglobin. However, when they zoomed in on closely related branches within the broad family tree – focusing in particular on the hummingbirds – they noticed a glycine-to-serine substitution that consistently appeared in the high-altitude hummingbirds at a site on the protein (amino acid 83 in the β chain) that could potentially enhance its affinity for oxygen. Intriguingly, a substitution at the same site also appeared in the high-altitude flowerpiercers.

This parallelism hinted at a common mechanism for enhancement of oxygen affinity within closely related species, but one that was not effective in more distantly related species. The team tested this hypothesis by reconstructing the ancestral haemoglobin sequences for hummingbirds, flowerpiercers, Neoaves (which are the common ancestor of all extant birds excluding the ducks, chickens and kin) and Neornithes (which are the common ancestor of all currently surviving birds). They used site-directed mutagenesis to introduce the key amino acid substitution in each haemoglobin sequence, and then tasked *Escherichia coli* with physically manufacturing these 'new' haemoglobins. After isolating the proteins, the team measured their oxygen affinities and found that the mutations significantly increased the affinities of ancestral hummingbird and flowerpiercer haemoglobins, but had no effect on the more distantly related haemoglobins. These results plainly showed that despite environmental consistency – all of the species were adapted to high-altitude hypoxia and so the proteins were adapted to increased oxygen affinity – the causal genetic mechanism was only similar among closely related species. When the team zoomed out to look at more distantly related species, they found that other differences dispersed throughout the haemoglobin chains precluded the mutation at β83 from having the same effect on oxygen affinity as it did within the closely related hummingbirds and flowerpiercers. Enhanced affinities therefore evolved in these more distantly related species through a different set of mutations.

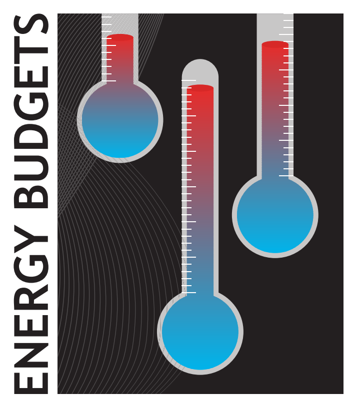
So, yes, there's more than one way to shine a penny, shoe a horse and catch a rabbit. Now we know that there's also more than one way to evolve a high-affinity haemoglobin. As idioms go, it's a good one, but it should probably be reserved for use among physiologists.

10.1242/jeb.147280

Natarajan, C., Hoffmann, F. G., Weber, R. E., Fago, A., Witt, C. C. and Storz, J. F. (2016). Predictable convergence in haemoglobin function has unpredictable molecular underpinnings. *Science* **354**, 336-339.

Matthew Regan
University of British Columbia
regan@zoology.ubc.ca

Flexible activity behaviour aids survival in the Arctic



Activity is an essential part of life for animals, whether it be to find food, shelter or a mate. And the key ingredient for such forays is energy, derived from the food they find. Animals need energy not only to be active, but also for a suite of basic life functions, including reproduction. In many mammals, males are often tasked with finding a mate, whereas females invest

Outside JEB is a monthly feature that reports the most exciting developments in experimental biology. 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.

energy in their young during pregnancy and lactation. Consequently, there is often a mismatch in the energetic needs of the sexes, resulting in differences in activity across the year. On top of managing their energy expenditure during the reproductive period, animals also have to deal with general day-to-day energetic constraints, such as bad weather, which can be particularly demanding for mammals inhabiting freezing Arctic habitats.

Arctic ground squirrels hold the title of the northern-most small mammal hibernators. They have to successfully raise and prepare their young for the long winter hibernation period over the short spring/summer breeding season, which requires an enormous energy investment from the animals. This is why Cory Williams, from Northern Arizona University, USA, and his group were interested in the activity behaviour and energy expenditure of Arctic ground squirrels between April and August.

Braving the Alaskan wilderness, Williams and his team travelled to two field sites in Alaska, one near Atigun River and a second, colder site at Toolik Lake. There they secured collars to male and female ground squirrels to measure how much time they were inactive in the burrow and active above the surface (using light loggers), and then recorded what the animals got up to when they were out and about (using accelerometers). The team also collected a wealth of environmental data from weather stations at the two field sites, including ambient temperature, solar radiation, wind speed, rainfall and major snowfall events. Finally, they calculated an index of the animals' energy expenditure from the accelerometer recordings to discover how their energetic needs correlated with the weather conditions and their reproductive state.

Arctic ground squirrels often encounter freezing conditions, even in the summer, and Williams and his group revealed that their activity patterns were indeed strongly influenced by the weather conditions. The ground squirrels preferred to be above ground and active when it was warmer and, not surprisingly, they also liked staying dry, displaying less activity on days with rain or snow. This meant that individuals at the cooler and wetter Toolik Lake site spent less time above ground than their counterparts at Atigun River. For Arctic ground squirrels, such flexible adjustments of activity behaviour are really important as they lose lots of body heat in cold and wet conditions, dramatically increasing the amount of energy they need.

The team also observed that the females' maternal responsibilities ensured that they occupied their underground burrows more often than males, particularly during early lactation. However, while the males spent more time above ground, they expended just as much energy as the active females. It seems that the females employed more effort while foraging above ground in comparison to the males, which would help them to obtain the energy required for lactation while allowing for more time with their pups: newborn ground squirrels need their mothers for food and body warmth, especially as their burrows are enclosed by frozen soil. So, female Arctic ground squirrels really have it tough, trying to balance nursing their pups and foraging activity to successfully raise young in frosty Arctic summer conditions.

10.1242/jeb.147272

Williams, C. T., Wilsterman, K., Zhang, V., Moore, J., Barnes, B. M. and Buck, C. L. (2016). The secret life of ground squirrels: accelerometry reveals sex-dependent plasticity in above-ground activity. *R. Soc. Open Sci.* **3**, 160404.

Clare Stawski
University of New England
cstawsk2@une.edu.au

Heavily armed snails on the attack

Natural selection has equipped predators with an amazing armoury – including teeth, claws and venom – and, in turn, prey can defend themselves with toxins, shells and spikes. Of all the weapons displayed by predators on the attack, and the defensive counter-measures evolved by prey, a snail's shell might not look very impressive. But as revealed in a recent paper by Yuta Morii, from the Hokkaido University in Japan, and an international team of collaborators, we may have underestimated this piece of armour and, more importantly, what it can tell us about speciation.

Karaftohelix land snail shells vary in their size, colour and shape. Because they look so different, some *Karaftohelix* species were assigned to different genera until researchers realised that these snails are anatomically and genetically indistinguishable. Given that these snails live in almost identical microhabitats and can coexist in some regions, how did such diverse shell forms evolve? Morri and colleagues suspected that predation may play a vital role.

To find out whether predation promotes phenotypic radiation in these snails, the team studied several *Karaftohelix* species from populations in Japan and Russia. The team observed the behaviour of snails when they were nudged with a pair of fine tweezers or housed with a predatory beetle. Two very different defensive strategies emerged. Some species behaved just like many snails in our gardens, retreating into their shells and using them like shields to keep marauding beetles at bay. But other species did something surprising: they fought back, rhythmically swinging their shells to-and-fro, bashing the beetles onto their backs.

If predator–prey interactions helped drive the phenotypic radiation we see in these snails, then we would expect these differences in behaviour to correlate with morphological variation in shells. To see whether this is the case, Morii and colleagues characterised several shell traits, such as height and diameter, and then related these traits to defensive behaviour. The team found that beetle-bashing snails have wider relative shell apertures. This makes sense given that a narrow shell entrance may be great for keeping predators out, but wider apertures allow for a muscular body capable of swinging a shell like a battle club. Additionally, shells used like shields tended to have a smaller diameter overall, suggesting that a narrow shell offers a more secure retreat.

So far, this study tells us that shells can serve as shields or clubs and that snails can go on the attack. But the key advance made by this paper is in showing how predator–prey interactions might drive speciation. Phylogenetic analyses showed

Journal of Experimental Biology (2017) 220, 735-737

that the passive (retreating) and active (beetle-bashing) strategies of defence, and the associated shell forms, evolved independently in island and continental populations of snail. This parallel evolution of similar shell shapes and behaviours suggests that selection for anti-predation defence is responsible for these behavioural and morphological differences. While researchers have long suggested that predation pressure could drive speciation, it has been unclear how this might work. Morii and colleagues show us how anti-predator adaptations can promote phenotypic radiation, which may be a key step towards speciation.

10.1242/jeb.147264

Morii, Y., Prozorova, L. and Chiba, S. (2016). Parallel evolution of passive and active defence in land snails. *Sci. Rep.* **6**, 35600.

C. Ruth Archer
University of Exeter
c.archer@exeter.ac.uk

An ancient origin for the diaphragm

Lungs provide air-breathing vertebrates with an efficient and often intricate surface for the exchange of oxygen and carbon dioxide. However, they require a mechanism for the air within them to be repeatedly replenished. Animals achieve this in a variety of different ways, but most suck air into the lungs by generating a negative thoracic pressure. The simplest way to do this is to expand the rib cage with intercostal muscles, and many vertebrates do just that. But in mammals, the primary respiratory driver is the diaphragm: a large crescent-shaped muscle that separates the lungs from the abdomen. When it contracts, it draws in air and inflates the lungs. This effective breathing mechanism, partnered with alveolar lungs, equips mammals with a high capacity for oxygen consumption and may have been key to the evolution of high metabolic rates and warm blood. But when the diaphragm first evolved in the mammalian lineage has remained a mystery.

In search of the earliest evidence of a mammalian diaphragm, Markus Lambertz, from the University of Bonn, Germany, and his colleagues examined fossils of caseids – enormous mammal-like reptiles that occupy the earliest branch of the synapsids, the lineage from which mammals ultimately descended. But even if the caseids had diaphragms, there is little chance it would be written in the stone directly, as muscle is too delicate to fossilize. Thus, the authors reconstructed the respiratory paleobiology of these half-tonne behemoths.

Based on the dimensions and morphology of the caseid trunk, Lambertz and colleagues suggest that these animals had a very limited capacity for directly expanding the rib cage. If they were sluggish and had low oxygen demands, they may have just about managed, but if they had oxygen consumption rates similar to those of active modern reptiles, they would have had to pant over 50 times a minute.

However, the team encountered a game changer when they closely analysed the caseid bones. They were spongy and almost osteoporotic. If, as had previously been assumed, these animals were terrestrial, their fragile bones would have been vulnerable to damage. Further, their short necks would have made the menial tasks of eating and drinking difficult. The team therefore suggest that the caseids were predominantly aquatic beasts.

If these aquatic assumptions are correct, the caseid ventilatory insufficiencies would be tremendously exposed. All known diving animals require large ventilation volumes and are characterised by effective ventilation mechanics. If the caseids relied on their limited rib movements alone, they would be left short of breath. In the authors' own words, 'the impossible meets the improbable'. These ancient ancestors must have relied on accessory breathing apparatus. The caseids could have 'swallowed' air, pushing it from the mouth to the lungs, like frogs, but their mouths are too small to make a meaningful contribution to ventilation. They also could have possessed specialised muscles like turtles, or something completely unique, but this seems far-fetched and lacks evidence. Instead, Lambertz and colleagues suggest the most likely scenario is that these ancient mammal-like reptiles probably had mammal-like diaphragms to help them catch their breath.

10.1242/jeb.147298

Lambertz, M., Shelton, C. D., Spindler, F. and Perry, S. F. (2016). A caseian point for the evolution of a diaphragm homologue among the earliest synapsids. *Ann. N. Y. Acad. Sci.* **1385**, 3-20.

William Joyce
Aarhus University
william.joyce@bios.au.dk