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Title: Why Zebra Finches Don't Get Hypercholesterolemia

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In his 1994 classic, “Why Zebras Don’t Get Ulcers” (1), Robert Sapolsky chronicles how the pathological effects of chronic stress commonly seen in humans are much rarer in non-human animals. A new paper by Velho et al. (2) in this issue of Proceedings of the National Academy of Sciences reports a fascinating discovery that provides an update to this theme. In this paper, the authors investigate the evolution of a receptor involved in cholesterol uptake by cells that is also exploited by the vesicular stomatitis virus (VSV) to enter cells during infection. They find that in songbirds—including the popular lab model, the zebra finch—this low-density lipoprotein receptor (LDLR) is missing several key protein coding regions that are found in many other avian groups as well as in mammals. This apparent case of evolutionary loss of function raises interesting questions about how songbirds manage to balance their cholesterol levels and avoid hypercholesterolemia (hence the title of this commentary). It also has important implications for a variety of other fields, ranging from efforts to use VSV-G pseudotyped lentiviruses as a tool to manipulate gene expression, to efforts to understand and control vesicular stomatitis, a major agricultural disease caused by VSV.

Not surprisingly, the path to this fascinating discovery was far from straightforward. Songbirds represent important model organisms for the study of learned vocal communication because they, like humans but unlike typical lab mice or rats, are robustly capable of vocal imitation (3). To date, however, the genetic tools for testing candidate genes for learning processes have lagged behind those available in mice and rats. To address this gap, researchers have strived over many years to optimize viral transduction in zebra finches to test the functional effects of experimentally altered gene expression levels. Early forays focused on testing VSV-pseudotyped lentiviruses due to their high success rate in mammals and some birds including quail and chickens (neither of which are songbirds). Puzzlingly, the same high titer, clean lentiviral preparations that gave beautiful infectivity in quail, mouse brain or chicken egg yielded abysmally low infectivity in zebra finch tissues, a conundrum for the field, including the authors of this study (2).

In 2013, Finkelshtein et al. (4) provided a clue to the mystery by identifying the highly ubiquitous low-density lipoprotein receptor (LDLR) as the entry portal for these viruses in human and mouse cells. While the Covid-19 virus gains entry to host cells via its spike protein (whose coding regions provide the sequence for the mRNA vaccines), VSV entry is mediated by the virus’s coat glycoprotein, hence its VSV-G moniker. This clue prompted Velho and colleagues (2) to search for the gene encoding LDLR in the zebra finch genome. Early searches yielded no hits and it was not until the most recent zebra finch genome was assembled by the international Genome 10K Consortium (5) that the gene was found. It turns out that LDLR lies in a difficult region to sequence and assemble, with lots of repetitive sequence and very high GC content. Only with improved sequencing and updated assembly algorithms were the gene and surrounding regions located.

While the full LDLR gene appears to be present across most branches of the avian phylogeny, startlingly, the zebra finch and four other oscine songbirds (another finch, one tit, one thrush, and one crow) lack three key structural protein domains, including one known as CR2 that is

exploited by the VSV coat protein for gaining entry into cells (6). This domain loss could plausibly explain the low infectivity of VSV-G pseudotyped lentivirus in zebra finch tissue. To flesh (or fluff) out their bioinformatic inference, Velho et al. (2) conducted clever functional tests using embryonic cells and fibroblast cultures. At laying, the chicken egg contains ~60,000 cells of which ~50 contribute to primordial germ cells (7). Early efforts to transduce this germ line had success rates akin to finding a needle in a haystack (7). Recent developments that isolate primordial germ cells from blood and then culture them have enabled lentiviral-driven genetic manipulation and selection with transformative success (8, 9). Given the new insights into zebra finch LDLR, the efficiency of this process might be improved by differentially pseudo-typing the virus, to exploit more conserved entry portals.

Here, Velho et al. (2) conducted a side-by-side comparison of the ability of VSV-G lentivirus to transduce embryonic cells in freshly laid eggs as well as cultured embryonic fibroblasts from chickens and zebra finches. As expected, transduction rates differed dramatically with robust transgene expression of green fluorescent protein (GFP) in chicken embryos and fibroblasts. In striking contrast, a zebra finch embryo appears as a darkened silhouette against a background of glowing egg albumen and virtually no fluorescence is apparent in the zebra finch fibroblasts. The authors then went one step further to bring their chickens home to roost. They transduced the fibroblasts with a human version of LDLR. This manipulation boosted GFP expression 10-fold in the zebra finch cells whereas the high fluorescence previously seen in chicken cultures was unaltered, supporting the author's inference that the lost gene regions were inhibiting functionality. This finding solves the puzzle of the low neuronal infectivity of VSV-G pseudotyped lentiviruses when injected into zebra finch brain (10) and the relatively rare success in using them for songbird transgenics (11).

The discovery of an apparently non-functional LDLR receptors in songbirds then led Velho et al. (2) to ask yet another intriguing question: how are songbirds managing their levels of low-density cholesterol? In most vertebrates, LDLR internalizes circulating low- and very low-density lipoproteins carried by apolipoprotein E for subsequent degradation (12). Impaired LDLR function in humans results in large increases in low-density lipoproteins and is associated with severe hypercholesterolemia (13). Velho et al. (2) noticed that another protein-coding domain missing from the songbird LDLR gene is critical for receptor folding and trafficking. This raised the question of how lipid transport systems of zebra finches and other songbirds have adapted to maintain their cardiovascular health. To begin to address this second puzzle, the authors measured cholesterol and triglyceride levels in blood samples from adult male zebra finches, with remarkable findings. For we humans, maintaining a balance between our levels of HDL and LDL is a key feature of metabolic homeostasis, and a typical visit to the doctor will include a conversation about our ratio of the "good" cholesterol (HDL) to the "bad" cholesterol (LDL). When Velho et al. (2) examined levels of cholesterol and triglycerides in the serum of both chickens and zebra finches, they found a striking contrast between the two species. While chickens had a balance of HDL and LDL lipid fractions similar to that found in humans, zebra finches almost entirely lacked the LDL fraction. Instead, virtually all of both the cholesterol and triglycerides were carried in the HDL fraction, an unprecedented pattern. This finding raises a flock of questions about songbird metabolism functions without LDL and how they apparently evolved to fly right by hypercholesterolemia.

The fact that songbirds apparently lack a fully functional LDLR and are resistant to infection by VSV has intriguing implications in fields ranging from disease ecology to neurobiology to genome evolution. Vesicular stomatitis, a disease caused by VSV, is a major agricultural malady afflicting primarily horses and cattle whose basic life cycle and epidemiology is poorly understood. It is endemic to southern Mexico, Central America and northern South America, but occasionally erupts into the western United States, resulting in quarantines that cause severe economic disruption to livestock producers (14). As disease ecologists seek to understand how a disease that is apparently vectored by small blood-feeding insects like blackflies can sweep so rapidly across the entirety of Mexico, interest has focused on a potential role of migratory vertebrates as intermediary hosts (15) The fact that songbirds are apparently resistant to infection by VSV

suggests that migratory songbirds should be low on the list of suspects. It also raises the interesting chicken-and-egg question of whether the loss of functionality of LDLR was driven by the selective pressure of lentivirus infection or was a serendipitous side effect.

One longstanding challenge for songbird researchers has been the lack of tools for transgenic experiments. As described above, many studies have used direct injections of VSV pseudotyped lentiviruses (16) containing relevant transgenes into the vocal control circuitry of zebra finches, and have reported both altered gene expression and behavioral changes. Given what we now know about the zebra finch LDLR, how did neural transduction and the resultant behavioral effects reported in these studies happen? While the canonical mammalian LDLR is missing, other members of the low-density lipoprotein receptor family are expressed in zebra finch tissue and could provide an alternative entry portal to cells (9). In addition to hijacking receptors, many viruses have generalized entry mechanisms. Determining the details for different cell types and tissues, in addition to different species, will provide insight into the biological arms-race between viruses and their hosts.

Many questions remain outstanding regarding the major genome changes discovered by Velho et al. (2). Is the loss of the three LDLR exons, and the apparent absence of LDL cholesterol, shared by all songbirds? What function, if any, does this newly discovered LDLR form have in songbirds? Is it restricted to the songbird lineage or might it be shared with other branches of the avian tree? Has the apparent loss of function of the canonical LDLR in songbirds been compensated for by the gain of new functionality encoded elsewhere in the genome? This question is especially intriguing because in addition to its roles in metabolism and viral entry described above, the LDLR is also involved in steroid hormone synthesis in many vertebrates (17). And finally, does this loss of the LDLR exons bear any relationship to the evolution of vocal learning in the songbird lineage? The answer to these questions will require improved sequencing and assembly of additional avian genomes, a goal that the Vertebrate Genomes Project has set out to accomplish for all ~70,000 extant vertebrate species (5). Based on those already available, the canonical LDLR form is present in both non-vocal learners such as chickens and quails and vocal learners like the parrots and hummingbirds (2), suggesting there may not be a general connection with the evolution of vocal learning. It is clear, though, that while many of us 'chickens' may eventually suffer the ill effects of our so-called Covid 19 pounds gained during the pandemic, songbirds at least have managed to out-fox hypercholesterolemia.

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