The value of comparative animal research: Krogh's principle facilitates scientific discoveries.

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Abstract

Biomedical research is dominated by relatively few animal models. Research has over-relied on these models due to their well-described genomes, genomic manipulations and short generation times. However, recent advances in large scale molecular sequencing experiments have revealed, in some cases, the limited similarities in experimental outcomes observed in common rodents (i.e. mice) compared to humans. The value of more varied comparative animal models includes examples such as long-term body weight regulation in seasonally breeding hamsters as a means to help understand the obesity epidemic, vocal learning in songbirds to illuminate language acquisition and maintenance, and reproduction in cichlid fish to discover novel genes conserved in humans. Studying brain peptides in prairie voles and cichlids advanced knowledge about social behavior. Taken together, experiments on diverse animal species highlight non-traditional systems for advancing our understanding of human health and well-being.

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Introduction

During Charles Darwin's travels on the Beagle, he conversed with a Spanish lawyer and a German naturalist, Renous. Renous asked the Spanish lawyer '*what he thought of the King of England sending out a collector to their country, to pick up lizards and beetles, and to break stones?* To which the Spanish lawyer replied '*No man is so rich as to send out people to pick up such rubbish*' (Darwin, 1839). Investment in basic research has a long and distinguished tradition, and we are fortunate that governments continue to provide the essential financial support. Every year, nations throughout the world invest substantial amounts into basic and biomedical research with an aim of generating long-term translational outcomes that will benefit humankind. From 2000-2014, research spending adjusted for purchasing power has increased approximately 64% in the United States (~$290 to $450 billion), 57% in the European Union overall (~$200 to $350 billion) and 66% in the United Kingdom (~$20 to $30 billion Research Council United Kingdom) (van Noorden, 2016). One of the key indicators of successful returns on research investment is the number of scientific publications. As evidenced by the MEDLINE database, the United States, European Union and the United Kingdom RUK contribute to approximately 32%, 26% and 8% of the world's scientific advancement through publications, respectively (van Noorden, 2016). These patterns highlight a healthy balance between government expenditure and major scientific advances that in turn facilitate innovative technologies and knowledge that benefits human health and wellbeing.

The majority of biomedical research seeks to enhance knowledge at basic mechanistic and applied translational levels to better inform healthy and pathological conditions in humans. However, our methods under-use animal models to understand health and disease and to develop medicines. Most animals in biomedical research are mice and rats. Comparing the

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proportion of human publications in 2016 for a range of animal models shows that publications using mice accounted for approximately 25% compared to human research. The summed percentage of the non-traditional animals accounted for less than 2%, illustrating a massively disproportionate amount of research funding on mice models. Yet, 'non-traditional' animal models— hamsters, songbirds, voles, and cichlid fish—have aided discoveries. These non-traditional biomedical species offer specialized, adapted genomic, physiological, immunological and behavioral traits. Comparing these traits across animals,helps identify the common underlying causes of many human conditions (see also Remage-Healey et al., 2017). Here we provide novel evidence to show that comparative animal studies yield major scientific advancements.

Biomedical models for scientific research

Granted the exponential increase in publications using 'human models' from 1968 to 2016 in PUBMED, the two most common biomedical research animal models include mice and rats. The ability to generate genetically modified mice in the 1990s ushered in a new era of research that identified the functional significance of specific genes. Consider the traditionally female hormone estogen: generating mice with a deleted estrogen-receptor gene illustrated how estrogen controls reproductive health in both females *and* males (Rissman et al., 1997). Subsequently, the capability to delete specific genes selectively in mice has increased the rate of using this model species.

However, mice are a limited model species to understand human function, so some findings do not translate well to human research, particularly in immunology (Bolker, 2012; de Souze, 2013; Drake, 2013). The comparative approach—going beyond mice, rats, and human—

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can identify commonalities and differences across organisms. Unfortunately, scientific funding is substantially lower for non-traditional animal species.

Krogh's Principle and the comparative approach for biomedical animal models

August Krogh was a Danish physiologist awarded the Nobel Prize in Physiology or Medicine in 1920 for his discovery of the circulatory systems' ability to carry oxygen to muscles. In addition, Krogh proposed that '*For a large number of [scientific] problems there will be some animal of choice or a few such animals on which it can be most conveniently studied … we must apply to the zoologists to find them*' (Krogh, 1929). As noted, mice have contributed to the major scientific advancements in the last twenty years. However, as Krogh advised, many human conditions—physiological, immunological, neural, and behavioral— are best studied in an alternative model. Despite the comparative approach's advantages to understand fundamental biomedical science or many pathological or disease conditions, research continues to over-rely on mice (Beach, 1950). Substantial genomic developments such as genome-sequencing (Koboldt et al., 2013) and genome-editing (Lee et al., 2016) now allow respectively sequencing whole genomes in a matter of days and conducting precise genomic manipulations. These two tools permit genomic analyses in a range of mammals, birds, and fish.

Below, four representative animal species illustrate physiology, immunology or behaviors different from other common biomedical models (i.e. mice) but facilitate our understanding of the human condition using Krogh's principle. The comparative perspective below generates fundamental biological knowledge that can complement other biomedical models to provide a comprehensive understanding for human health and disease conditions.

Brain and hormonal control of long-term body weight regulation in hamsters.

The World Obesity Federation estimates the prevalence of obesity is approximately 35-40% of the United States population. Healthy body weight regulation involves a complex interplay between behavioral (i.e. diet), physiological, and neural pathways (Yeo & Heisler, 2012). Most research has investigated short-term regulation, balancing food intake and energy expenditure. Specific neuropeptides (e.g. agouti-related peptide, neuropeptide Y) signal energetic states: either low (undernourished) or high (over-fed) body-weight conditions. These systems determine short-term timing of meal intervals and compensatory responses to acute energy insufficiency, so genetic manipulation of such pathways often produces a clear phenotype. However, problems of healthy body weight maintenance extend beyond this well-defined neural system. Long-term hypothalamic mechanisms help regulate body weight (Ebling, 2015).

Seasonal animals provide a valuable opportunity to examine naturally occurring genomic, physiological, and behavioral changes with major translational implications for humans (Ebling, 2014; Morgan et al., 2006; Stevenson et al., 2015). For example, the Siberian hamster (*Phodopus sungorus*) has been a valuable model to study long-term changes in physiological systems, such as losing body weight (Stevenson & Prendergast, 2013) and immune function (Stevenson et al., 2014). Hamsters will show a reliable, robust, and repeatable, cycle in body weight. In the laboratory, long days similar to the summer maintain 'obese' hamsters. A simple change in the amount of light especially those that mimic short-winter days induces roughly 30% weight loss (Stevenson & Prendergast, 2015). The decrease in body weight represents a long-term change in homeostatic control of energy balance. Unfortunately, the common mouse models have lost the photoperiodic change in body weight and are therefore, not suitable for examining long-term body weight regulation. In the hamster brain, there is a discrete population of cells referred to as

tanycytes that are localized along the 3rd ventricle in the brain. These cells have been proposed to control the long-term changes in hamster body weight (Lewis and Ebling, 2017). In mice, tanycytes have been shown to be critical for the detection of plasma glucose, a strong measure of energetic state (Orellana et al. 2012). The prevailing idea is that the metabolic state of the animal is detected by tanycytes (Bolborea and Dale 2013), and then control body weight through a well-defined neuropeptide circuit (Yeo & Heisler, 2012). However, experiments conducted in mice predominantly examine the acute, short term impacts of diet on body weight regulation. The major advantage of the hamster model is the demonstration that long-term neuro-morphological changes by tanycytes that in turn govern key brain regions involved in energy balance. The current evidence indicates that the locally produced thyroid hormone, triiodothyronine, in tanycytes controls long-term changes in body weight (Murphy et al., 2012). How the environment and diet impacts the ability of tanycytes to produce triiodothyronine and the subsequent impact on the short-term brain circuits is poorly understood. A greater understanding of the role of thyroid hormone action in the hamster tanycytes will better inform how obesity is maintained over longer time scales in humans.

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Songbirds enhance our understanding of innately programmed learned behavior: language.

The National Institute on Deafness and Other Communication Disorders reports 6 to 8 million people in the United States have language impairments (NIDCD, 2016). These include: stutters, spasmodic dysphonia and autism. The majority of disorders develop during childhood or adolescence and show remarkable sex-biases (Bale et al., 2010). Research into the underlying genetic, molecular, and neural control of language is hindered by the lack of animals that learn the species vocalizations. Songbirds, and in particular the zebra finch, represent the model

system that has led to insights about the neurobiological control of learned vocalizations. A series of elegant experiments during the 1980s-1990s demonstrated that discrete brain regions are necessary for songbirds to learn their songs (Fee & Scharff, 2010; reviewed in Alvarez-Buylla et al., 1992). The two main brain regions are essential for producing songbird vocalization (hyperpallium, i.e. HVC, and caudal medial pallium; Bolhuis & Moorman, 2015). These two regions have functional analogy to areas in the human brain implicated in language (Broca's and Wernicke's areas), especially acquisition of language in infants and maintenance in adulthood (Bolhuis & Moorman, 2015).

Many songbirds, including the zebra finch, show a substantial neurogenesis in the adult brain, that is, new neurons born and recruited to HVC (Alvarez-Buylla et al., 1992; Alward et al., 2014). Locally produced hormones in the pallium (e.g. HVC) improve the perception of the species' own song (Remage-Healey et al., 2013) and improve the birds' ability to produce a high quality song (Alward et al., 2013, 2016; Rouse et al., 2015). The technological development of high-throughput analyses (such as microarray assays) has permitted the identification of large changes in gene expression in the songbird brain that are likely involved in vocal quality, a feature similar to how well humans produce speech (Replogle et al., 2008). These brain-derived hormones are likely regulating gene expression in brain regions involved in songbird vocalizations (Stevenson et al., 2012a). How the brain generates these new neurons, how the new neurons are recruited into HVC, and what triggers the functional outcome of these new neurons for songbird vocalizations remains unsolved. A deeper knowledge about these basic questions would aid treatment of language disorders in humans.

Elucidating the neural mechanisms of social behavior using the prairie vole

In the past few decades, prairie voles have become a valuable animal for understanding the complex brain networks that control social behavior (Insel & Shapiro, 1992; McGraw & Young, 2010). Prairie voles have received substantial attention, largely based on (1) a rich literature on their natural history and behavioral ecology, (2) leveraging tools developed in classic rodent models, due to their close genetic relationship, and (3) rare but defining behaviors shared with humans but absent in common biomedical models (e.g. mice), such as communal living, social monogamy, and biparental care (Carter, 1998; Lukas & Clutton-Brock, 2012; McGraw & Young, 2010). In this respect, prairie voles illustrate the Krogh Principle: for this species, general knowledge bridging behavioral ecology and neurobiology can expand. Moreover, this species is well suited to serve as a model for several aspects of human social behavior and dysfunction (Carter 2007; Young 2001).

By far, the most commonly studied form of social behavior in prairie voles is pair bonding. Comparative studies in the 1990s made the remarkable discovery that the neuropeptides, vasopressin and oxytocin, are critical for monogamous relationships (Carter et al. 1995). These peptides were identified in numerous brain regions now known as critical for social behavior and characterized as a 'pair bonding neural circuit' (Carter et al., 1997; Insel & Young, 2001; Young &Wang, 2004). Recent technological advances in molecular biology (e.g., epigenetic and optogenetic techniques) have expanded our understanding of the neural circuitry of reproductive decisions in prairie voles in a manner unavailable in common biomedical models (Amadei et al., 2017).

The value of prairie voles extends well beyond the study of pair bonding, and this species offers opportunities to address other pressing issues in behavioral neuroscience and general biology. For example, voles have been valuable to study biparental care (Kelly et al. 2017; Bales

et al., 2007, Wang and Novak, 1994; Prounis et al., 2015; Hammock, 2015), aggression (Gobrogge and Wang 2011), social recognition (Blocker & Ophir 2015; Zheng et al., 2013), neurogenomics of sociosexual behavior (McGraw et al., 2012), non-sexual relationships (Beery & Zucker, 2010), emotional regulation of the cardiovascular system and mind-heart interactions (Grippo et al., 2012), fMRI analysis of brain activity in awake animals (Yee et al., 2016), and reward and addiction (Aragona et al. 2007; Ryabinin & Hostetler, 2016). Recently, prairie voles have also emerged as a promising system for studies that integrate the role of cognition (i.e., learning and memory) in mating systems to provide a more comprehensive understanding of the suite of factors that drive reproductive strategies and social-decision making (Ophir et al. 2008; Ophir 2017; Rice et al. 2017; Okhovat et al. 2015; Phelps and Ophir 2009). Thus, prairie voles represent a species with numerous uses in basic and translational research. With a fully sequenced genome (McGraw & Young, 2010) and an established network of researchers interested in understanding its behavioral ecology, development, neurobiology, and molecular genetics, prairie voles hold extraordinary potential to address questions focused on the neural and genetic basis of social behavior.

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Cichlid fish species facilitate neurobiological discoveries of plasticity in social behavior

Behavior shaped by natural selection overwhelmingly results from selective pressures on social interactions. For example, group-living animals, including humans, form dominance hierarchies with obligatory social interactions. Nevertheless, most animal experiments are done on single individuals, typically in asocial, non-natural conditions. This is partly because keeping rat and mouse model colonies and observing them during their normal nocturnal activities is hard, expensive, and impractical.

In contrast, fish offer unusual opportunities for understanding social behavior, its mechanistic underpinnings, and its role in reproduction. Cichlid fish that have evolved in the African rift lakes comprise more than 2000 species, evolving a broad range of social systems including female, male, or bi-parental care; monogamous pairs with helpers; and polygamous harems with helpers (Awata et al., 2005). Because cichlids offer experimental access at several levels of biological organization, their analysis has uncovered many widely conserved neural, physiological, and molecular mechanisms (White et al., 1998; Fernald, 2006; Ma et al., 2015; Maruska and Fernald, 2014).

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Here we describe research on a well-studied African cichlid fish, *Astatotilapia burtoni*, from Lake Tanganyika, east Africa. *A. burtoni* males in nature congregate around food sources and are either dominant or non-dominant. Dominant males actively defend territories, court, and reproduce with females, while non-dominant males look like the females that they mimic, do not reproduce, and school together (Fernald & Hirata, 1977a). Crucially, these naturally occurring and reversible behavioral phenotypes can easily be observed in the laboratory, allowing experiments designed to answer questions at multiple biological levels.

Gonadotropin-releasing hormone (GnRH) is essential for regulating reproduction (Fernald & White, 1999; Stevenson et al., 2012b). Release of GnRH from the brain's hypothalamus controls the pituitary's production of gonadotropins responsible for gonadal (sex organ) development in all vertebrates; this process has been highly conserved during 500 million years of vertebrate evolution. Using *A. burtoni* allowed the first cloning of the gene controlling this peptide in non-mammalian vertebrates (White et al., 1995), revealing not one but three genes. This discovery in fish then facilitated the identification of a second GnRH gene in humans (White et al., 1998) and other mammals (Kasten et al., 1996). What is particularly striking in this

fish species are the observations that 1) GnRH containing neurons increase 8X in volume when an animal becomes dominant (Francis et al., 1993), 2) GnRH neurons are interconnected and fire synchronously in response to social ascent (Ma et al., 2015), 3) the GnRH production is regulated by social status (Soma et al., 1996), and 4) that males reared with adults show delayed maturation relative to those reared without adults (Davis & Fernald, 1990). The availability of new genetic techniques has enhanced the roles of non-traditional fish model systems for discoveries with direct relevance to human health and wellness (Juntti et al., 2013).

An individual's social status is important because the amount of GnRH in key brain regions is associated with the dominance rank, which is typically established via physical fights. Dominance is maintained through social signals including postures that demonstrate size or show teeth (Huntingford & Turner, 1987). In *A. burtoni*, non-dominant males attend closely to dominant males, demonstrating that they anticipate movements of dominant males (Desjardins et al., 2012). But what do these animals know about their environment? Is it possible for cichlids to recognize the relative strength of other fish? In the field, colonies of *A. burtoni* range in size from a few dozen to hundreds (Fernald & Hirata, 1977) meaning that they could use a strategy of fighting with every male in the colony to identify one they could beat. However, by watching other male-male interactions, cichlids can infer their chances of winning a fight by viewing (i.e. 'by-stander') pairwise fights of other fish (Grosenick et al., 2007). This skill, known as transitive inference, is a form of deductive reasoning that allows inference of a relationship among items that have not been explicitly compared. Piaget (1928) described this as a key milestone in the development of human infants older than 3 years, and it has also been described for non-human primates (Rapp et al., 1996), rats (Roberts & Phelps, 1994) and birds (Bond et al., 2003).

Social information causes profound genomic, neural, physiological, and behavioral responses in *A. burtoni* (reviewed in Fernald, 2012; Maruska, 2015). When non-dominant males have an opportunity to ascend in social rank, expression of the immediate early gene *egr-1* is upregulated within the POA, a critical hub within the social behavior network that mediates adaptive behavioral responses. Moreover, expression of gonadotropins and their receptors are higher in dominant males and increased in ascending males (Maruska et al., 2011). Within 30 minutes of social opportunity, expression levels of sex steroid hormones and their cognate receptors in the brain and gonads also increase (Maruska et al., 2013), as ascending males begin showing high levels of aggressive and reproductive behavior during social opportunity.

Many causal molecular mechanisms of *A. burtoni* behavior are unknown, but advances in genetic techniques have the potential to reveal new discoveries. For instance, recent work with gene editing technology (CRISPR-Cas9) identified a critical role of a receptor for one hormone (prostaglandin $F_{2\alpha}$) in regulating reproductive behavior in female *A. burtoni* (Juntti et al., 2016). New techniques have started a new era of animal research, allowing social behavior scientists to use diverse social systems present among many different taxa to understand the evolutionary trajectories of social behavior.

Conclusions and future directions

This paper highlights the comparative approach in animal experimentation to better understand human health and diseases. Common biomedical models, particularly mice and rats, are well-established systems that have made significant gains in basic and translational research for the benefit of human health and wellness. However, in some cases highlighted here, other specialized physiological responses and social behaviors produced in non-traditional animal models (i.e. hamsters, songbirds, voles, and cichlids) are more suited for scientific investigation.

Given the remarkable technological advances, such as whole genome sequencing and genome editing, the tools that were once the sole realm of mouse models can be readily applied to a range of animals. To successfully apply Krogh's principle, large-scale funders need to incorporate strategic priorities that focus on the scientific gains afforded by the comparative approach.

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