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SYMPOSIUM

Breaking Through the Bottleneck: Krogh's Principle in Behavioral Neuroendocrinology and the Potential of Gene Editing

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Synopsis In 1929, August Krogh wrote that for every question in biology, there is a species or collection of species in which pursuing such questions is the most appropriate for achieving the deepest insights. Referred to as "Krogh's Principle," these words are a guiding force for many biologists. In practice, Krogh's principle might guide a biologist interested in studying bi-parental care to choose not to use lab mice, in which the female does most of the parenting, but instead study species in which bi-parental care is present and clearly observable, such as in certain poison dart frogs. This approach to pursuing biological questions has been fruitful, with more in-depth insights achievable with new technologies. However, up until recently, an important limitation of Krogh's principle for biologists interested in the functions of certain genes, was certain techniques were only available for a few traditional model organisms such as lab mice, fruit flies (*Drosophila melanogaster*), zebrafish (*Danio rerio*) and *C. elegans* (*Caenorhabditis elegans)* , in which testing the functions of molecular systems on biological processes can be achieved using genetic knockout (KO) and transgenic technology. These methods are typically more precise than other approaches (e.g., pharmacology) commonly used in nontraditional model organisms to address similar questions. Therefore, some of the most in-depth insights into our understanding of the molecular control of these mechanisms have come from a small number of genetically tractable species. Recent advances in gene editing technology such as CRISPR (Clustered Regularly Interspersed Short Palindromic Repeats)/Cas9 gene editing as a laboratory tool has changed the insights achievable for biologists applying Krogh's principle. In this review, we will provide a brief summary on how some researchers of nontraditional model organisms have been able to achieve different levels of experimental precision with limited genetic tractability in their non-traditional model organism in the field of behavioral neuroendocrinology, a field in which understanding tissue and brain-region specific actions of molecules of interest has been a major goal. Then, we will highlight the exciting potential of Krogh's principle using discoveries made in a popular model species of social behavior, the African cichlid fish *Astatotilapia burtoni*. Specifically, we will focus on insights gained from studies of the control of social status by sex steroid hormones (androgens and estrogens) in *A. burtoni* that originated during field observations during the 1970s, and have recently culminated in novel insights from CRISPR/Cas9 gene editing in laboratory studies. Our review highlighting discoveries in *A. burtoni* may function as a roadmap for others using Krogh's principle aiming to incorporate gene editing into their research program. Gene editing is thus a powerful complimentary laboratory tool researchers can use to yield novel insights into understanding the molecular mechanisms of physiology and behavior in non-traditional model organisms.

Introduction

August Krogh proposed that for every question in biology there is a species for which research investigations are best suited for revealing the most precise, informative answers (Krogh 1929). The potential utility of Krogh's principle has reached new heights in the past decade, with new genome sequencing technologies and other genetic technological advancements for

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studying diverse organisms that are suited for particular questions in biology (Stevenson et al. 2018). For instance, one need not attempt to study biparental care in mice for which care is given mostly by the female; instead, these questions may be more well-suited for investigations in biparental poison frogs (Dulac et al. 2014). Krogh's principle has some limitations when it comes to certain biological questions, however. In traditional model organisms like lab mice, fruit flies (*Drosophila melanogaster*), zebrafish (*Danio rerio*), and *Caenorhabditis elegans*, testing the functions of molecular systems on biological processes can be achieved using genetic knockout (KO) and transgenic technology (Alward et al. 2023). These methods are typically more precise than other approaches commonly used in non-traditional model organisms to address similar questions, in which techniques like pharmacological or somatic genetic manipulations that have off-target effects are utilized (Bridge et al. 2003; Sledz et al. 2003; Knight and Shokat 2007; Alward et al. 2023). Therefore, while Krogh's principle has guided many researchers to achieve novel insights into fundamental biological mechanisms across species (Stevenson et al. 2018), some of the most in-depth insights into our understanding of the molecular control of these mechanisms have come from a small number of genetically tractable species.

Recent advances in gene editing technology, however, have begun to increase the functional molecular insights attainable using Krogh's principle. For example, the discovery of CRISPR (Clustered Regularly Interspersed Short Palindromic Repeats)/Cas9 gene editing and its subsequent application as a laboratory tool has increased the potential of non-traditional model organisms (Doudna and Charpentier 2014). In this review, we will provide a brief historical summary on how some researchers of nontraditional model organisms for decades have been able to achieve different levels of experimental precision with limited genetic tractability in their non-traditional model organism. Specifically, we will root our discussion in the field of behavioral neuroendocrinology, a field in which understanding tissue and brain-region specific actions of molecules of interest in the control of behavior has been a major goal. In behavior neuroendocrinology, experimental manipulations have ranged from the "general/broad" during early experiments to over time becoming highly precise in non-traditional model organisms. We will emphasize work done in a specific branch of behavioral neuroendocrinology, that of the role of steroid hormones on controlling behavior. After a brief discussion of the different approaches that have been employed in the study of behavioral neuroendocrinology, we will discuss how our lab species, the African cichlid fish *Astatotilapia burtoni*, is a powerful example of what can be achieved in terms of precise manipulations in behavioral neuroendocrinology during the gene editing era. We hope this review may function as a roadmap for others using Krogh's principle to perform experiments in non-traditional model organisms aiming to incorporate gene editing into their research program.

Bypassing limitations of genetic tractability: Examples from nontraditional model organisms in behavioral neuroendocrinology

Prior to the recent advances in gene editing technology as well as due to the limitations of genetic tractability in nontraditional model species, behavioral neuroendocrinology research utilized several other techniques such as pharmacological manipulations, site-specific regulation, and RNA-interference (RNAi) techniques to study molecular mechanisms of behavior. Here we will discuss how these techniques have yielded important insights into our understanding of the control of social behaviors by sex steroid hormones, but still suffer from certain limitations. Notably, the techniques we discuss are not exhaustive nor are they the only ones that have yielded the most impactful results in the field of behavioral neuroendocrinology. They were chosen to illustrate how a series of successively more precise methods yielded clearer insights into a phenomena of interest. For other reviews discussing other impactful and meaningful approaches in behavioral neuroendocrinology, please consult (Juntti et al. 2008; Bayless and Shah 2016; Juntti 2019; Thompson 2020; Ruiz-Ortiz and Tollkuhn 2021). Given the focus of this review, to ensure the relevance of the techniques and their implications are clear we will start by providing a brief background on how steroid hormones work.

Brief background on steroid hormones

Steroid hormones are lipophilic molecules that exert their effects by binding to membranous, cytoplasmic, or nuclear receptors (Pfaff et al. 2018). When bound by their ligand, steroid receptors modulate suites of cellular and physiological functions, such as homeostasis, growth and development, cellular signaling, sexual differentiation, and reproduction, as well as behavior. Steroid hormones can exert their effects through two mechanisms. The first, called the "genomic mechanism," occurs by the steroid diffusing across the cellular membrane, where it binds to its cognate cytoplasmic or nuclear receptor. For example, testosterone, an androgen, binds to the androgen receptor (AR). Once

bound, dimerization occurs, and this complex enters the nucleus where it modulates gene expression by binding to response elements in promoter regions of DNA. Another mechanism of action is considered "nongenomic," and occurs via the modulation of second messenger cascades or direct modification of membrane-bound ion channels. Notably, depending on the cascades that may occur following the activation of an ion channel or secondary messenger system, a "nongenomic" action of a steroid may eventually lead to alterations in gene expression indirectly (e.g., through a kinase dependent epigenetic modification). There are five major classes of steroid receptors—ARs, estrogen receptors (ERs), glucocorticoid receptors (GRs), mineralocorticoid receptors (MRs), and progesterone receptors (PRs)—that when bound by their ligand contribute to the modulation of homeostatic functions, physiology, and behavior. Steroid receptors are expressed throughout the body and in specific regions throughout the brain. This review focuses mostly on the sex steroid hormones androgens and estrogens and their receptors given their clear, strong links to the regulation of social behaviors.

Some steroids can be converted to metabolites that are more potent or serve a completely different function (Payne and Hales 2004). Testosterone can be converted through enzymatic reactions to both androgenic and estrogenic metabolites. The enzyme 5-α reductase converts testosterone to dihydrotestosterone or DHT, a more potent androgen found in most vertebrates; while the enzymes 11-hydroxysteroid dehydrogenase types 1 and 2 convert testosterone to 11-ketotestosterone (11-KT), an androgen considered fish-specific because it is present in very high levels in fish species compared to all other vertebrates (Tokarz et al. 2015). Aromatase converts testosterone to the estrogenic metabolite 17β-Estradiol (hereon referred to as estradiol) (Payne and Hales 2004; Tokarz et al. 2015). These enzymes are expressed in distinct tissues, including the brain, where specificity of function is achieved through precise region-specific and cellular-specific expression patterns (Forlano et al. 2006; Cornil et al. 2015; Cornil and de Bournonville 2018).

Peripheral and site-specific pharmacological manipulations

In neuroendocrinology research, castration, in which the testes are removed, has been used for decades to study the relationship between hormones and behavior in males as the gonads are a major source of sex steroid hormones. In rodents (Daan et al. 1975; David et al. 2022; Davidson 1966; Lofgren et al. 2012; Pasch et al. 2011; Vetter-O'Hagen and Spear 2012), birds (Alward et al. 2018; Ball et al. 2020; Balthazart et al. 1998; Nottebohm 1968), fish (Almeida et al. 2014; Mayer et al. 1990), lizards (Crews et al. 1978), and frogs (Wada and Gorbman 1977; Wetzel and Kelley 1983)castration typically causes a dramatic reduction in male-typical aggression and mating behaviors, which are restored in males treated with testosterone and/or its androgenic and estrogenic metabolites. Other experiments have used pharmacological manipulations targeting ARs and ERs, or aromatase, to gain deeper insight into the mechanisms through which androgens and estrogens control social behavior (Adkins-Regan 2005; Alward et al. 2023). ARs and ERs are expressed in numerous areas of the "social behavior network (SBN)," a network of highly conserved, interconnected brain regions that govern behavioral and physiological responses to the social environment (Goodson 2005; O'Connell and Hofmann 2011; O'Connell and Hofmann 2012). To increase the spatial specificity in determining the actions of steroid hormones, pharmacological agents for steroid signaling have been administered by a variety of research groups to specific sites of the SBN in the brain, across species, including mice (Lisciott et al. 1990; Nyby et al. 1992; Matochik et al. 1994; Sipos and Nyby 1998), rats (Christensen and Clemens 1974; Christensen and Clemens 1975; Antonio-Cabrera and Paredes 2014), hamsters (Takahashi and Lisk 1985; Sterner et al. 1992; Debold et al. 1989), birds (Balthazart and Surlemont 1990; Panzica et al. 1991; Riters et al. 1998; Alward et al. 2013; Alward et al. 2016; Alward et al. 2017), and lizards (Morgantaler and Crews 1978; Crews and Morgentaler 1979; Morrell et al. 1979; Wade and Crews 1991).For in-depth reviews highlighting the temporal and environmental dynamics of androgens and estrogens in the control of mating and aggression revealed through pharmacological manipulations see (Trainor et al. 2004; Balthazart and Ball 2006; Trainor et al. 2006; Trainor et al. 2008; Cornil et al. 2015; Heimovics et al. 2015; Alward et al. 2018).

Decades of work using pharmacological manipulations to better understand the role of steroid hormones in regulating social behaviors have yielded foundational insights. Nonetheless, unavoidable limitations of pharmacological approaches remained. For instance, pharmacological blockade of steroid receptors will never reach 100% efficiency and certain compensatory mechanisms may result. For example, flutamide, an AR antagonist, has been shown to increase AR expression as a compensatory response to treatment (Lai and Crews, 2017), further complicating interpretation of results. Moreover, peripheral pharmacological manipulations have a relatively low degree of molecular specificity due to potential off-target effects, limiting the degree to which precise hypotheses can be efficiently

tested (Knight and Shokat 2007). There are also species-specific limitations when it comes to using pharmacology to manipulate steroid receptors. For example, teleosts underwent a teleost-specific whole-genome duplication (TS-WGD) (Glasauer and Neuhauss 2014), which led to novel paralogs of genes encoding ARs, ERs, and aromatase. Therefore, while pharmacological interventions in teleost fishes have led to insights into the control of mating and aggression by androgens and estrogens (Godwin 2010; Munakata and Kobayashi 2010), they are less precise than findings in other vertebrates. Indeed, AR and ER agonists and antagonists cannot discriminate between novel teleost ARs and ERs, nor is it known if novel interactions occur between the paralogous genes that may be affected in response to pharmacological manipulations. Therefore, in addition to the issues of pharmacological interventions like off-target effects common to all vertebrates, interpreting results based on pharmacology in teleost fishes has special limitations that must be addressed by other methods.

RNAi techniques

Similar to the brain-region specific modulation of steroid actions using pharmacology, other studies have used genetic manipulations to affect steroid signaling in specific brain regions. The techniques we will discuss in this section involve genetic modifications in somatic cells; therefore, these changes are not heritable. This approach provided another level of spatial precision for manipulating steroid hormone mechanisms of behavior that could address the confounds of pharmacological approaches, as theoretically the manipulation should only affect the gene of interest. For example, Musatov et al used RNAi incorporating a viral mediated knockdown of ERα expression in the ventromedial hypothalamus (VMH) in female mice (Musatov et al. 2006). This manipulation reduced sexual behavior in females, highlighting a site-specific role of estrogen signaling in the VMH. Viral mediated RNAi of ERα in the POA or medial amygdala of pubertal male mice caused a reduction in both mating and aggressive behaviors (Sano et al. 2016). In adult male mice, viral mediated RNAi of ERα in three brain regions was performed and behaviors in male mice were investigated (Sano et al. 2013). Knock down of ERα in the VMH of these males reduced aggression and mating, while the same manipulation in the POA only reduced mating. Contrary to findings in pubertal mice, ERα knockdown in the medial amygdala of adults did not affect mating or aggression. These studies highlight how enhanced molecular and spatial specificity in disentangling a hormonal signaling system reveals precise, important insights relevant to our understanding of the control of social behavior. It is important to note, however, that RNAi approaches may cause unintended consequences, including an activation of interferon resposnes in the brain, confounding interpretation of experimental results (Bridge et al. 2003; Sledz et al. 2003).

In zebrafish (*Danio rerio*), similar knockdown studies have been performed using the "morpholino" (modified oligonucleotides) approach. Morpholinos are synthetic antisense oligonucleotides designed to block the translation initiation complex of target messenger RNA (mRNA) sequences (Bedell et al. 2011). This technology has been used to test the role of specific genes by transient blocking, particularly during development. In zebrafish, morpholino technology has been used to study the effects of certain genes throughout development and to model human genetic disorders (Nasevicius and Ekker 2000; Bedell et al. 2011). Morpholinos have also been used to study the role of steroid hormones in molecular and physiological processes in zebrafish (Hsu et al. 2006; Schmid et al. 2020), but to the best of our knowledge not behavior. Like site-directed pharmacological manipulations and other RNAi techniques, morpholinos may lack specificity and are capable of binding to other non-specific targets leading to additional phenotypes as a result (Bedell et al. 2011).

From lake to lab: *A. burtoni* provide in-depth insights into the hormonal control of social behavior

In the following sections we shift our focus to discuss *A. burtoni*. We will provide a general background of the species and how its social flexibility and overt behavioral and physiological changes have made it a premier organism in the study of behavioral neuroendocrinology. Furthermore, we discuss how gene editing has been used in *A. burtoni* to dissect more precisely the roles of novel ARs in the production of social behavior.

General background on *A. burtoni*

Astatotilapia burtoni are found in the wild in Lake Tanganyika off the coast of East Africa. One of the most striking observations of *A. burtoni* made during early field work was the coloration found among the males (Fernald and Hirata 1977a; Fernald and Hirata 1977b). Compared to female *A. burtoni*, which are drably colored, males are brightly colored in yellow, blue, or different combinations of these colors and possess a distinct black eye bar from the eye to their mouth (Fig. 1A). Most males in a community of these fish, however, do not possess these features of coloration (Fernald 2012). Indeed, the brightly colored males, which make up approximately 30% of a social environment, are territorial

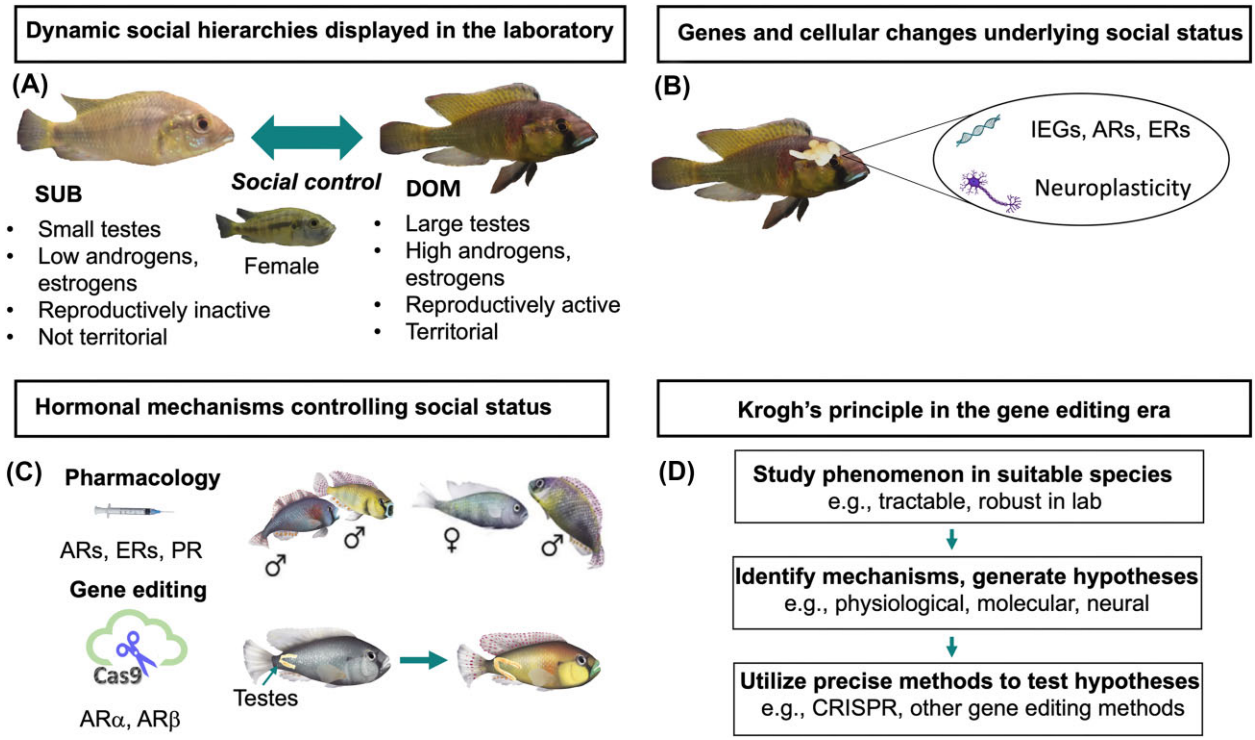

Fig. 1 Discoveries over decades of research in *A. burtoni* culminating in recent gene editing work illustrates the power of Krogh's Principle in the gene editing era. (A) The social hierarchies exhibited by male *A. burtoni* can be modeled in the laboratory. Studies from experiments in the lab yielded foundation information regarding the behavioral and physiological changes associated with social status in male *A. burtoni*. (B) With these foundational findings as a guide, experiments aimed at determining the molecular and neural mechanisms of social status in *A. burtoni* revealed specific neural activation (as measured by IEGs like egr1 and c-fos) and steroid receptor gene expression in the SBN may underlie recognition of social opportunity and changing social status. (C) The relationship between social status and steroid hormones led to pharmacology studies the revealed the importance of social status in determining the role of steroids. Because A. burtoni, like other teleosts, have novel AR paralogous genes, CRISPR/Cas9 was needed to reveal the precise roles of these receptors. AR mutant *A. burtoni* were instrumental in revealing the complex manner in which androgen signaling regulates social status in A. burtoni, with implications for understanding social status in other species. (D) Using the different phases of discoveries enhancing our understanding of the hormonal control of social status in *A. burtoni*, we propose a simple approach for applying Krogh's principle in the gene editing era. Acronyms: SUB: subordinate; DOM: dominant; IEGs: immediate early genes; ARs: androgen receptors; ERs: estrogen receptors; PR: progesterone receptor.

or dominant (DOM) males, while the remaining males are nonterritorial or subordinate (SUB) males. DOM males maintain their territories via physical aggression and nonphysical aggressive displays (Fig. 1A). Furthermore, these males perform a suite of reproductive behaviors like digging gravel or sand to create a mating site; and chasing and quivering at females that may lead to spawning. During spawning, the female releases the eggs into the water, puts them back into her mouth, and the male releases sperm to fertilize the eggs. The female carries the eggs in her mouth for about 2 weeks, until the larvae hatch, and she releases them from her mouth into the water. Therefore, female *A. burtoni* are called mouthbrooders.

SUB males do not display aggressive or reproductive behaviors; in fact, they tend to shoal with females (Fernald 2012; Maruska and Fernald 2013). However, neither SUB nor DOM social status is fixed in *A. burtoni*. SUB males observe their social environment waiting for an opportunity to attempt to become DOM. These opportunities arise when a larger DOM male is absent from the environment, leading to social ascent. During social ascent, a SUB male recognizes social opportunity and within minutes turns on DOM-typical bright colors and begins performing aggressive and reproductive behaviors. Social ascent can be controlled in the laboratory as well. In the social ascent paradigm, a suppressed focal male is housed with a larger suppressor male and three females in a central compartment. Flanking this compartment are identical social environments containing males and females smaller than the focal male. Social ascent is controlled by removing the larger suppressor male during the middle of the night so the focal suppressed male identifies this social opportunity and ascends. This approach has been used for nearly two decades to reliably induce social ascent in *A.*

burtoni (Burmeister et al. 2005; Fernald and Maruska 2012; Maruska and Fernald 2013; Maruska and Fernald 2018; Alward et al. 2019). Similarly, DOM males may become SUB through social descent, when in the same environment as a larger DOM male. Social descent appears to take longer than social ascent, but ultimately results in the male becoming SUB if a larger opponent appears (Maruska et al. 2013).

Discoveries on correlations between hormones, genes, the brain, and social status in *A. burtoni*

The complex social dynamics exhibited by *A. burtoni* made them especially appealing to experimental biologists. *Astatotilapia burtoni* are one of several species that exhibit robust social hierarchies that have caught the attention of researchers from fields such as biology, psychology, and neuroscience (Sapolsky 2005; Robinson et al. 2008; Fernald 2012; Fernald and Maruska 2012; Hofmann et al. 2014). Studying social hierarchies is important for understanding principles of flexible phenotypes but also for understanding the impacts of social rank on mental and physical health in humans and nonhuman animals (Sapolsky 2005). *Astatotilapia burtoni* were excellent for studying social hierarchies for a variety of reasons (Fernald 2012; Fernald and Maruska 2012; Fernald 2014; Maruska and Fernald 2018). For instance, seminaturalistic environments in the laboratory replicating their water chemistry, ambient temperature, photoperiod, and social environments can be set up readily using well-established aquatic housing protocols (Fernald 2012; Stevenson et al. 2018). In these environments the males quickly organize into social hierarchies, females participate in shoals, and males and females readily mate. These characteristics have allowed researchers ease of access to the physiological, molecular, and neural mechanisms of social status (Juntti 2019; Alward et al. 2023).

This ease of access has led to numerous fundamental discoveries on the biological mechanisms of social status in *A. burtoni*. For instance, over decades of research it has been shown that DOM males possess larger testes with more viable sperm compared to SUB males (Maruska and Fernald 2011; Fernald 2012; Fernald and Maruska 2012; Maruska 2015). Moreover, DOM males have higher levels of circulating androgens—including testosterone and 11-ketotestosterone—estrogens, cortisol, and progesterone compared to SUB males (Maruska and Fernald 2013; Maruska 2015). Social status also changes the brain as shown by numerous studies. Indeed, DOM males have larger somatostatin and gonadotropin releasing hormone (GnRH)-1 neurons in the preoptic area (POA), a brain region critical for transducing social information into coordinated physiological and behavioral responses (Fernald 2012; Fernald and Maruska 2012). Gene expression also differs dramatically in DOM versus SUB males. DOM males have higher expression of ARα and ARβ throughout their telencephalon as measured by qPCR (Maruska and Fernald 2013; Maruska 2015). A similar pattern has been observed for ERα and ERβ and GRα and GRβ. Therefore, in addition to the differences in coloration and behavior between DOM and SUB males, they also differ along multiple dimensions in terms of hormone levels, neuronal morphology, and steroid receptor expression, suggesting that dramatic physiological and brain changes underlie alterations in social status due to varying social cues.

Social ascent is also typified by dynamic changes in many of the same physiological and brain changes described above, suggesting that some of the mechanisms underlying the shift toward dominance occur rapidly. Ascending males experience a large increase in testosterone, 11-KT, cortisol, and progesterone within 30 minutes of social opportunity (Maruska et al. 2013). Gene expression is also altered in ascending males in nearly every region of the SBN, a conserved network of brain regions involved in regulating social behavior. For instance, the expression of the immediate early genes *egr-1* and *c-fos,* markers of neuronal activity, is higher in the SBN of ascending males compared to DOM and SUB males (Maruska et al. 2013), suggesting suites of cell populations respond to social opportunity specifically. Steroid receptor expression also changes during social ascent. Ascending males had higher levels of expression of ARβ in the dorsal medial telencephalon (Dm), a putative medial amygdala homolog, and the supracommissural nucleus of the ventral telencephalon (Vs), a putative homolog of the extended amygdala that also contains the bed nucleus of the stria terminalis (BNST). Both of these brain regions are involved in coordinated responses to winning a fight (Fuxjager et al. 2010; Maruska et al. 2013; Maruska 2015), suggesting enhanced sensitivity to androgens may underlie the response to social opportunity and subsequent social ascent.

Other cellular changes in the brain occur during social ascent. For example, Maruska and colleagues injected DOM, SUB, and ascending males with bromodeoxyuridine (Brdu), a marker that incorporates into newly dividing cells in the brain as well as other tissues (Maruska et al. 2012). Males housed alone were also injected with Brdu. DOM, ascending, and lone males had higher cellular proliferation throughout the SBN compared to SUB males. These results suggest brain regions involved in social behavior are remodeled after social opportunity, and new cells are made continuously after stable dominance is achieved. Neither the

phenotypes nor functions of these new cells are known, but Maruska et al. showed that these new cells can become mature neurons (Maruska et al. 2012). Moreover, the time-course of when these cells incorporate into functional cell populations and/or neuronal circuitry is not known. Additionally, it is not clear what molecular mechanisms control cellular proliferation as a function of social opportunity and social status, but based on the strong links of social status to steroid hormone levels (Fernald 2012; Fernald and Maruska 2012; Maruska 2015), and the known roles of steroid hormones in governing cellular proliferation, neurogenesis, and neuroplasticity (McEwen et al. 1991; Galea and McEwen 1999; Gould and Gross 2002; Ormerod and Galea 2003; Galea 2007; Spritzer and Galea 2007; Opendak et al. 2016; Alward et al. 2018), they may play a causal role in *A. burtoni*. Overall, these results suggest changes in social status involve drastic changes in cellular composition in the brain that could govern the dramatic changes in physiology, morphology, and behavior associated with distinct social ranks.

Navigating social hierarchies is a complex cognitive process. Male *A. burtoni* possess remarkable abilities in processing their social environment as has been revealed through laboratory studies (Fernald 2014). For instance, it has been found that *A. burtoni* assess their relative size using transitive inference, where they make decisions to fight another male based on whether that male won a fight against a male that was larger than it, a skill that, at one time, many thought was reserved for specific mammalian species like primates (Grosenick et al. 2007). Male *A. burtoni* are also keenly aware of social opportunity and can flexibly make decisions over short timescales. For example, it was found that SUB males turn on their colors and perform DOM-typical behaviors in an assay where larger DOM males cannot physically attack them but are in their visual environment (Chen and Fernald 2011; Desjardins et al. 2012). Recent work also showed resident male *A. burtoni* perform highly specific suites of behaviors depending on very small relative size differences between them and intruder males (Alward et al. 2021). For instance, resident males that were > 5% smaller or matched (resident between 0 and 5% smaller or larger in standard length) in size to the intruder performed more lateral displays, a type of nonphysical aggression, than resident males that were 5% larger in standard length than the intruder. However, physical aggression, such as chases and bites, did not differ as a function of relative standard length. These findings overall highlight the cognitive skills displayed by *A. burtoni* for navigating their social environments. It is especially intriguing to consider the complex "social calculus" displayed by *A. burtoni* considering behaviors related to enhancing social status in humans are at least in part controlled by steroid hormone signaling (Eisenegger et al. 2011; Dreher et al. 2016; Qu et al. 2017). In the next section we will discuss the studies done in *A. burtoni* to elucidate the causal hormonal mechanisms that govern social status.

Hormonal mechanisms regulating social status in *A. burtoni*

The first study testing the role of hormones on behavior in *A. burtoni* was performed by Fernald (Fernald 1976). Guided by the well-known principle at the time that testosterone is required for male-typical aggression and mating behaviors (Alsum and Coy 1974; Owen et al. 1974; Phoenix 1974; Larsson et al. 1975; Wada 1984), they injected male *A. burtoni* with the synthetic testosterone analog, testosterone propionate, to see if it could induce DOM-typical behaviors. Fernald (Fernald 1976) found that males injected with testosterone displayed enhanced eye-bar intensity, body coloration, and male-directed attack frequency but no change in courtship behaviors. This study was the first to demonstrate that testosterone injections impacted specific dominance behaviors in *A. burtoni*.

The testosterone injection study by Fernald, combined with the discovery of the strong links in *A. burtoni* between social status, steroid hormone levels, and steroid receptor expression in the brain implicated steroid hormone signaling as playing a crucial role in governing social rank and the associated physiological and behavioral traits. Hence, in *A. burtoni* experiments using a variety of pharmacological approaches to tease apart the role of steroid hormone signaling on social status have been performed. O'Connell and Hofmann (O'Connell and Hofmann 2012) injected DOM or subordinate males housed in community tanks with different ER, AR, and PR agonists and antagonists, and measured effects on aggressive, mating, and submissive behaviors. Social status was not controlled experimentally but instead was confirmed by visual assessment over several observational periods in community tanks before fish were injected with one of the different pharmacological agents. Stimulation of ER with injections of estradiol caused an increase in aggression in both DOM and SUB males, while ER antagonism with injections of the drug ICI182780 caused an increase in aggression in SUB males only. Interestingly, in the SUB males, after a slight reduction in aggression following ER antagonism, the rate of aggression mirrored that of the SUB males given the ER agonist. This pattern was not seen in DOM males.

Males were also injected with AR agonists and antagonists. ARs were stimulated using the potent an-

drogen dihydrotestosterone (DHT), while they were blocked using the steroidal AR antagonist cyproterone acetate (CA). CA was used over drugs like nonsteroidal AR antagonists like flutamide because it is thought to bind both ARs equally, whereas flutamide may have higher affinity for ARβ, an important approach to use in teleosts possessing two distinct ARs (O'Connell and Hofmann 2012; Alward et al. 2023). In DOM males, DHT stimulated courtship directed at females while CA reduced this behavior; neither manipulation affected aggression. In SUB males, however, neither DHT or CA altered the performance of courtship or aggression. PR manipulations also affected distinct behaviors in DOM and SUB males. PR stimulation with dihydroprogesterone increased courtship behaviors but not aggression in DOM males, while the PR antagonist ZK0112993 reduced courtship but not aggression in DOM males. PR manipulation did not affect these behaviors in SUB males; however, PR stimulation reduced fleeing behaviors in subordinate males. A follow-up experiment to the work by O'Connell and Hofmann further supported that idea that estrogenic signaling controls aggression but not courtship. For example, male *A. burtoni* injected with fadrozole, an inhibitor of aromatase, the enzyme that converts testosterone to estradiol, displayed less aggression than controls while courtship behaviors were unchanged (Huffman et al. 2013). All together, these results highlight the complex way distinct steroid hormone signaling systems control social behaviors in *A. burtoni* in a status-specific manner.

Based on the findings from O'Connell and Hofmann, Alward and colleagues (Alward et al. 2019) hypothesized that AR activation was particularly important for social ascent in *A. burtoni*. For instance, since ARs do not alter courtship in subordinate males, but ER manipulation alters aggression in both DOM and subordinate males, an interaction between social opportunity and androgen signaling may be key for a rise from subordinate to DOM social status. To test this hypothesis, experimentally suppressed subordinate males were injected with CA before a social ascent paradigm.Males that were given vehicle injections before social opportunity exhibited all of the expected traits of social ascent after removal of the suppressor male, including increased eye-bar intensity and body coloration and elevated aggression and courtship behaviors (Alward et al. 2019). Males injected with CA showed all the above behaviors except an increase in the performance of courtship behaviors. Indeed, CA males were statistically indistinguishable from subordinate males in the amount of courtship behaviors they performed. These results provided further support for the role of androgens controlling courtship but not aggression, and supported the hypothesis that social opportunity plus androgen signaling is key for exhibiting all of the behavioral features associated with social ascent.

While many studies provided evidence that steroid hormones underlie social status in *A. burtoni*, investigating their precise causal roles has been a challenge due to the presence of novel genes related to steroid hormone signaling (Alward et al. 2023). Indeed, as mentioned above, genomic and phylogenetic evidence strongly supports the placement of a TS-WGD approximately 350 mya in the common ancestor of all teleosts (Brunet et al. 2006; Glasauer and Neuhauss 2014). Consequently, while all other vertebrates possess one AR encoded by a single gene, most teleosts possess two distinct ARs, ARα and ARβ, encoded by two separate genes, *ar1* and *ar2* (Alward et al. 2023). Teleosts also possess extra copies of ERs. Like all vertebrates, teleosts possess ERα and ERβ encoded by distinct genes *esr1* and *esr2*. However, in many teleosts an extra copy of ERα has been discovered, resulting in ERβ1 and ERβ2 as their designations with *esr2a* and *esr2b* as the corresponding gene names. As opposed to most vertebrates, many teleosts are known to possess an extra GR. GRα and GRβ are encoded by the distinct genes *nr3c1a* and *nr3c1b*. Teleost fishes only have one MR just like other vertebrates. The vast majority of teleosts follow the vertebrate typical pattern of possessing a single PR; however, evidence exists suggesting goldfish have two PRs encoded by two distinct genes. Highly conserved across teleosts, however, is the presence of two distinct aromatase genes, *cyp19a1* and *cyp19a1a*, contrasting with all other vertebrates, which only possess one. Therefore, pharmacological approaches to disentangle the role of steroid hormones in controlling social status in teleost fishes are limited, as it is not known which receptors or enzymes are affected and what their specific roles are. This is a clear, robust example of one of the long-standing limitations mentioned above concerning the applicability of Krogh's principle in nontraditional model organisms.

To determine the roles of steroid hormones more precisely in regulating social status, genetic manipulation technologies are thus necessary. In several teleost fishes, gene editing has been used to create mutants lacking functional AR, ER, GR, and aromatase (Muto et al. 2013; Ziv et al. 2013; Tohyama et al. 2017; Yin et al. 2017; Yong et al. 2017; Crowder et al. 2018; Yan et al. 2019). In the subsequent section, we will highlight how gene editing was recently used to achieve novel insights into the control of social status by androgen signaling in *A. burtoni*.

Gene editing in *A. burtoni* to disentangle the androgenic control of social status

Using CRISPR/Cas9 gene editing, *A. burtoni* possessing non-functional genes encoding ARα, ARβ, or both were generated. These mutants were used to assess the role of ARs in controlling aspects of social dominance in males. Specifically, males were housed in "dominance induction" tanks in which established social housing paradigms were used that are known to induce the full suite of dominance traits in males (Alward et al. 2019; Alward et al. 2020). Males were then assayed for key traits related to dominance including coloration, reproductive physiology, and social behavior (Alward et al. 2020). Males lacking functional ARα produced significantly fewer aggressive displays and mating behaviors compared to WT males but possessed DOM-typical bright coloration and large testes. However, ARβ mutants possessed extremely small testes and were drably colored, but performed DOM-typical levels of aggressive displays and mating behaviors. Interestingly, both AR mutants performed normal levels of physical attacks directed at other males. However, males lacking both ARs did not perform any physical attacks towards males while also performing no aggressive displays and lacking DOM-typical coloration.

Thus, the presence of novel steroid receptor genes in a socially rich organism, combined with its genetic tractability, allowed for a novel discovery that provided insights into the hormonal control of social status. The results also highlighted the complex mechanisms regulating social status, suggesting different traits that collectively make up social status may be controlled by independent, androgen-controlled signaling pathways, a hypothesis that can be explored further in *A. burtoni* and other species. Results from AR mutant *A. burtoni* are thus a flagship example of the importance of using complimentary pharmacological and gene editing methods to understand the role of steroid hormones in regulating social behaviors across species. These results highlight a clear example of how gene editing can lead to important insights and lay the foundation for unexpected, testable hypotheses.

Summary

Krogh's principle has undoubtedly led to many advances in our understanding of how complex biological mechanisms can lead to specific biological phenotypes (Stevenson et al. 2018). This is exemplified in *A. burtoni* as a model of social status. Due to the complex social dynamics present in *A. burtoni* that are known to be controlled by steroid signaling systems, *A. burtoni* are particularly well-suited to answer questions of how hormones control behavior. Understanding such biological mechanisms using a species that responds to their social environment in a socially plastic manner like *A. burtoni* combined with advanced genetic manipulations, can thus aid in the understanding of similar mechanisms across species. This highlights the utility of Krogh's principle in nontraditional model organisms in the gene editing era and raises the question of what other complex mechanisms could be studied in the future.

In this review, we gave a brief overview of *A. burtoni* and how its unique social dynamics along with its genetic tractability allowed it to become an excellent model organism in the study of social and behavioral neuroscience. From its humble beginnings shoaling in Lake Tanganyika to the current studies in gene editing, *A. burtoni* have enriched our understanding of the molecular and biological basis of behavior. We have summarized these discoveries in Fig. 1, where we show major steps in discoveries made in *A. burtoni* to ultimately provide novel insights into the hormonal control of social status that were revealed through gene editing techniques. We distill these steps down into a suggested pattern for applying Krogh's principle in the gene editing era.

For example, we showed how the social hierarchy displayed by *A. burtoni* males can aid in our understanding of androgenic-controlled behaviors like aggression and mating (Fig. 1A), as well as how changes in social status can trigger various physiological changes including levels of androgens and estrogens, changes in gene expression and cell proliferation, male body coloration, and behavioral expression (Fig. 1A and B). Furthermore, we discussed the experimental progression through which *A. burtoni* researchers discovered how different steroid hormones and their receptors contribute to the production of behavior, and what previous manipulations could still not explain (i.e., the complex mechanisms through which multiple receptors act on different steroids leading to a complete suite of behavior) (Fig. 1C).

Finally, we briefly touched on how the advent of CRISPR/Cas9 gene editing technology in *A. burtoni* has allowed us to gain novel insight into the more complex mechanisms of steroid hormone mediated behaviors by disentangling the roles of each of the AR paralogs present in *A. burtoni*. The results of these gene editing experiments, along with future studies will expand our understanding of how behavior is produced and the complex mechanisms that play a part in its production. Additionally, the continuous use of novel and understudied organisms to study behavioral and other biological questions, can only add to Krogh's principle that there *is* a specific species to study for the most precise answers to our questions. With this in mind, we

have written a simple, 3-step guide for applying Krogh's principle (Fig. 1D).

Where to from here?

For each new method for studying the molecular and neural control of behavior, it is not just precision that is gained, but typically a new, or more nuanced, interpretation of a function of a particular molecule, cell-type, and/or brain region. In behavioral neuroendocrinology, early studies in several species using castration and/or pharmacology revealed broadly the impacts of steroids on social behaviors. Site-specific brain manipulations provided a road map for where in the brain steroids regulated social behavior in a diversity of species. But when it comes to the highest degree of molecular, spatial, and temporal precision in interrogating these steroid hormone signaling systems, no nontraditional model organism currently comes close to what has been achieved in mice. For example, while much of the groundwork for where to look in the brain for where certain hormones act to control mating and aggression came from pharmacology studies across species, more recent studies in mice were able to achieve extremely high precision in answering these questions using state-of-the-art technology like optogenetics and chemogenetics (Lee et al. 2014; Unger et al. 2015; Yang et al. 2017). Importantly, optogenetics and chemogenetics have been both incorporated into the methodological toolkit of zebrafish (Portugues et al. 2013; Godoy et al. 2015). Therefore, hopefully, these tools can become a regular part of non-traditional model organism research programs to reveal new mechanisms of the hormonal control of behavior.

The genomic revolution and gene editing era has cast nontraditional model organisms in an encouraging new light. Species like *A. burtoni* are not just fascinating models for elucidating the role of the social environment on behavioral and neural plasticity; we can now interrogate the genetic underpinnings that control these traits in a functional, mechanistic framework. Where mice are excellent for certain behavioral and neural questions, *A. burtoni* and other cichlid species exhibit profoundly complex social dynamics that may allow for a richer understanding of species-specific patterns for the control of social behavior (Santos and Salzburger 2012; Brawand et al. 2014; Heule et al. 2014). The same goes for species like voles, hamsters, lizards, frogs, birds, and other species where novel behavioral and physiological traits provide opportunities for identifying the fundamental molecular and neural mechanisms of social behavior (Adkins-regan 1990; Phelps et al. 2010; Stevenson et al. 2018; Thompson 2020).

While recent insights and new technologies give reasons for optimism, the road to achieving a level of of genetic tractability found in mice may be long and bumpy. For instance, mice genetics has been highly commercialized: mutant or transgenic animals can be ordered from companies like Jackson Labs. Will this be the model that emerges for nontraditional laboratory species? The closest to this level may be that of zebrafish, where a strong group of collaborative researchers share resources extensively, including on established websites (e.g., zfin.org). Perhaps there are lessons to be learned from the zebrafish community in establishing core collaborative research networks. Indeed, the field of zebrafish neuroscience has flourished and the number of investigators studying numerous diverse questions in this species is relatively large. Research groups, collaborative networks, and conferences aimed at fostering the collaborative approach in different species in the gene editing era may ultimately lead to foundational discoveries. Taking similar actions across diverse species may help August Krogh's principle be realized in a manner he could not have imagined at the time.

We realize that while CRISPR/Cas9 gene editing technology is theoretically functional in a manner that is agnostic to species type, technical limitations remain. A common theme among species in which gene editing is challenging, is they are internal fertilizers or their embryos are extremely small, making it difficult to administer genetic constructs using standard approaches. Chaverra-Rodriguez et al (2018) aimed to address this challenge by developing a technology called Receptor-Mediated Ovary Transduction of Cargo (ReMOT Control), in which a conjugate based delivery system transports genetic payloads to vitellogenin-positive cells, allowing manipulated genes to be transmitted in the germline in technically challenging species. Methods like this will push fields aiming to study social behavior in diverse species further to make novel insights.

The result of approaching the study of steroids in the control of social behavior with a deliberate view towards establishing collaborative research groups and resource sharing could allow for significant benefits for all researchers in neuroendocrinology. The more resources that become widely accessible and researchers across fields are aware of new developments, research groups may be motivated to go more in-depth on their specialty and reach out to other groups when their questions become relevant to them. Gene editing has increased the potential of more precise insights into the regulation by steroid hormones of aggressive and mating behaviors across vertebrates. The studies done thus far have begun a new phase in comparative work in which novel genes involved in androgen and estrogen

signaling across distinct phylogenies can now be interrogated for their functions using gene editing techniques to gain unprecedented insights. It has also provided unique opportunities from a practical standpoint for numerous experimental queries that are difficult or impossible to answer in traditional model organisms like mice. Overall, advancements in technology and the continued curiosity of researchers in diverse organisms have opened up new opportunities for novel answers into old questions to achieve a new comparative synthesis in behavioral neuroendocrinology.

Conclusion

Krogh's principle has been a guiding principle for biologists for decades (Krogh 1929; Stevenson et al. 2018). Many have used this principle to achieve deep insights into the role of genomic, molecular, physiological, and neural mechanisms without the use for gene editing technology (Stevenson et al. 2018). Also, just like pharmacology, RNAi and morpholinos, gene editing, optogenetics, and chemogenetics have all been shown to have off-target effects as well (Zhang et al. 2015; Li et al. 2019; Cho et al. 2020). With our review we are not suggesting that gene editing is necessary for deep insights on biological phenomena or going to solve the issues of other techniques for studying nontraditional model organisms; instead, we see gene editing as a complimentary method to add to one's toolkit. For example, gene editing can become a complimentary tool to answer questions as follow up to pharmacological, bioinformatics, or genomics studies (Warren et al. 2010; Brawand et al. 2014; Lorin et al. 2015; Feng et al. 2020).

Genetic models of other molecular systems, including for neuroendocrine functions, have been engineered using CRISPR/Cas9 gene editing for teleosts, including zebrafish (*Danio rerio*), *A. burtoni* (e.g., prostaglandin signaling, Juntti et al. 2016; Yabuki et al. 2016), and medaka (*Oryzias latipes*) (e.g., vasotocin and isotocin, Yokoi et al. 2015, 2020) and other vertebrate species, including prairie voles (e.g., oxytocin, Horie et al. 2019; Berendzen et al. 2023), lizards (e.g., tyrosinase, Rasys et al. 2019), and frogs (e.g., tyrosinase, Wang et al. 2015). We hope our focus on highlighting the potential of applying Krogh's principle in *A. burtoni* during the gene editing era for revealing principles of steroid hormone signaling in controlling social status will motivate others to pursue similar questions, yielding novel, testable hypotheses to reveal principles of physiology and behavior across species.

Author's contributions

All authors contributed equally. All authors conceived of the general idea of the review. All authors participated in discussions and development of the content, structure, and framing of the ideas in the manuscript. All authors wrote and edited original and final versions of the manuscript.

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Conflict of interest

The authors declare no conflict or financial interests.

Data availability statement

There are no data associated with this review.

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