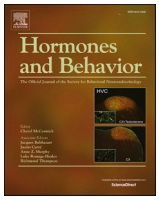
Hormones and Behavior 157 (2024) 105445

Contents lists available at ScienceDirect

Hormones and Behavior

journal homepage: www.elsevier.com/locate/yhbeh



Sex diversity in the 21st century: Concepts, frameworks, and approaches for the future of neuroendocrinology

Kristina O. Smiley [a,*,1], Kathleen M. Munley [b,1], Krisha Aghi [c], Sara E. Lipshutz [d,2], Tessa M. Patton [e,2], Devaleena S. Pradhan [f,2], Tessa K. Solomon-Lane [g,2], Simón(e) D. Sun [h,2]

[a] Department of Psychological and Brain Sciences, University of Massachusetts Amherst, 639 North Pleasant Street, Morrill IVN Neuroscience, Amherst, MA 01003, USA
[b] Department of Psychology, University of Houston, 3695 Cullen Boulevard, Houston, TX 77204, USA
[c] Department of Integrative Biology and Physiology, University of California Los Angeles, 405 Hilgard Ave, Los Angeles, CA 90095, USA
[d] Department of Biology, Duke University, 130 Science Drive, Durham, NC 27708, USA
[e] Bioinformatics Program, Loyola University Chicago, 1032 West Sheridan Road, LSB 317, Chicago, IL 60660, USA
[f] Department of Biological Sciences, Idaho State University, 921 South 8th Avenue, Mail Stop 8007, Pocatello, ID 83209, USA
[g] Scripps, Pitzer, Claremont McKenna Colleges, 925 North Mills Avenue, Claremont, CA 91711, USA
[h] Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor, NY 11724, USA

ARTICLE INFO

Keywords:
Androgens
Behavior
Birds
Estrogens
Fish
Gender
Multivariable
Rodents
Sex variability
Sex diversity
Statistical modeling

ABSTRACT

Sex is ubiquitous and variable throughout the animal kingdom. Historically, scientists have used reductionist methodologies that rely on *a priori* sex categorizations, in which two discrete sexes are inextricably linked with gamete type. However, this binarized operationalization does not adequately reflect the diversity of sex observed in nature. This is due, in part, to the fact that sex exists across many levels of biological analysis, including genetic, molecular, cellular, morphological, behavioral, and population levels. Furthermore, the biological mechanisms governing sex are embedded in complex networks that dynamically interact with other systems. To produce the most accurate and scientifically rigorous work examining sex in neuroendocrinology and to capture the full range of sex variability and diversity present in animal systems, we must critically assess the frameworks, experimental designs, and analytical methods used in our research. In this perspective piece, we first propose a new conceptual framework to guide the integrative study of sex. Then, we provide practical guidance on research approaches for studying sex-associated variables, including factors to consider in study design, selection of model organisms, experimental methodologies, and statistical analyses. We invite fellow scientists to conscientiously apply these modernized approaches to advance our biological understanding of sex and to encourage academically and socially responsible outcomes of our work. By expanding our conceptual frameworks and methodological approaches to the study of sex, we will gain insight into the unique ways that sex exists across levels of biological organization to produce the vast array of variability and diversity observed in nature.

1. Introduction

Sex is ubiquitous in the kingdom Animalia and has long captured the curiosities of both scientists and non-scientists alike. To study sex, scientists have historically defined "sex" as a binary categorical variable, in which organisms are designated as either *female* or *male* based on an observable (or set of observable) characteristic(s) associated with gamete type. However, it is becoming increasingly clear that two discrete sexes are insufficient to capture the sex diversity observed in nature. Indeed, many organisms – including humans – show an immense range of sex variability that supersedes binary categories. This complexity is due, in part, to the fact that sex is observable across many levels of biological organization, including genetic, molecular, cellular, physiological, behavioral, social, and ecological levels, which may or may not be congruent with one another. Conceptualizing sex as a discrete binary is further complicated by the reality that mechanisms

* Corresponding author.
E-mail addresses: ksmiley@umass.edu (K.O. Smiley), kmmunley@uh.edu (K.M. Munley), kaghi@g.ucla.edu (K. Aghi), sara.lipshutz@duke.edu (S.E. Lipshutz), tpatton4@luc.edu (T.M. Patton), devaleenapradhan@isu.edu (D.S. Pradhan), tsolomonlane@keckscl.claremont.edu (T.K. Solomon-Lane), sisun@cshl.edu (S.D. Sun).
[1] These authors share first authorship.
[2] These authors contributed equally. Authors are listed in alphabetical order.

https://doi.org/10.1016/j.yhbeh.2023.105445
Received 4 August 2023; Received in revised form 11 October 2023; Accepted 18 October 2023
Available online 17 November 2023

governing sex are embedded within complex biological networks, both affecting and being affected by other interconnected systems. We consider it within the purview of neuroendocrinologists to describe and model such complexity; however, it remains common practice to operationalize sex as strictly a binary variable, in our field and beyond. In addition, how we assign sex in our work is typically based on one defining characteristic, axis, or proxy of sex (e.g., chromosomes, genitalia, or plumage), which is constrained by the qualities that we (as human researchers) can observe and define as belonging to said sex category. We argue that to produce the most accurate and scientifically rigorous work examining the diversity and variability of sex, we must reconsider and advance the predominant frameworks, model systems, and analytical methods we are using to fully encompass the range of sex and its biology in animal systems.

In this perspective piece, we call special attention to the academic and socio-cultural importance of our scientific study of sex, even when using non-human animal models. This article draws both from empirical research and from the perspectives of several early career researchers with varied backgrounds in the field of behavioral neuroendocrinology who use a diverse range of organisms, levels of analysis, and techniques to inform their views on this subject. Together, we argue that current research standards for studying sex must be improved to inform more accurate and inclusive research. We believe this call to action is timely and urgent for several reasons, beyond our own personal scientific curiosities. First, the National Institutes of Health (NIH) requires the study of "both sexes" in all NIH-funded research (NIH, 2015). While this initiative was a necessary first step to address the male-centric bias in biomedical research, we argue it is necessary to go further and re-evaluate how sex-associated variables are integrated into neuroendocrinology and other biological research. This process will ensure proper reporting of sex differences and similarities (Garcia-Sifuentes and Maney, 2021), improve study interpretation and discussion (Miyagi et al., 2021), and broaden our perspectives on sex diversity and variability throughout the animal kingdom (McLaughlin et al., 2023). Second, we (the authors) envision the Society for Behavioral Neuroendocrinology (SBN) to be an exemplar for conducting science that engages with communities within and outside of academia. SBN holds a unique space for outstanding and expansive expertise in hormone-mediated traits, behaviors, brain development, morphology, physiology, genomics, and other molecular processes; thus, we are a society of scientists consistently on the forefront of sex-associated research, with wide translational potential (Aghi et al., 2022). This role comes with both academic and social responsibilities, exemplified by the use of our research to justify laws (Sudai et al., 2022), medical practices (Fausto-Sterling, 2000), and other regulations that impede on the lives and rights of our peers, particularly those in LGBTQIA+ communities (Gill-Peterson, 2018; Massa et al., 2023). If we, as scientists, want more inclusive and responsible research, then *we must start with our own*. Conscientiously accounting for these intersectional factors in the study of sex is necessary for: 1) producing rigorous research, 2) advancing our conceptual understanding, and 3) protecting our peers and other vulnerable communities.

The goal of this paper is to challenge how we conceptualize, frame, and use "sex" throughout our work to improve our research output while engendering a more inclusive and equitable scientific society. We acknowledge that sex is a multifaceted, complex phenomenon that consists of many intersecting variables and can be approached from different perspectives. It cannot be overstated that **we do not claim to have all the answers, nor do we purport to have a universal solution to these issues. Rather, we hope this perspective piece sparks constructive conversations on how to best approach this subject in our labs, classrooms, and scientific societies, each with their own unique contexts.** We encourage readers to keep an open *and* critical mind to identify aspects of this perspective piece that are relevant to their own research, while remaining aware of the sociopolitical impacts of this work, particularly on marginalized communities. If sex is not a part of their research, we still invite readers to apply these concepts to improve their critical evaluation of research on sex and to increase awareness of sex variables that may be relevant in their own studies.

In this perspective piece, we first provide conceptual background and scientific motivations for reevaluating the predominant operationalization of "sex." Next, we propose a new operationalization to better study sex in its full diversity and variability with an integrative, multidimensional, contextually adaptable framework that remains flexible for future changes. We then describe potential variables/factors, animal models, experimental approaches, and statistical analyses to consider in study design. Finally, we close with a brief discussion on actions that can be taken, both individually and collectively, to encourage responsible and positive academic and socio-cultural impacts of our research.

2. Conceptual background

2.1. What is sex and why do we need a new framework?

At its most broad, sex refers to a form of biological reproduction characterized by the recombination and division of parental genomic material that is unified in the next generation. Sex also refers to a summary category of individuals within a sexually reproducing species (i.e., males, females, hermaphrodites). In practice, these categories are assigned to individuals based on traits assumed to be associated, to varying degrees, with the production of haploid gametes that differ relatively in size: the larger "female" ova and the smaller "male" sperm. Classically, this categorization is then extrapolated to encompass other traits across different biological scales, or *Levels of Analysis*[3] (Table 1), without consideration of timing for the development, emergence, and maintenance of sex variable traits. This essentialist conceptualization of sex reduces all sex variable biology – development, genetics, anatomy, physiology, endocrinology, neurobiology, behavior, and ecology – to asymmetric gamete production (anisogamy) and privileges successful fertilization as a measure of fitness. While this definition of sex may be useful for modeling generalizable principles of sexual selection (De Vries and Lehtonen, 2023), it is overly deterministic in that it assumes anisogamy is ultimately causal for variation and diversity in sex biology (Fig. 1), including the genetics of gonadal determination, physiological and morphological divergences, behavioral and social differences, and sex roles (Goymann et al., 2023). This categorical, binary operationalization asserts *a priori* that sex differences arise from two distinct biologies within a species, as though "the two sexes" are complex machines with different mechanisms, or even as different as another species (Richardson, 2010). Collapsing the complexity of sex into a

[3] While one of the first uses of "levels of analysis" has been to study animal behavior and social plasticity within the framework of "proximate" and "ultimate" questions, many different perspectives have been used to categorize this concept (reviewed in MacDougall-Shackleton, 2011; Oliveira, 2012). To clarify what we mean by "levels of analysis" in reference to sex in this perspective piece, we are referring to the multiple biological levels of organization in which "sex" can present itself, including, but not limited to: genetic, morphological, hormonal, behavioral, and environmental (Table 1; McLaughlin et al., 2023).

Table 1
Definitions of terms associated with sex diversity and sex variability used throughout this perspective piece.

Term	Definition
Gender	Socially constructed roles, behaviors, and identities of individuals that occur in a particular historical, environmental, societal, and cultural context that can be embedded systemically, institutionally, ideologically, and relationally and may change over time.
Gonadal determination	The underlying mechanism(s) that initiate(s) the development of primary reproductive organs.
Levels of analysis	The multiple biological levels of organization in which "sex" can present itself, including, but not limited to genetic, morphological, hormonal, behavioral, social, and environmental levels.
Morph	A set of sex-associated correlated or covarying phenotypic traits belonging to an individual.
Sex	Of, or relating to, the process of biological reproduction characterized by the recombination and division of parental genomic material, unified at fertilization.
Sex (as summary category, pl. "sexes")	A subclass, categorical or continuous, in reference to a group of traits that are often, but not always, associated with reproduction. These include female, male, hermaphrodite, and intersex, among others.
Sex differentiation	The ontogenic processes by which sex variable traits emerge, are maintained, or changed.
Sex diversity	Variation – differences and similarities – in sex biology *between* and *across* species. Used to describe the variety of biological systems involved in sex.
Sex variability	Broadly refers to sex-associated variation – differences and similarities – *across* specific characteristics that correlate or covary with a sex category or other sex variables *within* a species.
Sex-stable species	Species in which the gonads and related traits, physiological systems, and behavior reach a steady state after development, but can change in response to perturbation.
Sex-dynamic species	Species in which the gonads and related traits, physiological systems, and behavior exhibit variability based on their (social) environment throughout the lifespan. These species display pronounced permanent or semi-permanent changes in these biological and physiological processes in response to perturbation, including sex change.
Sex convergence	A phenomenon in which a defined endpoint is measurably similar across sex, but the biological and physiological mechanisms underlying this endpoint are distinct (McCarthy et al., 2012).
Sex divergence	A phenomenon in which a defined endpoint is measurably different across sex, whether by similar or distinct biological and physiological mechanisms.

binary variable operationalizes sex categories – "a sex" or "the sexes" – as forms or substances independent from the influence of time rather than emergent states of interacting variables engaging in dynamic biological processes.[4] Herein lies the primary contention of this perspective piece: *operationalizing sex as a univariate, binary, categorical variable is insufficient to reveal the biology of sex*. If we assume the sexes are separate, we inevitably produce separate models: "Sex differences predict sex differences" (Gowaty, 2018).

Univariate, *a priori* operationalizations of sex severely limit our thinking and understanding of naturally occurring sex diversity, and assuming there are *only* two sex categories that span all levels of analysis is restrictive. Any variation observed outside of categorical archetypes is incorporated post hoc and are often interpreted as rare, exceptional, pathological, erroneous, ignored, or unimportant. These assumptions also bias data interpretation and encourage reports of spurious sex differences (Garcia-Sifuentes and Maney, 2021; Gowaty, 2018; Patsopoulos et al., 2007). Furthermore, this approach inherently disregards any naturally occurring sex diversity, despite the reality that individuals rarely fulfill archetypal sex categories, thereby hindering translation and comparison across different sexual systems in the animal kingdom (Bachtrog et al., 2014; DiMarco et al., 2022). Another consequence of this approach is that the historically binarized study of sex differences operationalizes "males" as baseline, whereas "females" are studied only in relation to males (Smiley et al., 2022; Massa et al., 2023). In the binary framework, the biologies of males and females are often considered so distinct that females are often relegated as a separate experimental condition or – as in some rodent studies – are studied following ovariectomy to control the influence of cycling gonadal hormones under the false pretense that endogenous hormone cycles cause increased behavioral variability (Levy et al., 2023; Shansky, 2019). The binary framework also overemphasizes an oppositional framing: if one variable value is male, then the other can only be female, precluding any applicability to animal sexual systems in which individuals can produce both, neither, or incomplete gametes. While the NIH initiative Sex as a Biological Variable (SABV) sought to remedy such systemic practices, it has not addressed the underlying fundamental issue of considering sex *only* as a binary variable (NIH, 2015; DiMarco et al., 2022).

The "sex as a binary" framework also collapses the multi-level, multi-scale nature of sex (McLaughlin et al., 2023), thereby flattening, erasing, and obscuring the influence of scale and time on sex biology. Many sex-associated traits have varying dynamics and occur at specific life stages, are influenced by developmental processes, or have effects seen later in life. Furthermore, these spatio-temporal properties can interact to produce sex variability (McCarthy, 2023). For example, gonadal steroid hormones, such as estrogens, contribute to the expression of adult sex variable territorial behaviors due to hormone surges during the perinatal period, with sex divergent effects dependent on the expression of enzymes in specific brain areas (Wu et al., 2009). In order to better investigate how sex, sex diversity, and sex variability (Table 1) arise from interacting variables spanning biological scale and time, a new, integrative framework that does not rely on categorical *a priori* assumptions that ignore or flatten the multidimensional nature of sex is required (Fig. 1A). Such a framework will enable scientists to describe sex differences with greater precision, incorporate instances of sex similarity without disregarding, oversimplifying, or overinterpreting results, and reduce reports of spurious differences. Ultimately, by centering sex variables and their scale-spanning relationships, this framework will guide studies toward identifying the specific biological mechanisms that generate sex variability and diversity.

2.2. Definitions & terminology

The language we use about sex greatly affects how we study it and interpret our findings. Here, we critically examine the historical basis of our current terminology and the ways it perpetuates a flawed univariate, binary approach to studying sex. We (re)define and refine these terms to improve the operationalizing of sex, enhancing the precision and inclusivity of our scientific language to better describe and understand the full range of sex diversity.

The largely binarized terminology of sex in behavioral neuroendocrinology can be traced back to the first seminal studies of hormones and sexual behavior, in which researchers administered hormones of the

[4] The predominant practice of operationalizing sex as a binary variable requires privileging certain biological properties over others when defining and assigning sex categories. This frames and influences nearly every aspect of a scientific study, from design and execution to analysis and interpretation, which we address here as practicing researchers (**Sections 3 and 4**). Philosophical perspectives on this discussion – such as whether biological phenomena, including sex, are best captured by a substance or process-centered ontologies – though relevant, are beyond the scope of this article. However, in the interest of encouraging continual discussion, we consider our proposed framework (dynamic sex variability, Fig. 1) as in line with similar dynamical perspectives on development, evolution (Waddington, 1957; Wilkins, 2008; Fabris, 2018), and sex differentiation (Sun and Tollkuhn, 2023).

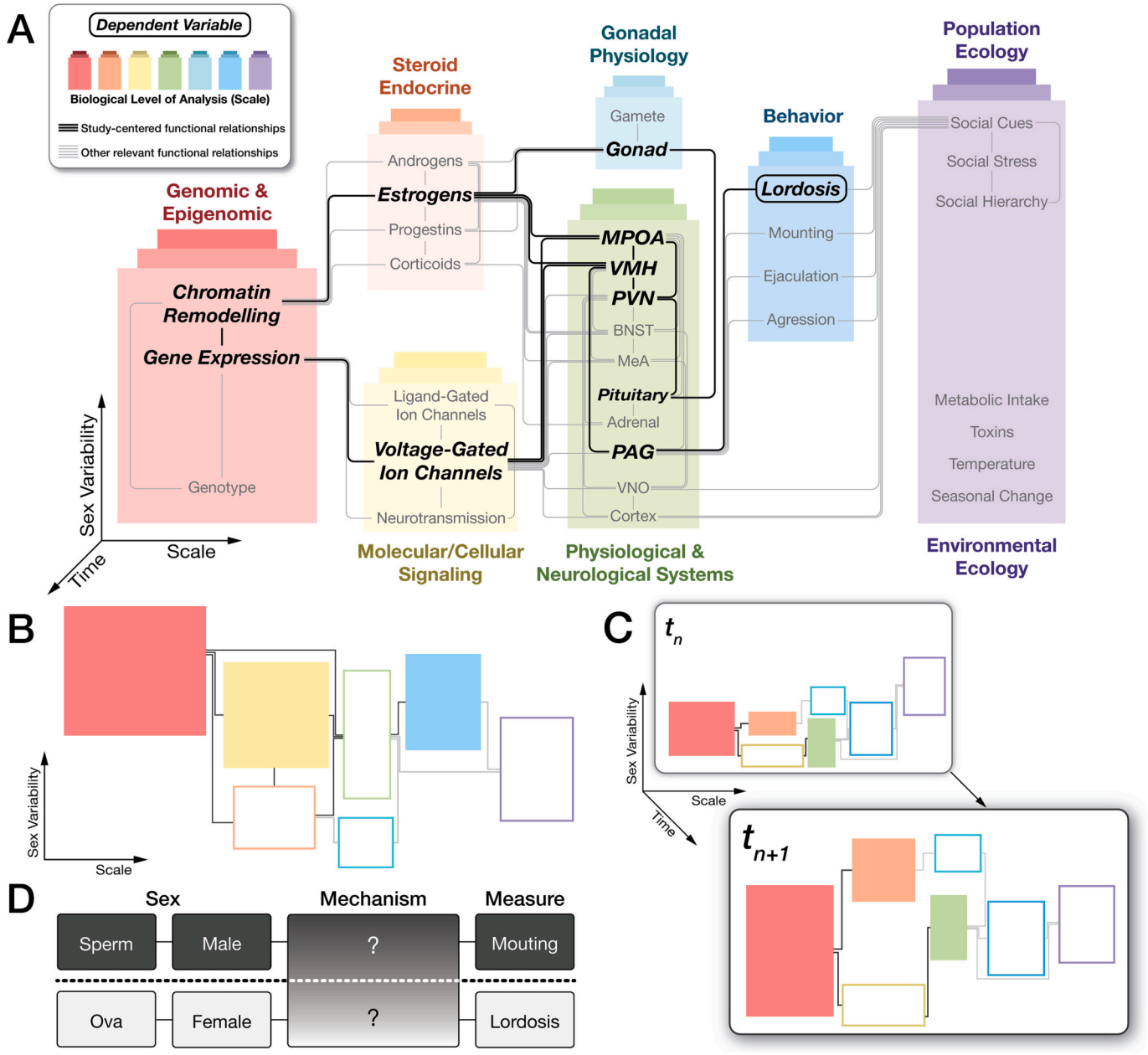


Fig. 1. Dynamic sex variability - An integrative, multidimensional framework. A framework that centers sex variability will enable the study of sex across multiple levels of biological analysis (colored boxes). **A)** *Visual schematic* representing the degree of sex variability (y-axis) across biological scale (x-axis) and time (z-axis), with example sex variables of interest (not all possible covariates and functional relationships are presented). In this example, to decipher mechanisms linking gonadal hormones to receptive reproductive behavior in adult rodents (lordosis), the functional relationships (black subset of possible gray relationships) between select variables (bold words) need to be identified. At each level of analysis, numerous variables differ across sex to contribute to reproductive behavior through these functional interactions. Importantly, the degree of variability across sex categories is dynamic and is affected by developmental stage, experience, and environmental factors. **B)** *An example of sex variable relationships across several levels of analysis.* For any given study of sex, there are limits to which levels of analysis (and variables within these levels of analysis) are experimentally tractable (colored boxes). The *study lens* encompasses "levels of analysis" with measured variables. Levels of analysis outside the study lens (outlined boxes) still contribute, in some manner, to the focus of the study (e.g., lordosis in A) and should be held as contextually relevant factors (Richardson, 2021). This example illustrates a hypothetical scenario in which sex variable behaviors (blue) are mediated primarily by high sex variability in genomic level of analysis (red) through variation in expressed proteins (yellow), but not from variability in steroid hormones (orange). Importantly, this framing acknowledges levels of analysis outside of the study lens, illustrated here by the assumptions made about neuroanatomical sex variability (green outlined box) that could shape the effects of differential gene expression on behavior at life stages where gonadal physiology does not exhibit high variability. **C)** *An example of sex variable relationships across several levels of analysis through time.* This hypothetical study lens seeks to identify changes in sex variability through biological time (e.g., development, aging, experience). This example illustrates a scenario in which there is little sex variability at time t_n at the genomic (red), endocrine (orange), molecular/cellular physiological (yellow), and neuroanatomical (green) levels. Sex variability emerges by time t_{n+1}, at which high sex variability is observable in certain levels of analysis. We propose that this framing captures the complexities of sex variable biology while aiding in study formulation, execution, and interpretation. **D)** This new, proposed framework (A-C) contrasts with the categorical binarized approach (D) that imposes *a priori* separation of biological mechanisms. *Anatomical abbreviations: BNST, bed nucleus of the stria terminalis; MeA, medial amygdala; MPOA, medial preoptic area; PAG, periaqueductal gray; PVN, paraventricular hypothalamic nucleus; VMH, ventromedial hypothalamus; VNO, vomeronasal organ.* (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

"opposite sex" and examined their effects on morphology, behavior, and physiology (e.g., Phoenix et al., 1959). These and other works mistakenly refer to androgens as "male hormones" and estrogens and progestins as "female hormones." While this terminology was most likely used for simplicity at the time, its continued use has contributed to a false dichotomy extended to other variables beyond hormones, gametes, and gonads. Recently, we have seen the idea of "sex" being a univariate trait institutionally mandated by the very language used in the NIH initiatives created to increase the study of sex differences – i.e., "sex as a biological variable" (NIH, 2015). The title alone states that sex is "*a* variable", inherently dismissing the fact that sex occurs at multiple levels of analysis (McLaughlin et al., 2023). Furthermore, the initiative states that studies must include "*both* sexes", assuming that there are only two sexes to consider. From this information, it is not clear how we should define sex in our studies or how we should group individuals and populations that fall outside of the binary framework. The solution to this problem is to first clearly operationalize and communicate how we are measuring sex in our studies (Miyagi et al., 2021). How "sex" as a summary category is operationalized in a given study depends on which trait(s) and/or "level(s) of analysis"> (Table 1) are measured and the goal(s) of the current study, as well as past studies. Ideally, a study of sex will include measures of multiple sex-associated variables at several biological levels to examine potential sex variability that may be occurring across levels of analysis, within and between species.

We must expand the terminology used to describe sex to be more precise and inclusive (Miyagi et al., 2021). In Table 1, we provide operational definitions for terms relevant to the study of sex, and we will refer to these terms throughout the paper. While we cannot define all possible relevant terms here, we encourage readers to thoughtfully consider the language being used in their own scientific writing and other forms of communication. Table 2 outlines a few examples of ways to reinterpret and update historically used terms into more inclusive and operational language. For instance, we propose using the term "gonadal determination" instead of the commonly used term "sex determination". Interpreted literally, "sex determination" refers to a mechanism that determines the sex category of an individual, meaning all sex-associated traits would be determined by such a mechanism. Thus, this term forgoes the multidimensionality of sex and continues the conflation of sex as originating by a single factor, reinforcing an immutable binary model. Similarly, some historically used terms suggest a categorical norm for which sex performs a behavior, despite few behaviors being exclusive to one sex. For example, the term "sex-role reversal" can more accurately be described by the actual behaviors that coincide within a population, such as high rates of male parental care and female competition for mates. Assuming a standard of sex roles is misleading and highlights a bias for how norms are defined (Ah-King and Ahnesjo, 2013). Indeed, paternal care evolved before maternal care (Gross and Sargent, 1985), and describing paternal care as a "reversal" does not add meaningful biological context. Additional examples of reinterpretation of harmful terms used in biology can be found in the EEB Language Project Repository.[5] Although these concepts may not directly apply to your own research, we encourage readers to use this exercise when reading other literature. Does re-examining or reframing the language used in published papers change the interpretation or call for a re-evaluation of the findings? This practice will increase awareness of biased language used in our scientific writing and in other media outlets, both for new trainees and well-established members of our field alike. Critical engagement with our own language usage is a foundational step toward improving the quality of our research and recontextualizing past works with present and future discoveries (Massa et al., 2023; Miyagi et al., 2021).

[5] The EEB Language Project: A repository for harmful terminology in EEB. URL: https://www.eeblanguageproject.com/repository (accessed July 25, 2023).

Table 2
Examples of how to redefine terms associated with sex diversity and variability to be operational, precise, and inclusive.

Historically used terminology	Updated terminology	Justification
Biological sex	Sex	"Biological sex" conflates the biology of sexed characteristics as determined and immutable. It has also been used to incorrectly draw distinctions between the genetic, molecular, physiological, and behavioral (nominally "sex") from the psychological, ecological, and sociological (nominally "gender") aspects of sex and gender diversity when, in fact, these elements are highly intertwined in unique and specific ways. In our integrative framework, sex goes beyond what is classically considered biological, thereby making the term "biological sex" overconstrained.
"Male hormones" (i.e., when referring to effects of androgens) and "female hormones" (i.e., when referring to effects of estrogens or progestins)	Androgens, estrogens, and progestins	This terminology presents a false binary that these hormones only have sex-specific functions while downplaying the role of other hormones. In reality, these hormones are functionally important in all sexes.
Masculinization (i.e., when referring to effects of androgens) or feminization (i.e., when referring to the effects of estrogens or progestins)	Androgenization (androgenic), estrogenization (estrogenic), or progestinization (progestigenic)	Using masculinizing/feminizing terminology in broad reference to outcomes of endocrine signals implies androgens, estrogens, and progestins only have sex-specific functions, when in reality, these hormones are relevant for all sexes. This terminology can be expanded to other molecules that have roles in sexual differentiation, such as oxytocin (oxytocinergic).
Sex determination	Gonadal determination	By definition, "sex determination" refers to a mechanism that determines the sex category of an individual, meaning all sex-associated traits are determined by one such mechanism. Thus, this term forgoes the multidimensional, multimodality of sex and continues the conflation of sex as ultimately originating from a singular process, reinforcing an essentialist binary operationalization.
Sex differences	Sex variability; subtyped: sex differences, sex similarities	As an umbrella term to describe sex, "sex differences" reinforces the search for discrete, binary

(*continued on next page*)

Table 2 (*continued*)

Historically used terminology	Updated terminology	Justification
		biological variation between sexes, when in reality, there are differences and similarities across sex categories. "Sex variability" better encompasses the range of possible outcomes and sex-associated phenomena to study, used in conjunction with "sex differences" and "sex similarities" in specific comparisons.
Sexual dimorphism or sexual polymorphism	Sexual heteromorphism; subtyped: sexual monomorphism, dimorphism, multimorphism	"Sexual dimorphism" is the primary categorization of sex variable morphs, due in part to its prevalence in the majority of animal species studied, but imprecise application of the term can confuse, erase, or hide intraspecies sex variable phenotypes. Instead, sexual heteromorphism should be used as the overarching category for multiple morphs within a species that relate to sex, and subtyped in appropriate contexts. This terminology encourages contextual specificity. For example, traits at one biological scale may be monomorphic, and another dimorphic. This approach forgoes the implied lack of overlap in traits between sex categories and appropriately emphasizes that there can be multiple modes in trait value within and across sexes (Anderson and Renn, 2023). We suggest the use of multimorphism when referring to more than two, as polymorphism has other uses in the biological sciences that may confuse rather than clarify.
Sex role reversal (i.e., in reference to a specific behavior or set of behaviors)	Behaviors that coincide within a population	This terminology assumes a standard of "typical" sex roles, which is misleading and highlights and perpetuates biases for how norms are defined.

Examples of how to reframe commonly used terminology in research to improve operationalization, precision, and inclusivity. As an exercise, we encourage readers to consider the following questions: 1) Where does this term come from? (i.e., what is the historical basis of this term; does it originate from a false dichotomy that is being perpetuated in some way?); 2) Is this term inclusive?; 3) Is this term precise or used vaguely?; 4) Is this term operationally defined in the text and is the definition consistent throughout?

2.3. Dynamic sex variability: An integrative, multidimensional framework

Our integrative framework (Fig. 1A) is centered on the emergence and degree of variability in specific, measurable sex-associated traits, both within a level of analysis (colored boxes, Fig. 1) and their interactions across levels. Focusing on measurable variables discourages definitions and practices that collapse and essentialize sex-associated traits across scales based on the assumption that sex categorizations necessarily coincide (compare to Fig. 1D; Massa et al., 2023; Massa and Correa, 2020). Biological scale (microscale: genetic, molecular, cellular, hormonal; macroscale: anatomical, behavioral, social, ecological) is represented on the x-axis (abscissa), with several example variables within a scale-specific level of analysis (text within colored boxes). Each level of analysis/variable exhibits differing degrees of sex variability, illustrated by its relative position on the y-axis (ordinate). How specific traits vary can be nonlinear, by qualitative or quantitative measure, or by distribution within and across sex categories. A critical third dimension to consider is biological time (e.g., development, aging, experience), represented on the z-axis (applicate). These "axes" are not meant to be precise or numeric values. Rather, they are heuristics for relating sex-associated variables across specific dimensions: scale, variability, and time. Importantly, this framework makes clear the contextual dependency of the variables used for sex categorization, stemming from both practical and conceptual constraints (Richardson, 2021). Overall, centering sex-associated variables within their spatio-temporal contexts emphasizes the limits of a variable's dynamic influence on other connected variables.

Neuroendocrine studies often encompass several sex-associated variables within and across biological levels and seek to identify the functional relationships and processes among those variables. For example: What neural circuits mediate behaviors that exhibit sex variability? Do specific hormones modulate these circuits holistically, or in specific brain areas to elicit sex variability in behavior? Do the mechanisms differ or change with age or across life stages? Do genotypic differences contribute to neurological sex variability? Do specific environmental conditions modulate the degree of sex variability? This framework can be used to map the variables relevant to a given research question, along with the functional relationships among them. With this framework, studies can be designed with more rigor to better tackle the question at hand without relying on assumptions about sex categorization. For example, Fig. 1A illustrates a map for a hypothetical study conducted in our Dynamic Sex Variability framework. This study focuses on sexually receptive behavior in rodents (lordosis). Importantly, lordosis is mapped as a behavior that greatly varies *across* sex (high sex variability) and does not explicitly tie the display of lordosis to a sex category or sex role. Notably, this approach forestalls false binary assumptions and incorrect interpretations, such as the belief that certain behaviors are exclusive to one sex category. In this example, lordosis is often described as a "female sexual behavior," despite decades-long observations of lordosis behavior in male rodents (Södersten et al., 1974; Södersten, 1976; Schaeffer et al., 1990). "Male mounting behavior" is also observed in female rodents (Hashikawa et al., 2017). If using a binary definition of sex, only rodents with ovaries might be included in the study, while rodents with testes may be excluded (Fig. 1D). Thus, the binary discourages the use of other sex categories to explore the biological underpinnings of lordosis. Such binarization also extends to hormones, with androgens often referred to as "male sex hormones" and estrogens and progestins as "female sex hormones" (Table 2; Massa and Correa, 2020). It should be noted that adult rodents possess all hormones to varying degrees that originate from several sources, not exclusively the gonads (Do Rego et al., 2009). Using the proposed sex variability framework (Fig. 1A), the limited utility and explanatory power of binarized sex categorizations is revealed (Fig. 1D), the specific mechanisms of interest are spotlighted, and important potential sex-atypical relationships remain in view. It encourages the inclusion of animals of varying sex categories, ages, and contexts, which are likely necessary to identify the relevant functional relationships between dynamically covarying traits at different biological levels (Fig. 1A, black lines).

This framework is appropriately flexible, allowing it to guide study design for a wide array of research questions on sex, while acknowledging practical limitations in research. For example, the parsing of

genetic and gonadal hormone contributions to sex variability is of central interest in neuroendocrinology (Sun and Tollkuhn, 2023). In some species of birds, sex variability in multiple behaviors exhibit complex associations with sex-associated heteromorphs, with differences in gene regulation independent of the gonads, gametes, or gonadal hormones (Horton et al., 2020; Prichard et al., 2022). This instance of sex-associated behavioral variability (Fig. 1B, filled blue box) is captured by the changing relationship between the genomic level of variability (Fig. 1B, filled red box: DNA methylation; Prichard et al., 2022), resulting in differential, sex-variable protein expression (filled yellow box) that drives variation in behavior. Importantly, this framework acknowledges and incorporates the practical limitations every study encounters. In this example, the study focus on the genomic origins of sex variable protein expression does not directly account for anatomical sex differences (Fig. 1B, outline green box) that could also contribute to behavioral variability (Fig. 1B, filled blue box). The same concept extends to aspects of gonadal physiology and endocrine, social, ecological, and environmental factors, which are outside of the scope or *study lens* (Fig. 1B, outlined boxes connected by gray relationships). Instead of dismissing, ignoring, or collapsing these interacting biological factors, as in the binarized framework (Fig. 1D), these variables and their functional relationships remain present in the conceptual model, even if data at that level are not collected. This approach places study results within their biological context, better informing the interpretation of results and identifying important implications.

This framework also accounts for the importance of time and its relationship to sex variability (Fig. 1C). Ontogeny, transition, and experience are fundamental for the emergence, maintenance, or convergence of sex variability within a species and sex diversity between species. Temporal differences of developmental steroid hormone signals among mammalian species are thought to contribute to variation in the degree of sexual heteromorphism of brain regions, such as the medial preoptic area (Wallen and Baum, 2002). Indeed, the predominant guiding model of the neuroendocrine regulation of behavior, the Organizational-Activational Hypothesis (Phoenix et al., 1959), describes two temporally distinct modes of gonadal hormone action on the brain. Although the Organizational-Activational Hypothesis has been influential, the dynamic sex variability framework makes its limitations more apparent, specifically by emphasizing that multiple covariates can unfold with varying time courses, giving rise to varying emergent outcomes (see also Arnold and Breedlove, 1985; Schulz et al., 2009). The incorporation of time in this framework encourages the explicit consideration of the dynamics of biological processes, including those that may occur in adulthood. Designing studies that hold biological time as a key factor also aids in placing single-time point results in a more accurate context while illuminating the specific limitations of studies that do not directly address development. This framework aids in the identification of dynamic processes among variables at different levels of analysis (Fig. 1C). For example, adolescence is a critical developmental period during which there is an increase in sex variability as animals mature (Schulz et al., 2009). While adolescence is marked by the onset of the pubertal gonadal hormone surge and initiation of the hypothalamic-pituitary-gonadal axis, other biological processes likely exhibit different dynamics. As adolescence progresses, several variables exhibit increased sex variability, such as gonadal hormones (Fig. 1C, orange filled box at t_n and t_{n+1}). Considering which other variables also change over time can illuminate possible functional relationships during maturation, while still acknowledging gaps not directly studied. For example, the gonadal hormone surge (Fig. 1C, orange filled box) could be directing increases in genomic sex variability (Fig. 1C, red filled box) that contribute to differences in neural circuit activity (Fig. 1C, green filled box). In this hypothetical example, the study is not formulated to identify molecular or cellular variability (Fig. 1C, yellow outline box) that may be mediating sex variability in neuronal activity, which may or may not exhibit variation to the same extent as other variables.

To summarize, this integrated multidimensional framework (Fig. 1A) is a heuristic to guide our studies of sex, its variability, and diversity, including study design, data analysis, and interpretation (further expanded upon in **Section 3**). It emphasizes the need to account for multiple variables to identify the dynamic processes between them, rather than collapsing traits assumed to be associated with sex categories. By actively considering sex variability, biological scale, and time, we are encouraged to recognize and integrate the contextual dependencies of measures and traits used for sex categorization, thereby better capturing the influences and changes of these variables over time. The framework is flexible, adaptable, and acknowledges practical limitations. By explicitly incorporating the dimension of time, the framework further aids in understanding the dynamics of sex variability within and between species and highlights the importance of considering developmental stages and experience. In contrast to a binarized framework (Fig. 1D), this integrative approach fosters a more comprehensive and targeted understanding of the complexities of sex variability and diversity across biological systems.

3. Experimental design and analysis

To better understand natural variation in sex across the animal kingdom and how sex influences physiology and behavior throughout the lifespan, it is critical that a diversity of organisms with different sexual phenotypes are represented in neuroendocrinology research, both in field and laboratory settings (reviewed in McLaughlin et al., 2023; Smiley et al., 2022). Moreover, because sex is dependent on the integration of multiple phenotypes and, thus, can have different effects on physiology and behavior (e.g., Munley et al., 2022c; Solomon-Lane et al., 2016; White et al., 2023), sex diversity and variability should be regularly incorporated into experimental approaches to enable researchers to disentangle whether and how sex influences each of these individual processes. Although “sex differences” have been investigated for decades (reviewed in Ball and Ketterson, 2008; Bangasser and Valentino, 2014; McCarthy et al., 2012; McCarthy and Nugent, 2015; Trainor, 2011; Yan and Silver, 2016), there is not a consensus on how to study sex in animal models, both with respect to experimental design and statistical analysis. To date, sex diversity and variability in neuroendocrinology has primarily been studied in sex-stable species (i.e., species in which the gonads and related traits, physiological systems, and behavior reaches a steady state after development; Table 1; reviewed in McLaughlin et al., 2023; Smiley et al., 2022). Relatively little is known, however, about how these mechanisms may differ in sex-dynamic organisms (i.e., species in which the gonads and related traits, physiological systems, and behavior exhibit variability based on their environment throughout the lifespan; Table 1). There is also considerable variation in which variables are measured in experiments, how these variables are quantified, and whether and how sex is incorporated into statistical modeling and testing, making it challenging to explicate the role of sex in modulating neuroendocrine mechanisms and behavior. In this section, we provide recommendations for best practices when designing experiments that examine or integrate sex variability and diversity, including: 1) factors and variables to consider measuring, 2) implementing diverse model organisms, 3) mechanistic approaches for studying multi-leveled traits, and 4) integrating sex-associated variables into data and statistical analyses. Collectively, these guidelines will not only allow researchers to answer central questions about how the brain, behavior, and other attributes are influenced by sex, but will also enhance our understanding of how these processes may differ across species with diverse sexual systems.

3.1. Factors and variables to consider when studying sex diversity and variability

In order to take a truly integrative approach to studying sex-associated traits, we must be aware that the variables we are using to determine sex (e.g., morphological, hormonal, genetic) not only interact

with and influence each other (Fig. 1), but also occur and fluctuate in the context of other factors that we may or may not be able to measure. As integrative biologists who examine multiple traits and factors that relate to sex, we use this framework with the understanding that they are not hierarchical separations of importance (reviewed in MacDougall-Shackleton, 2011). It is important to note that this section is not intended to be an exhaustive list of factors to consider when designing experiments, but as a launching point for discussion and reflection of past and present experimental design which aims to study sex (either as an independent or dependent variable). We also note that in many cases, it will be impossible to control for every factor/variable in an experiment, so part of our aim is to increase awareness of factors/variables that are important in study design and may affect sex-associated traits that are being measured.

3.1.1. Timing factors

Broadly, we encourage scientists to *always* consider the role of temporal dynamics in their studies of sex, even when development or experience is not the focus of a study. 'Post'-developmental time points, such as adulthood, are predominantly considered within a static frame, reinforcing essentialist and reductive interpretations of dynamic biological processes (Dupré and Nicholson, 2018). In turn, such views of biological stasis congeal with essentialist binary logic described in the previous sections and can contribute to the dismissal of sex variables that are dynamic in nature (Smiley et al., 2022). Instead, *all biological processes have temporal components*, even though a system may superficially appear static. Our proposed framework emphasizes this point through the explicit consideration of spatiotemporal dimensions within sex variability (Fig. 1). In this view, phenotypically stable – or matured – sex categories, such as male, female, and hermaphrodites, can be understood as states of homeorhesis, a steady state (homeostasis) that is actively maintained by ongoing processes extended through time and is robust to certain perturbations (Waddington, 1957), while stages of development and transition are more plastic and dynamic states. Thus, sex variability within a species and diversity across species are natural outcomes of the many dynamic systems involving numerous interacting sex variables (Fig. 1A), which are able to simultaneously exhibit contradictory properties of robustness and plasticity to enable adaptation and evolution (Fabris, 2018).

More practically, the consideration of dynamics involves timed sampling that includes both cross-sectional and sequences for longitudinal studies, hallmarks of neuroendocrinology experimental design. Choosing relevant end points and/or timepoints for repeated measures can be critical for understanding both the initiation of timing of expression and mechanisms that regulate the expression of sex-associated traits. For example, during development, the timing of expression of hormonal and other specific transcription factors can fluctuate in a dose-dependent way; therefore, measuring hormonal changes during development may reveal periods of divergence across sexes, such as the appearance of distinct reproductive organs. Often, experimental timepoints are chosen based on logistical constraints of the experimenter in an attempt to keep time of day consistent. However, conducting fundamental experiments to determine the biologically meaningful timepoints that are relevant to the organism being studied is an essential first step before designing studies and will help reveal periods of divergence between the phenotypes that define sexes. For example, in studies investigating endocrine mechanisms during sex change, which is often observed in sex-dynamic organisms (see Section 3.2.2), it is important to determine the timing of when important behavioral and morphological markers appear *before* deciding the timing for sacrificing animals to obtain tissue samples for molecular markers. Molecular actions can take minutes, hours, or days to manifest; thus, it is critical to determine whether these effects are non-genomic or genomic and act under the appropriate social context before investigating the effects of drugs or specific molecules on sex-associated traits. These considerations become even more important when expensive treatments or molecular tools are being used and may increase the costs of both personnel and other resources. Below, we describe several timing-related factors which should be taken into consideration when designing experiments.

3.1.1.1. Developmental timing. The organization, patterning, differentiation, and subsequent specialization of cells that make up multicellular organisms occur at specific times during the life cycle. These processes are orchestrated by suites of regulatory genes that encode transcription factors and signaling molecules, such as the 'Gene Regulatory Network' (Li and Davidson, 2009; Emmert-Streib et al., 2014), which are activated by maternal transcription factors during embryonic and perinatal development (Davidson et al., 2002; Paraiso et al., 2019; Poulat, 2021). There is immense plasticity in these mechanisms during development, even in species in which gonadal physiology is stably determined by chromosomes or genes. For example, the regulatory genes involved in shaping the final expression of sexual characteristics and reproductive organs are 'dispersed' in a gradient, and the process of development proceeds in response to the dose-dependent expression of these genes (Barresi and Gilbert, 2023). Furthermore, activation of one particular gene typically leads to expression of other downstream genes and may regulate more than one biological pathway based on when it is expressed. For example, the gene *Sox9* is responsible for craniofacial and musculoskeletal development, in addition to testis patterning (Vaillant et al., 2001; Leung et al., 2011). Similarly, Anti-Müllerian hormone (AMH) is necessary for ovarian and follicle development later in life (Baba et al., 2017), beyond its role in breaking down the Müllerian ducts. Gene expression is also influenced by other biomolecules that may be present in the endogenous and/or exogenous environment surrounding an embryo or larva (Barresi and Gilbert, 2023). Furthermore, sex variables such as gonadal hormones direct sex differentiation of hormone-responsive neurons throughout development and maturation by regulating gene expression. During adulthood, the same gonadal hormones produce dramatically different responses than during perinatal development (Gegenhuber et al., 2022); thus, other temporally-constrained factors may be interacting with these hormones to regulate sex differentiation at various life stages.

3.1.1.2. Life history and cycle. Many processes, such as growth, regeneration, tissue repair, and cellular function, continue throughout an organism's lifespan and are influenced by its life history. For example, amphibians and insects have complex life history patterns because they undergo dramatic physiological changes that are orchestrated by the reactivation of developmental processes during metamorphosis (Tata, 1993). Similarly, mammals undergo pronounced changes during puberty, when the neuroendocrine processes for reproductive maturity are activated and secondary sexual characteristics begin to develop concurrently with cognitive and emotional changes (Laube et al., 2020; Vijayakumar et al., 2021; Brooks-Gunn and Warren, 1988; Schulz et al., 2009). These processes are regulated by combinations of both endogenous factors and signals from the exogenous environment (e.g., the presence of a possible mate or toxins) that are transduced to modify biochemical signaling pathways and/or gene expression. Explicitly considering the dynamics of these processes during and after significant perturbations, such as environmental toxin exposure, is an important factor to consider in study design. Thus, taking an integrative approach (Fig. 1C) can help identify potential variables and processes that contribute to the expression of sex variability at various life history stages. For example, studies in primate, rodent, and avian species have shown that estrogens have wide-ranging effects in shaping the sex-associated cellular architecture of the developing brain (MacLusky et al., 1986; McCarthy, 2008; Holloway and Clayton, 2001), as well as neuronal electrophysiological properties (McHenry et al., 2017). In utero, whether estrogens originate from maternal or placental circulation, or from gonads or brain of the developing embryo itself,

downstream signaling mechanisms of estrogens are regulated by receptors located in the nucleus or cytoplasm (McCarthy, 2008). Understanding the simultaneous expression and functional activation of key biomolecules (see Section 3.1.3.1) that initiate the expression of sex-associated traits can provide convergent evidence for the initiation and maintenance of phenotypes.

3.1.1.3. Biological rhythms. Biological rhythms, or the natural cycle of change in endogenous chemicals or functions, are predominantly influenced by the master "clock" located in the suprachiasmatic nucleus of the mammalian brain (Gillette and Tischkau, 1999). While it is unclear that a central "clock" is present in all vertebrates, the whole body or cells in specific organs are sensitive to a biological clock that regulates physiological functions and responses in a rhythmic manner (reviewed in Steindal and Whitmore, 2019). For example, hormones are rarely secreted at a constant level throughout the day, but instead undergo diurnal or phasic fluctuations (Williams et al., 1990). Rhythms may be circadian, fluctuating on a 24-h cycle that the body initiates as early as development (Carraco et al., 2022), or can change on a seasonal basis (Wingfield and Farner, 1978). For example, temperate-dwelling vertebrates, such as songbirds and rodents, undergo seasonal changes in gonad size; thus, more pronounced levels of reproductive hormones are secreted from the gonad during the breeding season. However, during the non-breeding season, the reproductive tissues regress and circulating levels of gonadal steroids (e.g., testosterone, estradiol, and progestins) are low. During this time of the year, behaviors are predominantly regulated through extragonadal steroids, such as those produced by the brain or adrenal glands (Munley et al., 2018; Pradhan et al., 2010; Do Rego et al., 2009). Thus, animals with different gonadal physiologies (e.g., testes, ovaries, ovotestes) may exhibit distinct degrees of sex variability that covary with the seasons. When individuals are not actively producing gametes, there may be substantially less sex variability in other sex-associated traits, whereas during the breeding season, there may be more nuanced effects of hormones on sex-asscociated traits that do not differ in the same manner as the gonads (Smiley et al., 2022). Similar behaviors can also be regulated by different mechanisms based on the season and may influence, be influenced by, or act independently of gonadal physiology or hormones (Munley et al., 2022b; Pradhan et al., 2010; Quintana et al., 2021).

3.1.2. Contextual factors

3.1.2.1. Testing conditions. When designing studies to measure sex variable social behavior, several considerations should be made, including explicitly stating experimental contexts and conditions that may influence outcomes. Such factors, from experimenter identity (Georgiou et al., 2022) to the time of day, can impact complex behavioral interactions and should be acknowledged when discussing experimental results. All investigators, regardless of whether they study traditional or non-traditional animal models, should present their findings and discuss the associated limitations of their experiments in light of controlled and uncontrolled variables. Sex-associated traits, especially concerning reproduction and aggression, involve interactions with conspecifics and usually occur only under specific circumstances or contexts within an organism's lifespan (Pradhan et al., 2015a). Within the boundaries of a laboratory setting, experiments in behavioral neuroscience are typically designed to focus on one behavioral paradigm to uncover neural circuits. For example, in rodents, aggression is typically studied using resident-intruder assays (Fuxjager et al., 2009; Munley et al., 2022a, 2022c), social recognition is assessed using habituation/dishabituation tests, long-term social bonding/affiliation is measured through partner preference tests, and social approach or avoidance tests are used to investigate fear and anxiety (reviewed in Lee and Beery, 2019). While these approaches are well-established, these experiments are rather simplistic and usually dichotomized when considered within a larger social context, when multiple variables (e.g., social experience, gonadal hormones) are known to influence the expression of sex-associated behaviors (Guthman and Falkner, 2022). We recommend that at least two complementary behavioral tests be chosen in light of ecological relevance for the particular species being investigated. When possible, the implementation of new machine learning-assisted behavior tracking tools (Pereira et al., 2022) can further disentangle these complex relationships in multiple social contexts while minimizing experimenter bias.

3.1.2.2. Housing conditions. Another important issue that should be addressed and considered when drawing conclusions is how organisms are obtained and housed – whether they are wild-caught and brought to the laboratory, laboratory-bred, or studied completely in the wild (Calisi and Bentley, 2009). The type of housing conditions, such as the size of an enclosure, housing enrichment provided in the design of the enclosure, availability of territories and nesting sites, and whether organisms are socially isolated or socially housed, may also affect sex-associated traits. Moreover, if animals are socially housed, the degree of visual and pheromonal interaction with conspecifics and whether they are in sex-segregated groups, mixed sex groups, mixed age groups, or pair housed are all important points to consider. For example, if an individual generally lives in social hierarchies in mixed sex/age groups, but is solitarily housed after a pharmacological manipulation or moved to a testing chamber for a battery of tests with conspecifics for 5 min, its behavior might be different than if the observations occur in its home cage in a semi-natural environment, where resources are provided *ad libitum* and there is minimal competition. Further, group size might strongly affect the complexity of social interactions, such that a greater repertoire of behaviors might be expressed in organisms that are group housed rather than pair housed. For example, in multi-female groups of rhesus monkeys (*Macaca mulatta*), males direct their reproductive behavior toward females only during the peri-ovulatory phase; however, in male-female pair housed conditions, males attempt copulations during both the follicular and peri-ovulatory phases (Wallen and Winston, 1984). Thus, environmental context can affect social group dynamics and the expression of sex-associated traits and should be considered as testing variables.

3.1.3. Variables to consider measuring

3.1.3.1. Key biomolecules. To date, two classes of hormones have been a major focus of research investigating sex variability in the neuroendocrine regulation of behavior: neuropeptides (especially the nonapeptides arginine vasopressin and oxytocin) and steroids (reviewed in Balthazart et al., 2018; Caldwell and Albers, 2016; Carter, 2017; Donaldson and Young, 2008; McCarthy et al., 2009; Remage-Healey, 2014). Neuropeptide and steroid production and their signaling mechanisms can be assessed at multiple levels, including the concentration of hormone present, the activity or expression of synthetic or metabolic enzymes, and the abundance of receptors. In general, tissue- and/or region-specific quantification of hormone production or receptors are preferable over systemic measurements (e.g., blood, fecal, saliva, urine, and hair samples), as they provide greater insight into how these metabolic pathways and signaling mechanisms are changing locally within an organism. This concept is especially relevant for neuropeptides and steroids: changes in the production of these biomolecules and their receptors are often restricted to specific tissues, and their underlying mechanisms are plastic and can shift rapidly based on an organism's external environment, which may not be detected using systemic measures of hormones (reviewed in Balthazart et al., 2018; Cornil and Charlier, 2010; Do Rego et al., 2009; Pradhan et al., 2015a; Schmidt et al., 2008).

3.1.3.2. Morphological characteristics. The outward (e.g., size, shape,

color, pattern, structure) and inward (e.g., bones, organs) appearance of body parts, especially form-function relationships as they pertain to social communication and reproduction, has been of interest to many neuroendocrinologists. For example, external morphological indicators of reproductive condition have been used as proxies to plan experimental timelines and endpoints in many species, such as brood patches in songbirds (Lea and Klandorf, 2002), gravidity in fish and frogs (Reyer and Bättig, 2004; West, 1990), sexual swelling and red coloration around the buttocks and vulva of primates (Nunn, 1999), and anogenital distance in rodents (Flores et al., 2018). Further, morphology, as it pertains to organismal anatomy, has been historically separated into four different phenomena based on variability and plasticity: developmental plasticity, polyphenism, phenotypic flexibility, and life-cycle staging (reviewed in Piersma and Drent, 2003). In the context of the neuronal networks that mediate social behavior, anatomical and functional network distinctions are key considerations to hold while designing and interpreting studies of sex variable behavior (Kelly, 2022).

3.1.3.3. Epigenetic influences. The regulation of fixed versus plastic sexual differentiation is a fundamental question in reproductive biology and sexual selection. Both genetically- and environmentally- mediated specifications could explain the developmental processes that regulate gonadal determination and sexual phenotypes (Gegenhuber and Tollkuhn, 2019). Interplay of the hormonal milieu and other environmental factors during critical periods of development can lead to activation or repression of genes involved in sex differentiation. Epigenetic mechanisms, such as DNA methylation (Auger et al., 2011), microRNAs (Morgan and Bale, 2012), chromatin accessibility (Gegenhuber et al., 2022), CpG binding proteins (Kurian et al., 2008), X chromosome inactivation (Jeon et al., 2012), and histone modification (Matsuda et al., 2012; Murray et al., 2009), regulate key gonadal activation genes during development and/or adulthood. Given that steroid hormone receptors are highly expressed in the mammalian brain (Denney et al., 2023) and act primarily through epigenetic regulation of gene expression, the identification of epigenetic modifications and gene expression profiles in various hormone contexts remains a central question in neuroendocrinology (Sun and Tollkuhn, 2023). These mechanisms culminate in brain and gonadal differentiation, along with the expression of sex variable behavior that is dynamic across the lifespan, even in sex-stable species (Schwarz et al., 2010).

3.2. Model organisms for studying sex diversity and variability

Sex diversity in the neuroendocrine regulation of various phenotypes can arise in many ways and may have distinct functional consequences across species. Although studying sex variability and diversity in sex-stable species has been a major focus of neuroendocrinology research for decades, other animal models that utilize diverse reproductive systems and life-history strategies have become more prevalent in recent years, yielding critical insight into the neuroendocrine mechanisms underlying phenotypes across sex. In this section, we highlight several examples of animal models that can be used to study sex diversity in neuroendocrine processes and behavior, including both sex-stable and sex-dynamic species. Characterizing sex diversity and variability using animal models that represent the range of reproductive and sexual systems displayed in nature is essential for revealing how neural and hormonal mechanisms vary across sex and among species.

3.2.1. Sex-stable species

For some species, sex-associated traits are relatively stable across the lifespan. In these cases, three different phenotypic variants are generally observed across sex (as summary categories) – Type I: Phenotypes that consist of multiple forms, one of which is more prevalent in one sex and less prevalent or entirely absent in other(s), Type II: Phenotypes that exist on a continuum and the average is different across sex, and Type III: Phenotypes that are the same or similar across sex, but the neuroendocrine underpinnings are distinct (sex convergence; Table 1; based on definitions in McCarthy et al., 2012). Here, we present several examples that illustrate how variability in sex-associated phenotypes can be observed in animals with sex-stable systems and discuss future directions that can be pursued using sex-dynamic organisms.

3.2.1.1. Type I: Phenotypes which are more prevalent in one sex and less prevalent or absent in the other(s). "Sexual heteromorphism" (Table 2) is the occurrence of two (dimorphism) or more (multimorphism) qualitatively distinct morphs in a sexually reproducing species, where a morph is a set of sex-associated correlated or covarying phenotypic traits belonging to an individual (Table 1). This phenomenon is commonly observed for traits that are directly associated with reproduction, such as courtship singing and displays and mating behavior. For example, male manakins (subfamily *Piprinae*) perform elaborate courtship displays that include colorful plumage and high-speed acrobatics, and these displays are not typically observed in female manakins (reviewed in Fuxjager et al., 2023; Schlinger et al., 2013). In these species, rapid limb movements are regulated by exceptionally fast wing displays that are driven by muscle kinetics, which are likely controlled by steroid receptors and enzymes present in the brain, muscles, and spinal cord (Eaton et al., 2018; Feng et al., 2010; Fusani et al., 2014; Fuxjager et al., 2012, 2016). Female manakins also express the same steroid-related genes in the brain, muscles, and spinal cord as males, but generally show lower levels of expression (Feng et al., 2010; Fuxjager et al., 2012). While they do not naturally perform these complex displays, testosterone treatment can activate acrobatic movements in females to some extent, although not with the complete repertoire observed in males (Day et al., 2007). These findings demonstrate that physiological sex variability can be subtle and highlight the immense plasticity within the neuroendocrine pathways that modulate the expression of these traits. Type I sex variability is also observed in species with diverse mating systems. For example, jacanas (family *Jacanidae*) have a socially polyandrous mating system, in which females mate with multiple males simultaneously in one breeding season and males perform the majority of parental care (Emlen and Wrege, 2004). Female jacanas tend to be larger and more competitive than males, but do not have higher levels of circulating androgens (Lipshutz and Rosvall, 2020). Similarly, in the cichlid fish *Julidochromis marlieri,* females are larger and more territorial, whereas males are primarily responsible for parental care (Schumer et al., 2011). Including model organisms with diverse mating systems and studying differences in traits that span multiple levels of biological organization will be essential for elucidating how mechanistic variation can produce sex diversity and variability.

3.2.1.2. Type II: Phenotypes that exist on a continuum and the average is different across sex. To date, Type II sex variability, in which a physiological or behavioral endpoint exists along a quantifiable continuum and the average differs across sex, has been revealed in several species. One of the most well-studied examples of Type II sex variability is stress responsivity (reviewed in Bale and Epperson, 2015; Bangasser and Valentino, 2012). Broadly, female vertebrates tend to be more sensitive to stress manipulations, including exhibiting more pronounced changes in neural activity, neuroanatomy, hypothalamic-pituitary-adrenal axis function, and displaying more anxiety-like behavior than similarly aged male conspecifics (reviewed in Bangasser and Wiersielis, 2018; Heck and Handa, 2019; Trainor, 2011; Shepard et al., 2016; Wellman et al., 2020). Sex variability in stress responses have been demonstrated in rodents and birds across a variety of contexts, such as social stressors (e.g., social defeat, social isolation) and restraint stress. These responses have also been characterized across life-history stages, from development to adulthood (e.g., Marasco et al., 2012; Spencer et al., 2009; reviewed in Bale and Epperson, 2015; Bangasser and Valentino, 2014;

Laman-Maharg and Trainor, 2017; Zilkha et al., 2021), suggesting that these mechanisms may be evolutionarily conserved across species. Collectively, Type II sex variability presents an excellent opportunity to explore how differences in neuroendocrine circuits and their regulation can culminate in diverse physiological and behavioral phenotypes.

3.2.1.3. Type III: Phenotypes that are the same or similar across sex, but the neuroendocrine underpinnings are distinct. In contrast to Type I and Type II sex variability, there are relatively fewer cases in which distinct neural or hormonal mechanisms converge on similar behaviors. This phenomenon, which is referred to as sex convergence (Table 2), has been proposed as a means to prevent overt differences in behavior by compensating for naturally occurring sexual variation in physiology (reviewed in De Vries and Boyle, 1998; De Vries and Södersten, 2009; De Vries, 2004). The neuroendocrine regulation of seasonal aggression in Siberian hamsters (*Phodopus sungorus*; reviewed in Demas et al., 2023; Munley et al., 2022b) is an excellent example of sex convergence. Unlike most rodents, in which adult males are primarily responsible for territory defense and adult females often limit aggression to pregnancy and lactation, both male and female Siberian hamsters are highly territorial and exhibit an increase in aggressive behavior during the non-breeding season (Jasnow et al., 2000; Scotti et al., 2007). Although male and female hamsters exposed to short-day photoperiods display equivalent increases in aggression (Munley et al., 2023; Munley et al., 2022c), there is emerging evidence that this behavioral phenotype is associated with distinct changes in steroidogenesis in the adrenal glands and brain. Short-day male hamsters exhibit an increase in 3β-hydroxysteroid dehydrogenase activity in the adrenal glands relative to long-day males, whereas short-day females have lower 3β-hydroxysteroid dehydrogenase activity in the adrenals and anterior hypothalamus than long-day females (Munley et al., 2022c). In addition, while short-day hamsters show similar changes in estrogen receptor 1, aromatase, and 5α-reductase mRNA expression in the arcuate nucleus, a brain region that controls reproduction, there are sex variable effects of short days on gene expression in brain regions associated with aggression (e.g., medial preoptic area, anterior hypothalamus, and periaqueductal gray; Munley et al., 2023). Thus, these findings suggest that male and female hamsters exhibit different neuroendocrine responses that converge at a similar behavioral endpoint: increased aggression during the non-breeding season.

It is important to note that, although sex convergence appears to be a less common phenomenon than Type I and II sex variability, it is likely that these mechanisms are overlooked in endocrine studies, because researchers often assume that if a physiological or behavioral phenotype is similar across sex, then they are modulated by the same neuroendocrine mechanisms. Thus, there is probably a far greater number of sex convergent processes that exist throughout the animal kingdom than those currently described in the literature. Characterizing the neuroendocrine control of sex convergent traits will provide valuable insight into how distinct compensatory mechanisms can evolve to maintain social behaviors that are important for survival and reproductive success, despite sex variability in physiology. More broadly, future research that focuses on elucidating these mechanisms will be important for facilitating a shift to a more inclusive and accurate description of sex as a biological phenomenon.

3.2.2. Sex-dynamic species

Sex change (also referred to as sequential or serial hermaphroditism) is an adaptation that allows organisms to increase their reproductive success by transforming to another sex in response to changing environmental conditions (reviewed in Ghiselin, 1969; Munday et al., 2006; Policansky, 1982). Sex-dynamic organisms are valuable model systems because these species allow us to naturally recapitulate the development of sex organs and sex variable traits in reproductively mature individuals. Historically, most studies examining mechanisms of sex change have focused on gonadal reorganization, because this process is essential for producing viable gametes and indicate a functional sex change (reviewed in Nagahama et al., 2021; Vega-Fruits et al., 2014). Complete expression of an alternative gonad, however, involves expression of sex-biased behavior, secondary sex characteristics, and synchronous orchestration of external morphological features. Because these traits manifest at different rates, organisms that change sex exist across a spectrum of phenotypes that are in flux and, over time, arrive at a new steady state. Thus, the expression of sex-associated traits at the molecular, physiological, and behavioral levels, including the rewiring of internal anatomical structures and neural systems, can occur asynchronously (reviewed in Capel, 2017; Gemmell et al., 2019; Todd et al., 2016). To date, few species have been used to study the physiological basis of sex change and its associated phenotypes; thus, sex-dynamic species are currently underutilized as models of sex variability.

3.2.2.1. Protogynous species. In most protogynous species, individuals are born with female reproductive anatomy and are capable of transitioning to reproductive males during adulthood. This strategy is the most common form of sequential hermaphroditism in teleost fishes and is especially prevalent in species exhibiting polygynous mating systems, in which there is intense competition between males for mating opportunities (reviewed in Gemmell et al., 2019). Protogyny has been described in many species of wrasses (family *Labridae*), parrotfishes (family *Scaridae*), groupers (family *Epinephelidae*), and angelfishes (family *Pomacanthidae*) and in some species of gobies (family *Gobiidae*), crustaceans [e.g., isopods (order *Isopoda*), and tanaidaceans (order *Tanaidacea*); reviewed in Gemmell et al., 2019; Godwin, 2019; Subramoniam, 2017]. In particular, the neuroendocrine mechanisms regulating protogyny have been well-studied in the bluehead wrasse (*Thalassoma bifasciatum*). In the absence of the dominant, terminal-phase male in a given social group, transitioning individuals of this species exhibit behavioral changes within hours, attain dominance status, and can produce functional testes within 7–10 days (Warner and Swearer, 1991). Furthermore, in the absence of a terminal-phase male, large female wrasses whose gonads were surgically removed attain dominance status and display male-typical spawning behavior (Godwin et al., 1996). This discovery was foundational because it demonstrated that behavioral changes can occur independently of gonadal factors and led to the hypothesis that the brain, not the gonads, regulates behavioral sex change. This shift in paradigm allowed scientists to re-evaluate and expand the definition of "sex" to not only the production of viable gametes, but also other key characteristics and traits, such as reproductive behavior or coloration. Transcriptomic analysis of both brain and gonadal tissue of wrasses during the process of sex change shows that genes that favor male-biased development exhibit increased expression early in the sex change process [e.g., *amh*, *doublesex* and *mab-3 related transcription factor 1* (*dmrt1*), *sox9*, and gonadal soma-derived factor (*gsdf*)]. Concurrently, the expression of female-promoting genes [e.g., folliculogenesis specific bHLH transcription factor (*figla*), aromatase (*cyp19a1*), 17β-hydroxysteroid dehydrogenase (*hsd17b*)] is downregulated later in the process. Sex change in this species also involves epigenetic reprogramming, which allows for the re-evaluation of the relative plasticity of genes that regulate gonad determination (Todd et al., 2019). Additional studies in other protogynous species will provide further insight into the molecular and neural regulation of sexual plasticity in vertebrates.

3.2.2.2. Protandrous species. Protandrous species consist of individuals who reproductively mature as males and are capable of transitioning to functionally reproductive females during adulthood. Protandry is generally less common than protogyny and typically occurs in small, stable groups with either a monogamous mating pair or a random mating system (i.e., a system in which an individual is equally likely to mate with any other individual in a population), such that territorial

defense and/or intense sperm competition is absent (reviewed in Gemmell et al., 2019; Munday et al., 2006). This strategy has been documented in several species of sea bream (e.g., *Acanthopagrus*, *Sparus*, and *Lithognathus* sp.; reviewed in Gemmell et al., 2019), crustaceans [e.g., mole crabs (*Emerita asiatica*), Manning grass shrimp (*Thor manningi*), and prawns (genus *Pandalus*); reviewed in Chiba, 2007; Ye et al., 2023], and mollusks [e.g., marine snails (family *Calyptraeidae*), freshwater mussels (*Elliptio complanata*), and common limpets (*Patella vulgata*); reviewed in Lesoway and Henry, 2019; Wright, 1988]. The neuroendocrine regulation of protandrous sex change, however, has been most extensively studied in anemonefishes (*Amphiprion* and *Premnas* sp.; Casas et al., 2022; Godwin, 2019; Hattori and Casadevall, 2016). Male clownfish (*Amphiprion* sp.), for example, transition to female when the largest individual in a social group, the dominant female, is lost (Fricke and Fricke, 1977; Godwin, 2019). This process is associated with a suite of physiological and behavioral changes, including invagination, changes in circulating steroid levels, alterations in arginine vasotocin and isotocin immunoreactivity and steroidogenic gene expression in the brain, and increased aggression toward the male and subordinate non-breeding individuals within a social group (Casadevall et al., 2009; Casas et al., 2016; Godwin, 1994; Iwata et al., 2010; Parker et al., 2022). In contrast, sex change is age and/or size-dependent in some species of sea bream and most protandrous crustaceans and mollusks (reviewed in Gemmell et al., 2019; Wright, 1988; Ye et al., 2023). For example, black porgy (*Acanthopagrus schlegeli*), Australian barramundi (*Lates calcarifer*), and gilthead seabream (*Sparus aurata*) sexually mature and reproduce as males for their first few years of life before changing sex to female (Guiguen et al., 1994; Liarte et al., 2007; Wu and Chang, 2013), but the precise mechanisms that trigger this transition have yet to be characterized. These species can enhance our understanding of how an organism's external environment, as well as its internal state, control the timing of sex change during adulthood.

3.2.2.3. Bidirectional sex changing species. Most hermaphrodites are only capable of changing sex once. Some sex-dynamic species, however, are bidirectional sex changers and retain sexual plasticity (i.e., the ability to change from male to female or from female to male) for some or all of their lifetime. Bidirectional sex change (also referred to as serial sex change) is an especially useful reproductive strategy for animals that experience limited mating opportunities and has been reported in some species of gobies [e.g., coral-dwelling gobies (genera *Gobiodon* and *Paragobiodon*), bluebanded gobies (*Lythrypnus dalli*); reviewed in Black and Grober, 2003; Pradhan et al., 2015a], wrasses [e.g., bluestreak cleaner wrasse (*Labroides dimidiatus*), star-bamboo leaf wrasse (*Pseudolabrus sieboldi*)], dottybacks (family Pseudochromidae), groupers [e.g., coral grouper (*Cephalopholis miniata*), orange-spotted grouper (*Epinephelus coioides*), Hong Kong grouper (*Epinephelus akaara*)], angelfishes (genus *Centropyge*; reviewed in Gemmell et al., 2019; Munday et al., 2010), and mushroom corals (family *Fungiidae*; Loya and Sakai, 2008). Specifically, bluebanded gobies have been instrumental for understanding social hierarchies and behavioral changes (Rodgers et al., 2007; Solomon-Lane and Grober, 2015; Solomon-Lane et al., 2015), morphogenesis of external genitalia and internal reproductive organs (Carlisle et al., 2000; Pradhan et al., 2014a; Schuppe et al., 2016), and the neuroendocrine processes underlying sexual plasticity in juveniles and adults (Black et al., 2004; Solomon-Lane and Grober, 2012; Solomon-Lane et al., 2013; Pradhan et al., 2014b; Solomon-Lane et al., 2016; reviewed in Perry and Grober, 2003). The earliest measurable marker of sex change in this species is often behavioral because it is triggered by a change in social structure that group members immediately respond to (Pradhan et al., 2014a, 2014b; White et al., 2023). While body size can be an important factor, fish likely use multiple cues to assess their rank in a social hierarchy (Rodgers et al., 2007). During the sex change process, gobies may display sex-specific behaviors [e.g., 'male-typical' behaviors (courtship jerk movements, parenting) or 'female-typical behaviors' (courtship solicitation displays, agonistic behavior; Pradhan et al., 2014a; Pradhan et al., 2015b; A.M. Jirik and D.S. Pradhan, unpublished results)] after physiological changes have been initiated, but they may not yet be capable of reproducing because complete gonadal transformation and/or the expression of behaviors that require morphological features might take longer to manifest (Lorenzi et al., 2012; Pradhan et al., 2014a; Solomon-Lane et al., 2014). The mechanisms underlying these rapid behavioral changes, especially those related to agonistic behavior, are likely similar to those of sex-stable species, such as changes in steroidogenic enzyme activity in the brain (Pradhan et al., 2010; Black et al., 2005). Together, these findings underscore how behavior and reproduction can be discordant in sex-stable and sex-dynamic species, a phenomenon that cannot be accounted for in traditional binary operationalizations of sex.

3.3. Mechanistic approaches for studying multi-leveled traits

3.3.1. Pharmacological and genetic manipulations

Traditionally, pharmacological approaches, which involve the administration of hormone receptor agonists or antagonists, have been used to investigate sex variation in neuroendocrine mechanisms. While these methodologies are still widely used and have provided invaluable insight, they can lack specificity in their targets [e.g., tamoxifen, an estrogen receptor antagonist that non-selectively binds to both subtypes of estrogen receptors (estrogen receptor α and β)] and have varying degrees of effectivity, depending on the model organism that is being studied (reviewed in Cunningham et al., 2012; Novick et al., 2020). In recent years, the advancement of molecular genetic techniques has enabled researchers to study the sex-associated effects of neuroendocrine substrates on physiological and behavioral phenotypes with greater precision and in a greater range of model systems (reviewed in Alward et al., 2023; Boender and Young, 2020; Juntti, 2019; Woodcock et al., 2017). Genome editing approaches, such as CRISPR/Cas9, provide an excellent opportunity to examine the functional significance of a gene of interest throughout an organism's lifespan (reviewed in Barrangou and Doudna, 2016; Juntti, 2019). Because CRISPR/Cas9 gene constructs are typically inserted early during development and result in genetic deletion at the organismal level, this methodology allows researchers to examine how the absence of a gene of interest affects physiology and behavior at different life-history stages. CRISPR/Cas9 gene editing has been used in numerous non-traditional model systems, including teleost fishes [African cichlid fish (*Astatotilapia burtoni*; Alward et al., 2020; Juntti et al., 2016), medaka (*Oryzias latipes*; Nishiike et al., 2021; Yokoi et al., 2020), Nile tilapia (*Oreochromis niloticus*; Jiang et al., 2017; Yan et al., 2019)], birds [Japanese quail (*Coturnix japonica*; Lee et al., 2019)] and rodents [Syrian hamsters (*Mesocricetus auratus*; Taylor et al., 2022), prairie voles (*Microtus ochrogaster*; Berendzen et al., 2023; Horie et al., 2019), California mice (*Peromyscus californicus*) and African spiny mice (*Acomys cahirinus*; Boender et al., 2023)]. Other molecular genetic manipulations, such as chemogenetics [e.g., Designer Receptors Exclusively Activated by Designer Drugs (DREADDs)], optogenetics (e.g., channelrhodopsin), and viral vectors [e.g., lentiviruses, adeno-associated viruses (AAVs)], are typically administered during adulthood and cause a rapid, but relatively long-term perturbation in the tissue in which it is inserted (reviewed in Fernandez-Ruiz et al., 2022; Haggerty et al., 2020; Smith et al., 2017). Such techniques are particularly useful for organisms that do not have a fully sequenced genome and have been successfully developed for some non-traditional species [e.g., Japanese quail (Scott and Lois, 2005), Syrian hamsters (Been et al., 2013; Hedges et al., 2009), and Siberian hamsters (Munley et al., 2022a)]. Methodologies have also been developed to enable intersectional viral manipulations, allowing for interrogation of multiple genetic variables simultaneously to better dissect interacting sex variables, such as gene regulatory networks (Pouchelon et al., 2022). Collectively, these approaches will open novel avenues to explore how different biomolecules regulate neural and hormonal processes and how these mechanisms vary

across sex.

3.3.2. *Techniques for measuring biomolecules and characterizing their synthetic and signaling pathways*

Examining the synthesis, metabolism, and signaling mechanisms of neuroendocrine substrates (e.g., steroid hormones, neuropeptides) is essential for elucidating sex-associated effects on physiology and behavior. Concentrations of hormones or the activity of individual synthetic enzymes can be measured in circulation and tissues using antibody-based techniques, such as enzyme immunoassays (EIAs) and radioimmunoassays (RIAs). Alternatively, levels of multiple biomolecules or enzymes of interest can be quantified simultaneously using mass spectrometry approaches such as liquid chromatography-tandem mass spectrometry (LC-MS/MS), a highly sensitive technique that is capable of quantifying minute concentrations of biomolecules from various types of biological samples. LC-MS/MS can also be used to screen for, detect, and measure concentrations of novel biomolecules, an approach that is particularly useful for researchers studying non-traditional model organisms (reviewed in Munley et al., 2022d; Taves et al., 2011). In contrast, the expression of neuropeptide and steroid

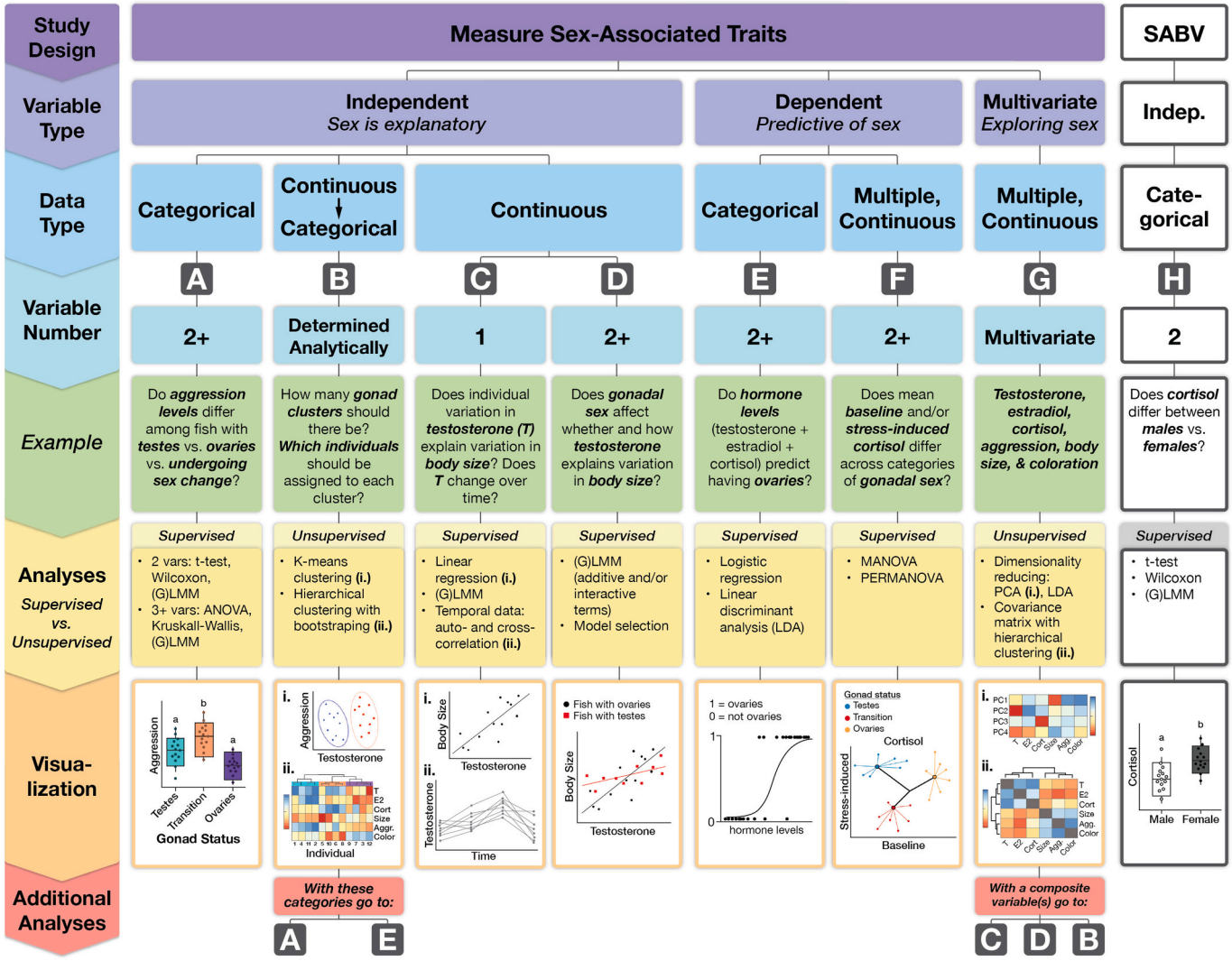


Fig. 2. Approaches for the statistical analysis of sex-associated traits. Flow chart of example approaches for analyzing datasets with sex-associated variables. These analyses answer questions about whether and how sex-associated variables explain variation in dependent variables of interest, how independent variables of interest explain variation in dependent sex-associated variables, and how sex-associated variables (within and/or across levels of analysis) relate to one another. The examples match those in the text where possible, but the approaches are widely applicable and flexible. The choice(s) of analyses should follow directly from the research question(s), and it is likely that multiple approaches will be used in the analysis of an integrative dataset. **A)** Analyzing sex as an independent, categorical variable with two or more levels. Note that depending on the research question, study design and limitations, and model system, it may still be appropriate to analyze a single sex-associated variable with two levels (i.e., univariate and binary), for example, if sex is explicitly defined as gonadal sex and all experimental subjects have either ovaries or testes. **B)** Taking continuous sex-associated variable(s) (one or more) and creating a categorical variable (two or more levels). **C)** Analyzing sex as an independent, continuous variable with one level or **D)** more than one level (additive and/or interactive terms). **E)** Analyzing two or more dependent, categorical sex-associated variables. **F)** Analyzing two or more dependent, continuous sex-associated variables (additive and/or interactive terms). **G)** Exploratory approaches for how multiple sex-associated variables relate to one another for an integrative, comprehensive description of sex as a multivariate phenotype. This approach can be used to create fewer composite variables [e.g., principal components (PCs)], which can then be analyzed as independent sex-associated variables. **H)** Typical analyses of sex as a biological variable (SABV) include a univariate, binary sex-associated variable, with SABV as the independent variable, explaining variation in a dependent variable of interest. *Abbreviations: Agg, aggression; Cort, cortisol; E2, estradiol; (G)LMM, (generalized) linear mixed model; LDA, linear discriminant analysis; PCA, principal components analysis; T, testosterone; vars, variables.*

receptors is typically assessed using histological methods, such as *in situ* hybridization, immunohistochemistry, and receptor autoradiography, which enable researchers to identify sites of anatomical localization within tissues and provide insight into whether the downstream actions of receptors are non-genomic or genomic. The expression of genes encoding enzymes and receptors or their proteins can also be measured using molecular biology approaches, such as quantitative polymerase chain reaction (PCR) or western blotting, or using more broad-scale approaches to gain a more comprehensive view of changes in gene or protein expression or epigenetic markers within a particular tissue, such as transcriptomic, proteomic, and epigenomic profiling (reviewed in Crews, 2010; Marguerat and Bähler, 2010; Patterson and Aebersold, 2003; Ruiz-Ortiz and Tollkuhn, 2021). Moreover, the effects of hormones on living cells can be assessed using electrophysiology, in which specific cells within a tissue of interest are stimulated and their electrical activity measured (e.g., Remage-Healey and Bass, 2005; Spool et al., 2021), or *ex vivo* tissue preparations, such as organotypic or slice cultures (e.g., Holloway and Clayton, 2001; Tam and Schlinger, 2007). Because researchers typically focus on one of these levels of analysis, the integration of these tools will be essential for providing a more holistic understanding of the mechanisms underlying variation and fluidity across sex-associated traits. Additionally, it is important to note that many of these techniques provide static snapshots of an organism's internal state and, thus, are unable to account for the dynamics of synthesis, metabolism, and signaling of relevant biomolecules. These limitations must be considered and openly discussed in both experimental design and interpretation.

3.4. How to statistically analyze sex

Analysis of sex-associated (and non-sex-associated) traits follows from the research question(s), experimental design, variable(s) measured, and methods used for measurement, all of which have implications for the interpretation of results. Here, we provide a practical discussion of ways to incorporate sex variable study design and analyze datasets with sex-associated traits using a range of explanatory, predictive, and exploratory approaches (supervised and unsupervised, Fig. 2). These approaches can be generalized across study species and experiments. First, although we focus on sex-associated traits because of the topic of this paper, these integrative approaches are appropriate for non-sex-associated traits and/or traits with an unknown or variable relationship/relevance to sex. Second, there are approaches that we discuss separately for clarity, but are often used together, in parallel, or sequentially. In the text and Fig. 2, we give examples of how analyses can be used together. Third, as we include a breadth of approaches, we encourage readers to visit the cited references for a deeper understanding of the mathematical basis, assumptions, and applications of these methods. Finally, this section is not an exhaustive review. There are other useful analytical approaches (e.g., Beltz et al., 2019; Rich-Edwards et al., 2018; Miller et al., 2017; Clayton, 2016, 2018; Maney, 2016; Joel, 2021; Maney and Rich-Edwards, 2023), as well as other ways to use these analyses.

3.4.1. Addressing sex variability requires a sex variable study design

When designing a new experiment, our integrative, sex variable framework can aid in identifying the study lens, measurable variables, and experimental design (see **Section 2**; Fig. 1). The research question (s) will determine how "sex" is treated in subsequent statistical analyses (e.g., independent vs. dependent variable, single vs. multivariate) and the interpretation of the results. For example, finding a statistically significant difference among sexes is descriptive, but not yet causal, with an interpretation such as "males are more likely to exhibit…" or a conclusion that "there is a sex difference" (Gowaty, 2018). Finding a sex difference is a starting point rather than an ending point, and secondary or exploratory analyses can then be conducted to address the possibility of sex variability. These tests can be followed by power analyses to assess categorical sex variability, in line with previous Sex as a Biological Variable initiatives (Diester et al., 2019). We caution against over-interpretations; categories of sex should not become a proxy for specific traits, nor should the state of belonging to a sex category be interpreted as an independent causal variable (Miyagi et al., 2021). Additionally, researchers should opt to include all individuals in the study design, sampled randomly from the population, independent of sex. By measuring operationally-defined sex-associated variables across levels of analysis, we can apply a more rigorous and unbiased approach to determining how sex-associated traits are influenced by one or multiple variables, in interaction within and across levels (Fig. 1). Such studies have more interpretive power: if "sex" has an effect, by what means? Where do we think that sex variability originates from - genetic factors, hormones, social context, anatomy?

3.4.2. Sex as explanatory: Sex-associated trait(s) as the independent variable(s)

There are multiple ways to test whether variation in sex-associated traits significantly explains variation in a dependent variable of interest, including what is perhaps the most common question: does this dependent variable of interest differ significantly across sex?

3.4.2.1. Analyzing sex-associated trait(s) as categorical or continuous. How sex is measured, and at which levels, affects how the data can be analyzed. Whereas a categorical variable must be analyzed with discrete categories, a continuous variable may be analyzed as continuous or by forming clusters (Fig. 2B, see below). The first step should always be to visualize the distribution of the sex-associated trait. Sex-associated traits may follow a binary, bimodal, or a more complex distribution. If there is an obvious bimodal or multimodal distribution, it may be reasonable to cluster data by eye. The distribution of the data may also be informed by the biology of the organism. For example, in a population of sex-changing fish, there may be two major peaks for gonadal morphology, measured as percent ovarian tissue: approximately 100 % for ovary and 0 % for testis. We would also expect some individuals (e.g., those undergoing sex transition) to have gonads with both ovarian and testicular tissue in more equal balance. In this scenario, the data could be analyzed as a continuous variable (percent ovarian tissue) or as a categorical variable (ovaries, testes, and transitioning/intermediate). Potential differences in sample size among these categories (e.g., in sex-skewed populations) is a consideration for certain statistical tests. If the sample size in one category is too low for statistical analysis, a description of that category could be included instead. Creating categories from a continuous distribution can lead to a loss of precision, or may even be actively misleading, especially if the data are not well suited to categorization. For example, if there are two data clusters based on gonadal morphology — 50 % or more ovarian tissue versus <50 % ovarian tissue — fish with 49 % and 51 % ovarian tissue are more similar to each other compared to fish with 0 % or 100 % ovarian tissue, respectively. There are multiple statistical approaches for analyzing sex-associated traits as continuous variables (see below). Although it may be less common to measure certain sex-associated traits as continuous in some fields, we encourage doing so when it is feasible and relevant because of the precision it can add to the analysis and the information it can provide for data interpretation.

3.4.2.2. Categorical sex-associated independent traits(s). If analyzing a sex-associated trait as a categorical variable, common approaches include a *t*-test (if two categories) or a one-way ANOVA (if three or more categories) to determine whether a dependent variable differs significantly between those categories (Fig. 2A). For example, a t-test can evaluate if there are significant differences in body size (dependent variable) between fish with different gonadal types (independent variable). Non-parametric approaches, including Wilcoxon signed-rank and Kruskal-Wallis tests, can also be used to compare a dependent variable

among categorical groups. It is valuable, and increasingly required by journals, to report effect sizes (e.g., Cohen's D, eta-squared), which measure the magnitude of the effect of the sex-associated independent variable on the dependent variable (Nakagawa and Cuthill, 2007). If multiple statistical analyses are run to individually test the effects of different sex-associated independent traits on a dependent variable of interest, it is recommended to apply a false discovery rate or Bonferroni correction to reduce the probability of Type I errors (Noble, 2009).

3.4.2.3. Transforming continuous variables into categorical variables. If appropriate for the data and research question, there are multiple methods that take a continuous variable and separate experimental subjects into discrete clusters (i.e., every individual is assigned to only one grouping; Fig. 2B). These clusters are formed without relying on *a priori* categorizations that can introduce bias, especially with respect to groupings of sex. Importantly, whether the resulting clusters have biological meaning must be interpreted by the researchers. We describe two methods below, but there are many approaches that can be used (Charrad et al., 2014; Murtagh and Legendre, 2014).

3.4.2.3.1. K-means clustering. K-means clustering (Fig. 2B.i) is one of the most common clustering approaches, and it uses Euclidean distance between experimental subjects to define groupings that, together, contain all subjects. K-means can create a specific, predetermined number of clusters of subjects. For example, if a sex-associated trait is expected to be binary (e.g., ovaries versus testes), two clusters would be appropriate. However, K-means can also be used to create increasing numbers of clusters, which can then be evaluated [e.g., using the elbow method (Syakur et al., 2018), silhouette coefficient (Tambunan et al., 2020), and/or gap statistic (Tibshirani et al., 2001)] to determine the optimal number of clusters for a given dataset. K-means clustering can also be used to create clusters based on a single independent variable (e.g., percent ovarian tissue) or a composite independent variable calculated from a dimension-reducing technique like principal components analysis [PCA, e.g., principal component (PC) 1; Fig. 2G, see below]. K-means clustering can also be used to create clusters based on multiple independent variables, such as percent ovarian tissue and body size, or PC1 and PC2. This multidimensional clustering works best in low dimensions (i.e., fewer variables). There are important limitations for K-means clustering (and other methods that use Euclidean distance) in higher dimensions (see Shukla, 2014 and Ikotun et al., 2023 for further discussion).

3.4.2.3.2. Hierarchical clustering. In contrast to K-means, hierarchical clustering (Fig. 2B.ii) is an approach that does not require an *a priori* number of clusters. In this application of hierarchical clustering, the experimental subjects are assigned to groupings based on the values of one or more sex-associated traits. A bottom-up (i.e., agglomerative) or top-down (i.e., divisive) approach can be used, and both approaches are visualized in a dendrogram. In agglomerative clustering, all sex-associated traits start individually in their own cluster. In this analysis, variables that are more similar (based on a distance measure) are merged up the hierarchy. In divisive clustering, all variables start together in one cluster, and clusters that are more heterogeneous (based on a distance measure) are split down the hierarchy. Multiscale bootstrap resampling can be used to identify significant clusters of experimental subjects (Suzuki and Shimodaira, 2006). For example, in a study of male cichlid social and reproductive phenotypes, there was a binary expectation for dominant males to cluster separately from subordinate males. However, hierarchical clustering of dominance behavior and morphology revealed a third, intermediate cluster (Fulmer et al., 2017). These clusters can then be used to test for statistical differences in dependent traits of interest. For example, a one-way ANOVA (or Kruskal-Wallis test) could be used to test whether hormone levels differ significantly between subordinate, intermediate, and dominant males (Fig. 2A). Bootstrapping is important because hierarchical clustering will identify clusters even if none are expected.

3.4.2.4. Continuous independent sex-associated traits(s)

3.4.2.4.1. One independent variable. Linear or non-linear regression analyses are common approaches for testing whether variation in an independent sex-associated variable significantly explains variation in a continuous dependent variable (Fig. 2C). For significant associations (i.e., $p < 0.05$ or another appropriate alpha level), the r^2 value gives the percentage of variation in the dependent variable explained by the independent variable. Many experimental designs require more complex models (e.g., generalized linear mixed effects models, GLMMs) that can handle non-normal distributions, which are common in biology, one or more independent variables (fixed effects, which can be additive or interactive), and random effects (typically a grouping variable, e.g., clutch, social group; Harrison et al., 2018; Kumle et al., 2021). For example, linear regression could be used to test whether plasma testosterone levels (independent variable) significantly explain variation in cichlid fish body size (dependent variable; Fig. 2C). If another variable, such as the clutch that the experimental subject comes from or their social group, could influence the hormone-behavior association, that variable could be included as a random effect. For temporal data with time or development as the independent variable and a sex-associated variable as the dependent variable or interaction term, auto- or cross-correlation analyses are appropriate (Veldhuis et al., 2008; Hefley et al., 2016; Fig. 2C.ii.).

3.4.2.4.2. Multiple (additive or interactive) independent variables. Multiple linear regression and (G)LMMs can both be used to test whether multiple independent variables together (additively or interactively) significantly explain variation in the dependent variable (Fig. 2D). This approach is appropriate particularly if the independent variables of interest are known *a priori* (Harrison et al., 2018). If there are multiple potential independent variables of interest and it is not known which subset of variables to analyze, data exploration (Zuur et al., 2010) and/or model selection (e.g., Portet, 2020) approaches can be used to identify the best, or set of best, linear models. How to select the best model is beyond the scope of this paper (see Baayen et al., 2008; Bates et al., 2015; DeBruine and Barr, 2021; Harrison et al., 2018), but both model fit (indicated by r^2) and model adequacy should be evaluated and reported (Harrison et al., 2018). Forward or backward model selection approaches, in which independent variables are added (forward) or deleted (backward) one-by-one until a "best" model is reached, have been criticized for overestimating effect sizes of significant predictor variables and unjustified focus on a single "best" model, which may not be meaningfully better than the second- or third-best (or more) models. Other combinations of predictor variables may effectively explain variation in the dependent variable of interest (Harrison et al., 2018). The Akaike information criterion (AIC, or AICc for smaller sample sizes) or another information theoretic approach can be used to identify the *set* of best models (Akaike, 1974; Portet, 2020; Harrison et al., 2018). The independent variables included in the top set can be further analyzed (e.g., model averaging) for their relative importance and more (Burnham and Anderson, 2004; Johnson and Omland, 2004; Grueber et al., 2011; Harrison et al., 2018). Multicollinearity, when several independent variables in a model are correlated, should be explicitly tested for and addressed, but not assumed (Graham, 2003).

One of the most common applications of interaction effects between multiple independent variables is to identify a sex difference, or whether a sex-associated trait (continuous or categorical) influences the effect of another independent variable (continuous or categorical) on the dependent variable (continuous). For example, does gonadal sex (ovaries versus testes, categorical independent variable) influence the effect of insulin-like growth factor 1 (IGF-1) levels (continuous independent variable) on cichlid fish body size (dependent variable), taking into account the clutch of the experimental subjects (random effect)? A statistical test — multiple linear regression, (G)LMM, or two-way ANOVA — with an interaction effect is necessary to conclude that there is a sex difference (Garcia-Sifuentes and Maney, 2021).

3.4.3. Sex as predictive: Sex-associated trait(s) as dependent variable(s)

It may also be of interest to analyze a categorical, sex-associated dependent variable. Logistic regression and linear discriminant analysis (LDA) are common classification models for understanding the relationships among one or more independent predictor variables and a categorical dependent variable that is binary (e.g., has ovaries versus does not have ovaries) or has more than two groups (Fig. 2E). The assumptions (e.g., predictor variables) must be normally distributed for LDA. Although the mathematical bases differ for these two approaches, they can be used in similar ways to assess which predictor variables best explain membership in a sex-associated group, as well as to estimate how good the classification rate is. For example, given circulating levels of testosterone, estradiol, and cortisol, logistic regression predicts the probability (odds) of having ovaries. LDA identifies the set of predictors that best discriminates among the sex-associated groups (Antonogeorgos et al., 2009; Lee et al., 2009). AIC, or other PCA information theoretic approaches (see above), can also be used with logistic regression to identify the set of best models. To analyze multiple sex-associated, continuous dependent variables simultaneously, multivariate analysis of variance (MANOVA), or a nonparametric permutational multivariate analysis of variance (PERMANOVA), can be used to identify significant effects and/or interactions, of one or more categorical independent variables (Anderson, 2014; Fig. 2F).

3.4.4. Exploring sex: Using multivariate analysis to understand sex as an emergent phenotype

3.4.4.1. Exploring sex as multivariate. When there are multiple sex-associated traits in a dataset, multivariate ordination approaches can provide a more integrative and comprehensive view of sex. Dimension reduction techniques can be used to create a smaller number of composite variables that each explain a portion of variation in the data (Fig. 2G). Reducing dimensionality is also valuable when the number of variables in the analysis exceeds the number of observations (i.e., sample size). Generally, correlated traits load together on a composite variable, while the different composite variables are uncorrelated with each other. There are multiple dimension-reducing techniques that differ in their approaches (e.g., supervised versus unsupervised), assumptions (e.g., linear versus non-linear), and interpretation (e.g., identifying the original variables that contribute to the composite variables; reviewed in Nanga et al., 2021). Common linear methods include PCA (Jolliffe and Cadima, 2016), LDA, and factor analysis. PCA maximizes variance in the data, whereas LDA maximizes between-group variance and minimizes within-group variance (Forkosh et al., 2019).

The pattern of dimension reduction can reveal how variables in the analysis relate to one another, which can provide meaningful biological insights. For PCA, the eigenvalue of each variable in the analysis indicates whether it loads strongly on a given PC (high positive values and low negative values). This can be visualized as a vector plot of PC1 x PC2 (or with higher PCs) or heatmap of eigenvalues for each of the top PCs (Fig. 2G.i.). The suite of sex-associated variables that load strongly together onto a PC, and in comparison with loadings of sex-associated variables on the other top PCs, can then be interpreted in the context of the model system and experiment. For example, do the sex-associated variables that load on PC1 have a shared underlying mechanism? Covariance matrices and hierarchical clustering can also be used to gain a multivariate understanding of sex. Above, we used hierarchical clustering to group individuals into clusters based on their values for each sex-associated trait in the analysis. Hierarchical clustering can also be used to group sex-associated traits in a covariance matrix (sex-associated variables x sex-associated variables), which can be visualized as a heatmap (Fig. 2G.ii). Bootstrap values can be used to identify significant clusters of traits that are the most similar. Similar to PCA, the suite of variables within a cluster, and comparisons among clusters, can be interpreted to reveal multivariate components of sex phenotype. Unlike PCA, each sex-associated trait can only be in one cluster. Clustering could also be carried out separately for the different sex categories, and the structure of the covariance matrix can be compared statistically (Cai et al., 2013; Li and Chen, 2012). Observing different patterns without a statistical test is not sufficient for reporting a sex difference (Garcia-Sifuentes and Maney, 2021).

3.4.4.2. Analyzing sex as multivariate. The composite variables resulting from dimensionality reducing techniques, such as PCA, can then be analyzed as independent and/or dependent variable(s) using the approaches described above (Fig. 2A-F). For example, in a PCA with the sex-associated traits testosterone, estradiol, cortisol, aggression, body size, and coloration (Fig. 2G), linear regression could then be used to test if PC1 (continuous independent variable) significantly explains variation in social network centrality (continuous dependent variable; Fig. 2C). A *t*-test could be used to test if PC1 (continuous dependent variable) differs significantly between categories of sex (e.g., ovaries versus testes) or between experimental treatment groups (categorical; Fig. 2A). K-means could be used to create clusters from PC1 (Fig. 2B) and analyzed further (Fig. 2A or E).

3.4.4.3. Defining sex phenotype for individuals in multiple dimensions. PCA and other dimension-reducing techniques can, in some ways, lead a researcher back to the initial challenge that led to using a dimension-reducing technique in the first place: each experimental subject is still defined by multiple variables, and the researcher wants to understand how these variables relate to one another. For example, how do PC1 and PC2 together contribute to phenotype or explain how subjects respond to an experimental treatment? For simplicity, we will discuss a scenario with just PC1 and PC2, but these approaches can also be used in higher dimensions (i.e., with more composite variables). Visualizing the data (e.g., as a scatterplot with PC1 on the x-axis and PC2 on the y-axis) may reveal clusters that are distinct enough that individuals can be assigned to a group by eye, and subsequent analyses can ask if there is sex variability or interaction effects for dependent variables of interest (see above). A clustering algorithm (e.g., K-means, see above) can also be used to create groups. As described above, if data are continuously distributed (here, in multiple dimensions), clustering may not be appropriate or as accurate.

An alternative (or additional) approach for understanding sex as a multivariate, emergent phenotype is to define its geometry and calculate continuous, descriptive measures of sex that take multiple sex-associated traits (or multiple composite variables) into account. One example of this approach is Pareto task inference (Hart et al., 2015; Forkosh et al., 2019; Zilkha et al., 2023). Using the top PCs as axes (e.g., PC1 x PC2), each individual is a point on the two dimensional plane, and the cloud of points together (all individuals in the analysis) has a shape. Principal convex hull analysis can be used to identify the best and simplest geometric shape (a polytope) that significantly encloses all of the points. The sex-associated variables that are enriched at each vertex of the polytope describe an "archetype," and an individual's distance from each vertex reflects phenotypic trade-offs. Distance from each vertex is a continuous variable, which can be further analyzed to answer questions such as: Does distance from vertex A (e.g., enriched for testosterone, aggression, and coloration) explain variation in social network position?

3.4.5. How do sex-associated traits relate to other phenotypic traits?

Although we focus our discussion here on (presumed) sex-associated traits, these methods can also be used to understand how sex-associated variables relate to variables in a dataset that are not *a priori* considered related to sex. For example, a PCA can be used with both sex-associated variables and non-sex-associated variables, and the patterns of variables loading on the top PCs can reveal whether sex-associated variables load together, to the exclusion or inclusion of non-sex-associated variables.

Similarly, hierarchical clustering of a covariance matrix of sex-associated and non-sex-associated variables can reveal which variables significantly group together. Patterns of loading/clustering could suggest those variables have common underlying causes or related consequences.

4. Call to action

For the past century, it has been accepted (and on occasion, dogmatically asserted) that sex is a univariate, binary variable. While many efforts have been put forth to advance the study of sex variability in biomedical and other research fields, we propose an expansion of this framework. Sex can be expressed across multiple biological levels, many of which are interconnected and dependent on context and other environmental factors. Answering our scientific queries on the role sex plays in the expression of multiple phenotypes *requires* an integrative perspective that investigates how multiple sex-associated variables interact to influence specific phenotypes. By applying this integrative framework to our experimental approaches, we will gain invaluable insight into the unique ways that sex variability influences physiology and behavior over an organism's lifespan. Ultimately, we suggest that this multidimensional perspective of sex will better inform our understanding of the fundamental processes of biology: reproduction, development, adaptation, and evolution.

As an exercise, we (the authors) invite the reader to reflect on the ideas that have been discussed in this article. How have these topics – history, context, language, framework, experimental design, hypothesis generation, testing, analysis, and interpretation – affected your own thinking, attitudes, and outlook on your research practice, both within the field of neuroendocrinology and throughout the biological sciences? Below, we summarize some of the key "Call to Action" items presented in our paper. We hope that you integrate your own perspectives and thoughts, continue to reflect on them, engage in continual and open discourse, seek ways to apply these ideas to your research, and remain open and flexible to integrating new information that inevitably arises in the future as we continue our collective endeavor to scientifically understand sex.

4.1. Sex variability exists and should be measured

Now is the time that we take a more robust, rigorous, and flexible approach to studying sex in neuroendocrinology by appropriately accounting for the ways in which various levels of sex occur and interact within an individual in relation to the particular phenotypes we are measuring. One of our main goals is to increase awareness of this topic and the consequences of *not* including sex variable designs in our research. As a first step, we need to acknowledge that sex is a rich, various, and diverse phenomenon that can – and should – be measured across multiple levels of biological organization and can be variable within an individual, within a species, and across different species (Fig. 1). The study of sex diversity and variability in the animal kingdom has been hindered by imposing binary assumptions and limitations on what sex is, or can be, across species. By simply acknowledging that sex can, and does, exist outside a strict binary framework, we can evolve and improve how we define, measure, and analyze "sex" in our research.

One of the next steps to this approach is to critically evaluate the framework in which ours and others' research approaches the concept of sex. We view our framework as a starting point that informs our experimental questions, approaches, analyses, and interpretations of results and, ultimately, feeds back into our framework to be further improved. Fig. 3 describes a set of questions that can be used when reading and/or writing a paper that measures sex-associated phenotypes.

4.2. Language is important and has consequences

We must be cognizant of the terminology that we use when describing research on sex variability and diversity. Our language has history and context and can reinforce or create bias in the ways we perceive, interpret, or measure sex-associated variables. It is on us, as

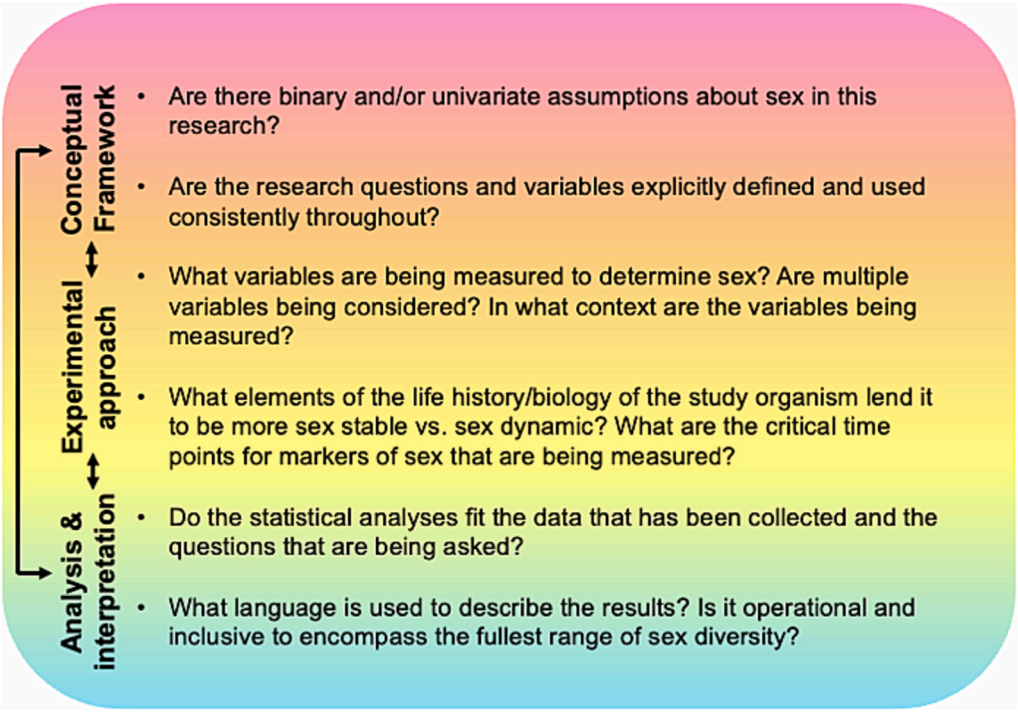


Fig. 3. Questions for evaluating frameworks and approaches which study sex variability and diversity. Here, we present a set of questions to ask when critically evaluating work (whether it is your own or others) that addresses sex diversity and variability. Note that these questions reflect the integration of the conceptual framework, experimental approaches, and analysis and interpretation, as each of these aspects informs the other.

researchers, to (re)educate ourselves about the relevant terminology and make serious efforts to be more operational, precise, and inclusive in our definitions and descriptions of sex-associated phenomena.

We must also be conscientious of the social responsibility we hold as members of a scientific society, as experts in neuroendocrinology, and as producers of scientific knowledge. We must be aware that our research will be used by others (e.g., politicians and activists) to justify the use of "binary sex" in laws and other regulations that impede on the lives and rights of our peers, particularly those in LGBTQIA+ communities. We must vocally and materially counteract those who use science to discriminate and oppress, as has historically occurred (Fausto-Sterling, 2000; Gould, 2006; Lewontin et al., 2017; Gill-Peterson, 2018) and continues to this day (Sudai et al., 2022; Sun and Ashley, 2023). Our work has immense impacts on how our culture understands both sex and gender, so we must be precise and appropriate in our usage, neither conflating the two nor outright dismissing their complex relationships (Garcia-Sifuentes and Maney, 2021; Miyagi et al., 2021).

4.3. Experimental and statistical approaches are important considerations when studying sex diversity and variability

As with all scientific research, it is critical to meticulously select which variables, model organisms, and experimental and analytical methodologies are utilized in studies that investigate sex variability and diversity. Although sex variability can result in sexual heteromorphism, in which pronounced differences in physiological and behavioral phenotypes are observed, this variation is nuanced, may only be revealed under certain social contexts, environmental conditions, or at certain time points, and varies based on the reproductive strategy and sexual system of the study species. Thus, to increase the likelihood that any sex variability that is present is detected experimentally, sex-associated traits should ideally be quantified at multiple levels of biological organization after careful consideration of timing and contextual factors that may influence the emergence of these traits. Finally, experiments must not only be rigorously designed and executed, but must also be statistically analyzed in a way that is appropriate for the variables measured, accurately reflects the research question, and accounts for the life history of the model organism used. Collectively, the complexities of sex variability and diversity make the selection of experimental approaches, animal models, and statistical testing especially important and contributes to responsible interpretation and dissemination of our work

4.4. Researchers and funding agencies should support the use to non-model organisms to study sex diversity

In order to broaden our understanding of sex diversity, scientists need to use appropriate model species. Non-traditional animal models and comparative species approaches offer unique insight into the array of dynamically interacting factors that contribute to the development and expression of sex diversity and variability. However, basic research progress in these systems is often slower because there are fewer researchers working in these systems and fewer resources are available to them (e.g., lack of sequenced and annotated genomes). As outlined above (**Section 3**), there are an increasing number of tools and technological advancements to study non-model organisms. More researchers are needed who are interested in exploring fundamental mechanisms of sex diversity and variability in these species, and federal funding sources, such as NIH, need to continue supporting this type of work (e.g., NIH NOT-HD-19-036[6]; RFA-OD-22-028[7]). Likewise, we encourage grant reviewers to be more open and advocate favorably when reviewing this boundary-pushing work in non-model species. If we take care not to anthropomorphize our species, we can appreciate the biology of the wide range of study species and the broad applicability of the questions we are asking, including their relevance to humans.

5. Positionality statement and conclusion

This paper brought together 8 early career researchers from U.S. universities who identify as cis- and trans- gender women and/or nonbinary at the time of formulating, discussing, and drafting this work. These scientists are graduate students, postdoctoral scholars, and faculty who have been trained in neuroendocrinology within the past 15 years – a period that has experienced a movement in institutional research initiatives and experimental design to include females (e.g., SABV). This paper developed out of the recognition that a radical re-evaluation of "sex" is necessary not only to continue this progress, but to increase awareness of the diversity and complexity of sex and to advance the surrounding scientific discourse. The process of writing this paper has helped the authors, many of whom had never collaborated before, to grow intellectually through recognizing and re-considering their traditional ways of thinking, critically reshape and extend their vocabulary and concepts, and inspire new avenues of inquiry and discovery. We encourage fellow scientists to continue challenging and revising the conventional ways we ask scientific questions. We also encourage scientific societies, institutions, and funding agencies to continue investing in opportunities that support these efforts.

To conclude, we believe these operationalized, integrative research approaches that move beyond the normative, essentialist, and reductive notions of the past will bring forth a fuller, more comprehensive understanding of sex. Sex is not merely a form of biological reproduction, but a natural phenomenon that explores the full variety of a species' phenotypic space that could emerge from a genotype to generate the vast, undeniable diversity throughout life as it adapts, explores, and evolves in the future.

Funding

This work was supported by a National Institute of Child Health and Human Development K99/R00 BRAIN Initiative Advanced Postdoctoral Career Transition Award to Promote Diversity Grant K99HD108800-01 (to K.O.S.), UCLA National Institute of Health Institutional Research and Academic Development Award 5K12GM106996 (to K.A.), National Science Foundation IntBIO Grant 2316364 (to S.E.L.), National Science Foundation CAREER Grant 2146398 (to D.S.P.), a Claremont McKenna College Faculty Research Grant (to T.K.S.L.), and a Howard Hughes Medical Institute Hanna H. Gray Fellowship (to S.D.S.).

CRediT authorship contribution statement

K.O.S., K.M.M., S.E.L., T.M.P., D.S.P., T.K.S.L., and S.D.S. conceived of the ideas and drafted the manuscript. K.A. contributed to the development of the sex variability framework. All authors contributed to revisions of the article. S.E.L., T.M.P., T.K.S.L., and S.D.S. developed the figures. K.O.S. and K.M.M. organized manuscript sections, directed editing, and finalized the manuscript. All authors approved the final version of the manuscript and agreed to be accountable for the content of the work.

Declaration of competing interest

The authors have no competing interests to declare.

Data availability

No data was used for the research described in the article.

[6] https://grants.nih.gov/grants/guide/notice-files/NOT-HD-19-036.html
[7] https://grants.nih.gov/grants/guide/rfa-files/RFA-OD-22-028.html

Acknowledgements

We thank Drs. Donna Maney and Kathleen Casto for the invitation to submit this perspective piece and Drs. Elizabeth Adkins-Regan and Peg McCarthy for their thoughtful discussions on the history of sex differences research in the field of behavioral neuroendocrinology. We are also grateful to Drs. Beau Alward, Art Arnold, Annaliese Beery, Kinsey Brock, Greg Demas, Geert de Vries, Matthew Grober, J. McLaughlin, Kimberly Rosvall, Rebecca Shanksy, and Irv Zucker for inspiration on the topics discussed in this perspective piece, Drs. Kristal Cain and Abby Kimmitt for inspiring the use of a modified Bechdel Test for evaluating frameworks and approaches for sex diversity and variability (Fig. 3), and Drs. Gautam Agarwal, Peter Dijkstra, Cody Kent, Hans Hofmann, and two reviewers for their helpful feedback on a previous version of this manuscript.

References

Aghi, K., Goetz, T.G., Pfau, D.R., Sun, S.E.D., Roepke, T.A., Guthman, E.M., 2022. Centering the needs of transgender, nonbinary, and gender-diverse populations in neuroendocrine models of gender-affirming hormone therapy. Biol. Psychiatry Cogn. Neurosci. Neuroimaging. https://doi.org/10.1016/j.bpsc.2022.07.002.

Ah-King, M., Ahnesjo, I., 2013. The "sex role" concept: An overview and evaluation. Evol. Biol. 40, 461–470.

Akaike, H., 1974. A new look at the statistical model identification. IEEE Trans. Autom. Control 19, 716–723.

Alward, B.A., Laud, V.A., Skalnik, C.J., York, R.A., Juntti, S.A., Fernald, R.D., 2020. Modular genetic control of social status in a cichlid fish. Proc. Natl. Acad. Sci. U. S. A. 117, 28167–28174.

Alward, B.A., Hoadley, A.P., Jackson, L.R., Lopez, M.S., 2023. Genetic dissection of steroid-hormone modulated social behavior: novel paralogous genes are a boon for discovery. Horm. Behav. 147, 105295.

Anderson, A.P., Renn, S.C.P., 2023. The ancestral modulation hypothesis: predicting mechanistic control of sexually Heteromorphic traits using evolutionary history. Am. Nat. 202, 241–259.

Anderson, M.J., 2014. Permutational Multivariate Analysis of Variance (PERMANOVA). Statistics Reference Online, Wiley StatsRef. https://doi.org/10.1002/9781118445112.stat07841.

Antonogeorgos, G., Panagiotakos, D.B., Priftis, K.N., Tzonou, A., 2009. Logistic regression and linear discriminant analyses in evaluating factors associated with asthma prevalence among 10- to 12-years-old children: divergence and similarity of the two statistical methods. Int. J. Pediatr. 2009, 1–6. https://doi.org/10.1155/2009/952042.

Arnold, A.P., Breedlove, S.M., 1985. Organizational and activational effects of sex steroids on brain and behavior: a reanalysis. Horm. Behav. 19, 469–498.

Auger, C.J., Coss, D., Auger, A.P., Forbes-Lorman, R.M., 2011. Epigenetic control of vasopressin expression is maintained by steroid hormones in the adult male rat brain. Proc. Natl. Acad. Sci. 108, 4242–4247.

Baayen, R.H., Davidson, D.J., Bates, D.M., 2008. Mixed-effects modeling with crossed random effects for subjects and items. J. Mem. Lang. 59, 390–412. https://doi.org/10.1016/j.jml.2007.12.005.

Baba, T., Ting, A.Y., Tkachenko, O., Xu, J., Stouffer, R.L., 2017. Direct actions of androgen, estrogen and anti-Müllerian hormone on primate secondary follicle development in the absence of FSH *in vitro*. Hum. Reprod. 32, 2456–2464. https://doi.org/10.1093/humrep/dex322.

Bachtrog, D., Mank, J.E., Peichel, C.L., Kirkpatrick, M., Otto, S.P., Ashman, T.-L., Hahn, M.W., Kitano, J., Mayrose, I., Ming, R., Perrin, N., Ross, L., Valenzuela, N., Vamosi, J.C., Consortium, T.T. of S, 2014. Sex determination: why so many ways of doing it? PLoS Biol. 12, e1001899.

Bale, T.L., Epperson, C.N., 2015. Sex differences and stress across the lifespan. Nat. Neurosci. 18, 1413–1420.

Ball, G.F., Ketterson, E.D., 2008. Sex differences in the response to environmental cues regulating seasonal reproduction in birds. Philos. Trans. R. Soc. Lond. Ser. B: Biol. Sci. 363, 231–246.

Balthazart, J., Choleris, E., Remage-Healey, L., 2018. Steroids and the brain: 50 years of research, conceptual shifts and the ascent of non-classical and membrane-initiated actions. Horm. Behav. 99, 1–8.

Bangasser, D.A., Valentino, R.J., 2012. Sex differences in molecular and cellular substrates of stress. Cell. Mol. Neurobiol. 32, 709–723.

Bangasser, D.A., Valentino, R.J., 2014. Sex differences in stress-related psychiatric disorders: neurobiological perspectives. Front. Neuroendocrinol. 35, 303–319.

Bangasser, D.A., Wiersielis, K.R., 2018. Sex differences in stress responses: a critical role for corticotropin-releasing factor. Hormones (Athens) 17, 5–13.

Barrangou, R., Doudna, J.A., 2016. Applications of CRISPR technologies in research and beyond. Nat. Biotechnol. 34, 933–941.

Barresi, M., Gilbert, S., 2023. Developmental Biology. Sinauer Associates, Oxford University Press.

Bates, D., Mächler, M., Bolker, B., Walker, S., 2015. Fitting linear mixed-effects models using lme4. J. Stat. Softw. 67 https://doi.org/10.18637/jss.v067.i01.

Been, L.E., Hedges, V.L., Vialou, V., Nestler, E.J., Meisel, R.L., 2013. ΔJunD overexpression in the nucleus accumbens prevents sexual reward in female Syrian hamsters. Genes Brain Behav. 12, 666–672.

Beltz, A.M., Beery, A.K., Becker, J.B., 2019. Analysis of sex differences in pre-clinical and clinical data sets. Neuropsychopharmacol 44, 2155–2158. https://doi.org/10.1038/s41386-019-0524-3.

Berendzen, K.M., Sharma, R., Mandujano, M.A., Wei, Y., Rogers, F.D., Simmons, T.C., Seelke, A.M.H., Bond, J.M., Larios, R., Goodwin, N.L., Sherman, M., Parthasarthy, S., Espineda, I., Knoedler, J.R., Beery, A., Bales, K.L., Shah, N.M., Manoli, D.S., 2023. Oxytocin receptor is not required for social attachment in prairie voles. Neuron 111, 787–796.

Black, M.P., Grober, M.S., 2003. Group sex, sex change, and parasitic males: sexual strategies among the fishes and their neurobiological correlates. Annu. Rev. Sex Res. 14, 160–184.

Black, M.P., Reavis, R.H., Grober, M.S., 2004. Socially induced sex change regulates forebrain isotocin in *Lythrypnus dalli*. Neuroreport 15, 185–189.

Black, M.P., Balthazart, J., Baillien, M., Grober, M.S., 2005. Socially induced and rapid increases in aggression are inversely related to brain aromatase activity in a sex-changing fish, *Lythrypnus dalli*. Proc. Biol. Sci. 272, 2435–2440.

Boender, A.J., Young, L.J., 2020. Oxytocin, vasopressin and social behavior in the age of genome editing: a comparative perspective. Horm. Behav. 124, 104780.

Boender, A.J., Boon, M., Albers, H.E., Eck, S.R., Fricker, B.A., Kelly, A.M., Ledoux, J.E., Motta, S.C., Shrestha, P., Taylor, J.H., Trainor, B.C., Triana-Del Rio, R., Young, L.J., 2023. An AAV-CRISPR/Cas9 strategy for gene editing across divergent rodent species: targeting neural oxytocin receptors as a proof of concept. Sci. Adv. 9, eadf4950.

Brooks-Gunn, J., Warren, M.P., 1988. The psychological significance of secondary sexual characteristics in nine to eleven-year-old girls. Child Dev. 59 (4), 1061–1069.

Burnham, K.P., Anderson, D.R., 2004. Multimodel inference: understanding AIC and BIC in model selection. Sociol. Methods Res. 33, 261–304. https://doi.org/10.1177/0049124104268644.

Cai, T., Liu, W., Xia, Y., 2013. Two-sample covariance matrix testing and support recovery in high-dimensional and sparse settings. J. Am. Stat. Assoc. 108, 265–277. https://doi.org/10.1080/01621459.2012.758041.

Caldwell, H.K., Albers, H.E., 2016. Oxytocin, vasopressin, and the motivational forces that drive social behaviors. In: Simpson, E.H., Balsam, P.D. (Eds.), Behavioral Neuroscience of Motivation. Springer Cham, Switzerland, pp. 51–103.

Calisi, R.M., Bentley, G.E., 2009. Lab and field experiments: are they the same animal? Horm. Behav. 56, 1–10.

Capel, B., 2017. Vertebrate sex determination: evolutionary plasticity of a fundamental switch. Nat. Rev. Genet. 18, 68–675.

Carlisle, S.L., Marxer-Miller, S.K., Canario, A.V.M., Oliveira, R.F., Carneiro, L., Grober, M.S., 2000. Effects of 11-ketotestosterone on genital papilla morphology in the sex changing fish *Lythrypnus dalli*. J. Fish Biol. 57, 445–456.

Carraco, G., Martins-Jesus, A.P., Andrade, R.P., 2022. The vertebrate embryo clock: common players dancing to a different beat. Front. Cell Dev. Biol. 10, 944016 https://doi.org/10.3389/fcell.2022.944016.

Carter, C.S., 2017. The oxytocin–vasopressin pathway in the context of love and fear. Front. Endocrinol. 8, 356.

Casadevall, M., Delgado, E., Colleye, O., Montserrat, S.B., Parmentier, E., 2009. Histological study of the sex-change in the skunk clownfish *Amphiprion akallopisos*. Open Fish Sci. J. 2, 55–58.

Casas, L., Saborido-Rey, F., Ryu, T., Michell, C., Ravasi, T., Irigoien, X., 2016. Sex change in clownfish: molecular insights from transcriptome analysis. Sci. Rep. 6, 35461.

Casas, L., Parker, C.G., Rhodes, J.S., 2022. Sex change from male to female: Active feminization of the brain, behavior, and gonads in anemonefish. In: Laudet, V., Ravasi, T. (Eds.), Evolution, Development and Ecology of Anemonefishes: Model Organisms for Marine Science. CRC Press, Boca Raton, pp. 117–128.

Charrad, M., Ghazzali, N., Boiteau, V., Niknafs, A., 2014. NbClust: An R package for determining the relevant number of clusters in a data set. J. Stat. Softw. 61 https://doi.org/10.18637/jss.v061.i06.

Chiba, S., 2007. A review of ecological and evolutionary studies on hermaphroditic decapod crustaceans. Plankton Benthos Res. 2, 107–119.

Clayton, J.A., 2016. Studying both sexes: a guiding principle for biomedicine. FASEB J. 30, 519–524. https://doi.org/10.1096/fj.15-279554.

Clayton, J.A., 2018. Applying the new SABV (sex as a biological variable) policy to research and clinical care. Physiol. Behav. 187, 2–5. https://doi.org/10.1016/j.physbeh.2017.08.012.

Cornil, C.A., Charlier, T.D., 2010. Rapid behavioural effects of oestrogens and fast regulation of their local synthesis by brain aromatase. J. Neuroendocrinol. 22, 664–673.

Crews, D., 2010. Epigenetics, brain, behavior, and the environment. Hormones 9, 41–50.

Cunningham, R.L., Lumia, A.R., McGinnis, M.Y., 2012. Androgen receptors, sex behavior, and aggression. Neuroendocrinology 96, 131–140.

Davidson, E.H., Rast, J.P., Oliveri, P., et al., 2002. A Genomic Regulatory Network for Development, 295, pp. 1669–1678.

Day, L.B., Fusani, L., Hernandez, E., Billo, T.J., Sheldon, K.S., Wise, P.M., Schlinger, B.A., 2007. Testosterone and its effects on courtship in golden-collared manakins (*Manacus vitellinus*): seasonal, sex, and age differences. Horm. Behav. 51, 69–76.

De Vries, C., Lehtonen, J., 2023. Sex-specific assumptions and their importance in models of sexual selection. Trends Ecol. Evol. https://doi.org/10.1016/j.tree.2023.04.01.

De Vries, G.J., 2004. Minireview: sex differences in adult and developing brains: compensation, compensation, compensation. Endocrinology 145, 1063–1068.

De Vries, G.J., Boyle, P.A., 1998. Double duty for sex differences in the brain. Behav. Brain Res. 92, 205–213.

De Vries, G.J., Södersten, P., 2009. Sex differences in the brain: The relation between structure and function. Horm. Behav. 55, 589–596.
DeBruine, L.M., Barr, D.J., 2021. Understanding mixed-effects models through data simulation. Adv. Methods Pract. Psychol. Sci. 4, 1–15.
Demas, G.E., Munley, K.M., Jasnow, A.M., 2023. A seasonal switch hypothesis for the neuroendocrine control of aggression. Trends Endocrinol. Metab. In Press.
Denney, K.A., Wu, M.V., Sun, S.D., Moon, S., Tollkuhn, J., 2023. Comparative analysis of gonadal hormone receptor expression in the house mouse, meadow vole, and prairie vole brain. bioRxiv. https://doi.org/10.1101/2023.05.12.540606. Preprint.
Diester, C.M., Banks, M.L., Neigh, G.N., Negus, S.S., 2019. Experimental design and analysis for consideration of sex as a biological variable. Neuropsychopharmacology 44, 2159–2162.
DiMarco, M., Zhao, H., Boulicault, M., Richardson, S.S., 2022. Why "sex as a biological variable" conflicts with precision medicine initiatives. Cell Reports Medicine 3, 100550.
Do Rego, J.L., Seong, J.Y., Burel, D., Leprince, J., Luu-The, V., Tsutsui, K., Tonon, M.-C., Pelletier, G., Vaudry, H., 2009. Neurosteroid biosynthesis: enzymatic pathways and neuroendocrine regulation by neurotransmitters and neuropeptides. Front. Neuroendocrinol. 30, 259–301.
Donaldson, Z., Young, L.J., 2008. Oxytocin, vasopressin, and the neurogenetics of sociality. Science 322, 900–904.
Dupré, J., Nicholson, D.J., 2018. A manifesto for a processual philosophy of biology. In: Nicholson, D.J., Dupré, J. (Eds.), Everything Flows: Towards a Processual Philosophy of Biology. Oxford University Press.
Eaton, J., Pradhan, D.S., Barske, J., Fusani, L., Canoine, V., Schlinger, B.A., 2018. 3β-HSD expression in the CNS of a manakin and finch. Gen. Comp. Endocrinol. 256, 43–49.
Emlen, S., Wrege, P., 2004. Division of labour in parental care behaviour of a sex-role-reversed shorebird, the wattled jacana. Anim. Behav. 68, 847–885.
Emmert-Streib, F., Dehmer, M., Haibe-Kains, B., 2014. Gene regulatory networks and their applications: understanding biological and medical problems in terms of networks. Front. Cell Dev. Biol. 2 https://doi.org/10.3389/fcell.2014.00038.
Fabris, F., 2018. Waddington's processual epigenetics and the debate over cryptic variability. In: Nicholson, D.J., Dupré, J. (Eds.), Everything Flows: Towards a Processual Philosophy of Biology. Oxford University Press.
Fausto-Sterling, A., 2000. Sexing the Body: Gender Politics and the Construction of Sexuality. Basic Books.
Feng, N.Y., Katz, A., Day, L.B., Barske, J., Schlinger, B.A., 2010. Limb muscles are androgen targets in an acrobatic tropical bird. Endocrinology 151, 1042–1049.
Fernandez-Ruiz, A., Oliva, A., Chang, H., 2022. High-resolution optogenetics in space and time. Trends Neurosci. 45, 854–864.
Flores, R.J., Cruz, B., Uribe, K.P., Carcoba, M., O'Dell, L.E., 2018. Sex differences and the role of ovarian hormones in modulating the behavioral effects of nicotine in rodent models. In: Becker, J.B., Tollkuhn, J. (Eds.), Sex Differences in the Brain: Balancing Sex in Preclinical Research. Society of Neuroscience, Washington, DC, pp. 59–67.
Forkosh, O., Karamihalev, S., Roeh, S., Alon, U., Anpilov, S., Touma, C., Nussbaumer, M., Flachskamm, C., Kaplick, P.M., Shemesh, Y., Chen, A., 2019. Identity domains capture individual differences from across the behavioral repertoire. Nat. Neurosci. 22, 2023–2028. https://doi.org/10.1038/s41593-019-0516-y.
Fricke, H., Fricke, S., 1977. Monogamy and sex change by aggressive dominance in coral reef fish. Nature 266, 830–832.
Fulmer, A.G., Neumeister, H., Preuss, T., 2017. Social plasticity in non-territorial male African cichlid fish *Astatotilapia burtoni*. J. Ethol. 35, 109–119.
Fusani, L., Donaldson, Z., London, S.E., Fuxjager, M.J., Schlinger, B.A., 2014. Expression of androgen receptor in the brain of a sub-oscine bird with an elaborate courtship display. Neurosci. Lett. 578, 61–65.
Fuxjager, M.J., Mast, G., Becker, E.A., Marler, C.A., 2009. The 'home advantage' is necessary for a full winner effect and changes in post-encounter testosterone. Horm. Behav. 56, 214–219.
Fuxjager, M.J., Schultz, J.D., Barske, J., Feng, N.Y., Fusani, L., Mirzatoni, A., Day, L.B., Hau, M., Schlinger, B.A., 2012. Spinal motor and sensory neurons are androgen targets in an acrobatic bird. Endocrinology 153, 3780–3791.
Fuxjager, M.J., Goller, F., Dirske, A., Sanin, G.D., Garcia, S., 2016. Select forelimb muscles have evolved superfast contractile speed to support acrobatic social displays. eLife 5, e13544.
Fuxjager, M.J., Ryder, T.B., Moody, N.M., Alfonso, C., Balakrishnan, C.N., Barske, J., Bosholn, M., Boyle, W.A., Braun, E.L., Chiver, I., Dakin, R., Day, L.B., Driver, R., Fusani, L., Horton, B.M., Kimball, R.T., Lipshutz, S., Mello, C.V., Miller, E.T., Webster, M.S., Wirthlin, M., Wollman, R., Moore, I.T., Schlinger, B.A., 2023. Systems biology as a framework to understand the physiological and endocrine bases of behavior and its evolution – from concepts to a case study in birds. Horm. Behav. 151, 105340.
Garcia-Sifuentes, Y., Maney, D.L., 2021. Reporting and misreporting of sex differences in the biological sciences. eLife 10, e70817. https://doi.org/10.7554/eLife.70817.
Gegenhuber, B., Tollkuhn, J., 2019. Sex differences in the Epigenome: a cause or consequence of sexual differentiation of the brain? Genes 10. https://doi.org/10.3390/genes10060432.
Gegenhuber, B., Wu, M.V., Bronstein, R., Tollkuhn, J., 2022. Gene regulation by gonadal hormone receptors underlies brain sex differences. Nature. https://doi.org/10.1038/s41586-022-04686-1.
Gemmell, N.J., Todd, E.V., Goikoetxea, A., Ortega-Recalde, O., Hore, T.A., 2019. Natural sex change in fish. Curr. Top. Dev. Biol. 134, 71–117.
Georgiou, P., Zanos, P., Mou, T.-C.M., An, X., Gerhard, D.M., Dryanovski, D.I., Potter, L. E., Highland, J.N., Jenne, C.E., Stewart, B.W., Pultorak, K.J., Yuan, P., Powels, C.F., Lovett, J., Pereira, E.F.R., Clark, S.M., Tonelli, L.H., Moaddel, R., Zarate, C.A., Duman, R.S., Thompson, S.M., Gould, T.D., 2022. Experimenters' sex modulates mouse behaviors and neural responses to ketamine via corticotropin releasing factor. Nat. Neurosci. https://doi.org/10.1038/s41593-022-01146-x.
Ghiselin, M.T., 1969. The evolution of hermaphroditism among animals. Q. Rev. Biol. 44, 189–208.
Gillette, M.U., Tischkau, S.A., 1999. Suprachiasmatic nucleus: the brain's circadian clock. Recent Prog. Horm. Res. 54, 33–58.
Gill-Peterson, J., 2018. Histories of the Transgender Child. U of Minnesota Press.
Godwin, J., 1994. Behavioural aspects of protandrous sex change in the anemonefish, *Amphiprion melanopus*, and endocrine correlates. Anim. Behav. 48, 551–567.
Godwin, J., 2019. Sex change in reef fishes: Behavior and physiology. In: Choe, J.C. (Ed.), Encyclopedia of Animal Behavior, 2 ed. Academic Press, pp. 364–372.
Godwin, J., Crews, D., Warner, R.R., 1996. Behavioural sex change in the absence of gonads in a coral reef fish. Proc. Biol. Sci. 263, 1683–1688.
Gould, S.J., 2006. The Mismeasure of Man (Revised and Expanded). W. W. Norton, United States.
Gowaty, P.A., 2018. Biological essentialism, gender, true belief, confirmation biases, and skepticism. In: Travis, C.B. (Ed.), APA Handbook of the Psychology of Women: History, Theory, and Battlegrounds, xxii. American Psychological Association, Washington, DC, US, pp. 145–164.
Goymann, W., Brumm, H., Kappeler, P.M., 2023. Biological sex is binary, even though there is a rainbow of sex roles: denying biological sex is anthropocentric and promotes species chauvinism. Bioessays 45 (2), e2200173.
Graham, M.H., 2003. Confronting multicollinearity in ecological multiple regression. Ecology 84, 2809–2815.
Gross, M.R., Sargent, R.C., 1985. The evolution of male and female parental care in fishes. Am. Zool. 25, 807–822.
Grueber, C.E., Nakagawa, S., Laws, R.J., Jamieson, I.G., 2011. Multimodel inference in ecology and evolution: challenges and solutions. J. Evol. Biol. 24, 699–711. https://doi.org/10.1111/j.1420-9101.2010.02210.x.
Guiguen, Y., Cauty, C., Fostier, A., Fuchs, J., Jalabert, B., 1994. Reproductive cycle and sex inversion of the seabass, *Lates calcarifer*, reared in sea cages in French Polynesia: histological and morphometric description. Environ. Biol. Fish 39, 231–247.
Guthman, E.M., Falkner, A.L., 2022. Neural mechanisms of persistent aggression. Curr. Opin. Neurobiol. 73, 102526.
Haggerty, D.L., Grecco, G.G., Reeves, K.C., Atwood, B., 2020. Adeno-associated viral vectors in neuroscience research. Mol. Ther. Methods Clin. Dev. 17, 69–82.
Harrison, X.A., Donaldson, L., Correa-Cano, M.E., Evans, J., Fisher, D.N., Goodwin, C.E. D., Robinson, B.S., Hodgson, D.J., Inger, R., 2018. A brief introduction to mixed effects modelling and multi-model inference in ecology. PeerJ 6, e4794. https://doi.org/10.7717/peerj.4794.
Hart, Y., Sheftel, H., Hausser, J., Szekely, P., Ben-Moshe, N.B., Korem, Y., Tendler, A., Mayo, A.E., Alon, U., 2015. Inferring biological tasks using Pareto analysis of high-dimensional data. Nat. Methods 12, 233–235. https://doi.org/10.1038/nmeth.3254.
Hashikawa, K., Hashikawa, Y., Tremblay, R., Zhang, J., Feng, J.E., Sabol, A., Piper, W.T., Lee, H., Rudy, B., Lin, D., 2017. Esr1+ cells in the ventromedial hypothalamus control female aggression. Nat. Neurosci. 20, 1580–1590.
Hattori, A., Casadevall, M., 2016. Sex change strategies and group structure of damselfishes. In: Frédérich, D., Parmentier, E. (Eds.), Biology of Damselfishes, 1 ed. CRC Press, Boca Raton, pp. 55–83.
Heck, A.L., Handa, R.J., 2019. Sex differences in the hypothalamic–pituitary–adrenal axis' response to stress: an important role for gonadal hormones. Neuropsychopharmacology 44, 45–58.
Hedges, V.L., Chakravarty, S., Nestler, E.J., Meisel, R.L., 2009. Δ FosB overexpression in the nucleus accumbens enhances sexual reward in female Syrian hamsters. Genes Brain Behav. 8, 442–449.
Hefley, T.J., Broms, K.M., Brost, B.M., Buderman, F.E., Kay, S.L., Scharf, H.R., Tipton, J. R., Williams, P.J., Hooten, M.B., 2016. The basis function approach for modeling autocorrelation in ecological data. Ecology 98 (3), 632–646.
Holloway, C.C., Clayton, D.F., 2001. Estrogen synthesis in the male brain triggers development of the avian song control pathway *in vitro*. Nat. Neurosci. 4, 170–175.
Horie, K., Inoue, K., Suzuki, S., Adachi, S., Yada, S., Hirayama, T., Hidema, S., Young, L. J., Nishimori, K., 2019. Oxytocin receptor knockout prairie voles generated by CRISPR/Cas9 editing show reduced preference for social novelty and exaggerated repetitive behaviors. Horm. Behav. 111, 60–69.
Horton, B.M., Michael, C.M., Prichard, M.R., Maney, D.L., 2020. Vasoactive intestinal peptide as a mediator of the effects of a supergene on social behaviour. Proc. Biol. Sci. 287, 20200196.
Ikotun, A.M., Ezugwu, A.E., Abualigah, L., Abuhaija, B., Heming, J., 2023. K-means clustering algorithms: a comprehensive review, variants analysis, and advances in the era of big data. Inf. Sci. 622, 178–210. https://doi.org/10.1016/j.ins.2022.11.139.
Iwata, E., Nagai, Y., Sasaki, H., 2010. Social rank modulates brain arginine vasotocin immunoreactivity in false clown anemonefish (*Amphiprion ocellaris*). Fish Physiol. Biochem. 36, 337–345.
Jasnow, A.M., Huhman, K.L., Bartness, T.J., Demas, G.E., 2000. Short-day increases in aggression are inversely related to circulating testosterone concentrations in male Siberian hamsters (*Phodopus sungorus*). Horm. Behav. 38, 102–110.
Jeon, Y., Sarma, K., Lee, J.T., 2012. New and Xisting regulatory mechanisms of X chromosome inactivation. Curr. Opin. Genet. Dev. 22, 62–71.
Jiang, D., Chen, J., Fan, Z., Tan, D., Zhao, J., Shi, H., Liu, Z., Tao, W., Li, M., Wang, D., 2017. CRISPR/Cas9-induced disruption of wt1a and wt1b reveals their different roles in kidney and gonad development in Nile tilapia. Dev. Biol. 428, 63–73.
Joel, D., 2021. Beyond the binary: rethinking sex and the brain. Neurosci. Biobehav. Rev. 122, 165–175. https://doi.org/10.1016/j.neubiorev.2020.11.018.
Johnson, J.B., Omland, K.S., 2004. Model selection in ecology and evolution. Trends Ecol. Evol. 19, 101–108. https://doi.org/10.1016/j.tree.2003.10.013.

Jolliffe, I.T., Cadima, J., 2016. Principal component analysis: a review and recent developments. Phil. Trans. R. Soc. A 374, 20150202. https://doi.org/10.1098/rsta.2015.0202.
Juntti, S.A., 2019. The future of gene-guided neuroscience research in non-traditional model organisms. Brain Behav. Evol. 93, 108–121.
Juntti, S.A., Hilliard, A.T., Kent, K.R., Kumar, A., Nguyen, A., Jimenez, M.A., Loveland, J. L., Mourrain, P., Fernald, R.D., 2016. A neural basis for control of cichlid female reproductive behavior by prostaglandin F2α. Curr. Biol. 26, 943–949.
Kelly, A.M., 2022. A consideration of brain networks modulating social behavior. Horm. Behav. 141, 105138.
Kumle, L., Võ, M.L.-H., Draschkow, D., 2021. Estimating power in (generalized) linear mixed models: An open introduction and tutorial in R. Behav. Res. Ther. 53, 2528–2543. https://doi.org/10.3758/s13428-021-01546-0.
Kurian, J.R., Bychowski, M.E., Forbes-Lorman, R.M., Auger, C.J., Auger, A.P., 2008. Mecp2 organizes juvenile social behavior in a sex-specific manner. J. Neurosci. 28, 7137–7142.
Laman-Maharg, A., Trainor, B.C., 2017. Stress, sex, and motivated behaviors. J. Neurosci. Res. 95, 83–92.
Laube, C., Van Den Bos, W., Fandakova, Y., 2020. The relationship between pubertal hormones and brain plasticity: implications for cognitive training in adolescence. Dev. Cogn. Neurosci. 42, 100753 https://doi.org/10.1016/j.dcn.2020.100753.
Lea, R.W., Klandorf, H., 2002. The brood patch. In: Deeming, D.C. (Ed.), Avian Incubation: Behavior, Environment, and Evolution. Oxford University Press, Oxford.
Lee, J., Tan, C.S., Chia, K.S., 2009. A practical guide for multivariate analysis of dichotomous outcomes. Ann. Acad. Med. Singap. 38, 714–719. https://doi.org/10.47102/annals-acadmedsg.V38N8p714.
Lee, J., Ma, J., Lee, K., 2019. Direct delivery of adenoviral CRISPR/Cas9 vector into the blastoderm for generation of targeted gene knockout in quail. Proc. Natl. Acad. Sci. U. S. A. 116, 13288–13292.
Lee, N.S., Beery, A.K., 2019. Neural circuits underlying rodent sociality: a comparative approach. Curr. Top. Behav. Neurosci. 43, 211–238. https://doi.org/10.1007/7854_2018_77.
Lesoway, M.P., Henry, J.Q., 2019. Sex determination, sexual development, and sex change in slipper snails. In: Tworzydlo, W., Bilinski, S.M. (Eds.), Evo-Devo: Non-Model Species in Cell and Developmental Biology, Results and Problems in Cell Differentiation. Springer International Publishing, Cham, pp. 155–182.
Leung, V.Y.L., Gao, B., Leung, K.K.H., Melhado, I.G., Wynn, S.L., Au, T.Y.K., Dung, N.W. F., Lau, J.Y.B., Mak, A.C.Y., Chan, D., Cheah, K.S.E., 2011. SOX9 governs differentiation stage-specific gene expression in growth plate chondrocytes via direct concomitant transactivation and repression. PLoS Genet. 7, e1002356.
Levy, D.R., Hunter, N., Lin, S., Robinson, E.M., Gillis, W., Conlin, E.B., Anyoha, R., Shansky, R.M., Datta, S.R., 2023. Mouse spontaneous behavior reflects individual variation rather than estrous state. Curr. Biol. 0 https://doi.org/10.1016/j.cub.2023.02.035.
Lewontin, R., Rose, S., Kamin, L.J., 2017. Not in Our Genes: Biology, Ideology, and Human Nature. Haymarket Books, United States.
Li, E., Davidson, E.H., 2009. Building developmental gene regulatory networks. Birth Defects Res. C Embryo Today 87, 123–130.
Li, J., Chen, S.X., 2012. Two sample tests for high-dimensional covariance matrices. Ann. Stat. 40 https://doi.org/10.1214/12-AOS993.
Liarte, S., Chaves-Pozo, E., García-Alcazar, A., Mulero, V., Meseguer, J., García-Ayala, A., 2007. Testicular involution prior to sex change in gilthead seabream is characterized by a decrease in DMRT1 gene expression and by massive leukocyte infiltration. Reprod. Biol. Endocrinol. 5, 20.
Lipshutz, S.E., Rosvall, K.A., 2020. Testosterone secretion varies in a sex- and stage-specific manner: insights on the regulation of competitive traits from a sex-role reversed species. Gen. Comp. Endocrinol. 292, 113444.
Lorenzi, V., Earley, R.L., Grober, M.S., 2012. Differential responses of brain, gonad and muscle steroid levels to changes in social status and sex in a sequential and bidirectional hermaphroditic fish. PLoS One 7, e51158.
Loya, Y., Sakai, K., 2008. Bidirectional sex change in mushroom stony corals. Proc. Biol. Sci. 275, 2335–2343.
MacDougall-Shackleton, S.A., 2011. The levels of analysis revisited. Philos. Trans. R. Soc. B 366, 2076–2085. https://doi.org/10.1098/rstb.2010.0363.
MacLusky, N.J., Naftolin, F., Goldman-Rakic, P.S., 1986. Estrogen formation and binding in the cerebral cortex of the developing rhesus monkey. Proc. Natl. Acad. Sci. 83, 514–516.
Maney, D.L., 2016. Perils and pitfalls of reporting sex differences. Philos. Trans. R. Soc. B Biol. Sci. 371, 20150119. https://doi.org/10.1098/rstb.2015.0119.
Maney, D.L., Rich-Edwards, J.W., 2023. Sex-inclusive biomedicine: are new policies increasing rigor and reproducibility? Women's Health Issues, 2023 Apr. Official Publication of the Jacobs Institute of Women's Health. https://doi.org/10.1016/j.whi.2023.03.004. S1049-3867(23)00072-5. 37087311.
Marasco, V., Robinson, J., Herzyk, P., Spencer, K.A., 2012. Pre- and post-natal stress in context: effects on the stress physiology in a precocial bird. J. Exp. Biol. 215, 3955–3964.
Marguerat, S., Bähler, J., 2010. RNA-seq: from technology to biology. Cell. Mol. Life Sci. 67, 569–579.
Massa, M.G., Correa, S.M., 2020. Sexes on the brain: sex as multiple biological variables in the neuronal control of feeding. Biochim. Biophys. Acta Mol. basis Dis. 1866, 165840.
Massa, M.G., Aghi, K., Hill, M.J., 2023. Deconstructing sex: Strategies for undoing binary thinking in neuroendocrinology and behavior. Horm. Behav. 156, 105441.
Matsuda, K.I., Mori, H., Kawata, M., 2012. Epigenetic mechanisms are involved in sexual differentiation of the brain. Rev. Endocr. Metab. Disord. 13, 163–171.
McCarthy, M.M., 2008. Estradiol and the developing brain. Physiol. Rev. 88, 91–134.
McCarthy, M.M., 2023. Neural control of sexually dimorphic social behavior: connecting development to adulthood. Annu. Rev. Neurosci. 46, 321–339.
McCarthy, M.M., Nugent, B.M., 2015. At the frontier of epigenetics of brain sex differences. Front. Behav. Neurosci. 9, 221.
McCarthy, M.M., Wright, C.L., Schwarz, J.M., 2009. New tricks by an old dogma: mechanisms of the organizational/activational hypothesis of steroid-mediated sexual differentiation of brain and behavior. Horm. Behav. 55, 655–665.
McCarthy, M.M., Arnold, A.P., Ball, G.F., Blaustein, J.D., De Vries, G.J., 2012. Sex differences in the brain: the not so inconvenient truth. J. Neurosci. 32, 2241–2247.
McHenry, J.A., Otis, J.M., Rossi, M.A., Elliott Robinson, J., Kosyk, O., Miller, N.W., McElligott, Z.A., Budygin, E.A., Rubinow, D.R., Stuber, G.D., 2017. Hormonal gain control of a medial preoptic area social reward circuit. Nat. Neurosci. https://doi.org/10.1038/nn.4487.
McLaughlin, J.F., Brock, K.M., Gates, I., Pethkar, A., Piattoni, M., Rossi, A., Lipshutz, S.E., 2023. Multivariate models of animal sex: breaking binaries leads to a better understanding of ecology and evolution. Integr. Comp. Biol. 63, 891–906.
Miller, L.R., Marks, C., Becker, J.B., Hurn, P.D., Chen, W., Woodruff, T., McCarthy, M.M., Sohrabji, F., Schiebinger, L., Wetherington, C.L., Makris, S., Arnold, A.P., Einstein, G., Miller, V.M., Sandberg, K., Maier, S., Cornelison, T.L., Clayton, J.A., 2017. Considering sex as a biological variable in preclinical research. FASEB J. 31, 29–34. https://doi.org/10.1096/fj.201600781r.
Miyagi, M., Guthman, E.M., Sun, S.D.-K., 2021. Transgender rights rely on inclusive language. Science 374, 1568–1569. https://doi.org/10.1126/science.abn3759.
Morgan, C.P., Bale, T.L., 2012. Sex differences in microRNA regulation of gene expression: no smoke, just miRs. Biol. Sex Differ. 3, 22.
Munday, P.L., Buston, P.M., Warner, R.R., 2006. Diversity and flexibility of sex-change strategies in animals. Trends Ecol. Evol. 21, P89–P95.
Munday, P.L., Kuwamura, T., Kroon, F.J., 2010. Bidirectional sex change in marine fishes. In: Cole, K.S. (Ed.), Reproduction and Sexuality in Marine Fishes: Patterns and Processes. University of California Press, pp. 240–271.
Munley, K.M., Rendon, N.M., Demas, G.E., 2018. Neural androgen synthesis and aggression: insights from a seasonally breeding rodent. Front. Endocrinol. 9, 136.
Munley, K.M., Dutta, S., Jasnow, A.M., Demas, G.E., 2022a. Adrenal MT1 melatonin receptor expression is linked with seasonal variation in social behavior in male Siberian hamsters. Horm. Behav. 138, 105099.
Munley, K.M., Han, Y., Lansing, M.X., Demas, G.E., 2022b. Winter madness: melatonin as a neuroendocrine regulator of seasonal aggression. J. Exp. Zool. A Ecol. Integr. Physiol. 337, 873–889.
Munley, K.M., Trinidad, J.C., Demas, G.E., 2022c. Sex-specific endocrine regulation of seasonal aggression in Siberian hamsters. Proc. R. Soc. Lond. Ser. B Biol. Sci. 289, 20220668.
Munley, K.M., Wade, K.L., Pradhan, D.S., 2022d. Uncovering the seasonal brain: liquid chromatography-tandem mass spectrometry (LC-MS/MS) as a biochemical approach for studying seasonal social behaviors. Horm. Behav. 142, 105161.
Munley, K.M., Sinkiewicz, D.M., Szwed, S.M., Demas, G.E., 2023. Sex and seasonal differences in neural steroid sensitivity predict territorial aggression in Siberian hamsters. Horm. Behav. 154, 105390.
Murray, E.K., Hien, A., de Vries, G.J., Forger, N.G., 2009. Epigenetic control of sexual differentiation of the bed nucleus of the Stria Terminalis. Endocrinology 150, 4241–4247.
Murtagh, F., Legendre, P., 2014. Ward's hierarchical agglomerative clustering method: which algorithms implement Ward's criterion? J. Classif. 31, 274–295.
Nagahama, Y., Chakraborty, T., Paul-Prasanth, B., Ohta, K., Nakamura, M., 2021. Sex determination, gonadal sex differentiation, and plasticity in vertebrate species. Physiol. Rev. 101, 1237–1308.
Nakagawa, S., Cuthill, I.C., 2007. Effect size, confidence interval and statistical significance: a practical guide for biologists. Biol. Rev. Camb. Philos. Soc. 82, 591–605. https://doi.org/10.1111/j.1469-185X.2007.00027.x.
Nanga, S., Bawah, A.T., Acquaye, B.A., Billa, M.-I., Baeta, F.D., Odai, N.A., Obeng, S.K., Nsiah, A.D., 2021. Review of dimension reduction methods. J. Data Anal. Inf. Proc. 09, 189–231. https://doi.org/10.4236/jdaip.2021.93013.
National Institutes of Health, 2015. Consideration of Sex as a Biological Variable in NIH-581 Funded Research (Notice 582 Number: NOT-OD-15-102). URL. https://grants.nih.gov/grants/guide/notice-files/NOT-OD-15-102.html. (Accessed 25 July 2023).
Nishiike, Y., Miyazoe, D., Togawa, R., Yokoyama, K., Nakasone, K., Miyata, M., Kikuchi, Y., Kamei, Y., Todo, T., Ishikawa-Fujiwara, T., Ohno, K., Usami, T., Nagahama, Y., Okubo, K., 2021. Estrogen receptor 2b is the major determinant of sex-typical mating behavior and sexual preference in medaka. Curr. Biol. 31, 1699–1710.
Noble, W.S., 2009. How does multiple testing correction work? Nat. Biotechnol. 27, 1135–1137.
Novick, A.M., Scott, A.T., Epperson, C.N., Schneck, C.D., 2020. Neuropsychiatric effects of tamoxifen: challenges and opportunities. Front. Neuroendocrinol. 59, 100869.
Nunn, C.L., 1999. The evolution of exaggerated sexual swellings in primates and the graded-signal hypothesis. Anim. Behav. 58, 229–246. https://doi.org/10.1006/anbe.1999.1159.
Oliveira, R.F., 2012. Social plasticity in fish: integrating mechanisms and function. J. Fish Biol. 81 (7), 2127–2150.
Paraiso, K.D., Blitz, I.L., Coley, M., Cheung, J., Sudou, N., Taira, M., Cho, K.W.Y., 2019. Endodermal maternal transcription factors establish super-enhancers during zygotic genome activation. Cell Rep. 27, 2962–2977.
Parker, C.G., Lee, J.S., Histed, A.R., Craig, S.E., Rhodes, J.S., 2022. Stable and persistent male-like behavior during male-to-female sex change in the common clownfish *Amphiprion ocellaris*. Horm. Behav. 145, 105239.
Patsopoulos, N.A., Tatsioni, A., Ioannidis, J.P.A., 2007. Claims of sex differences: an empirical assessment in genetic associations. JAMA 298, 880–893.

Patterson, S.D., Aebersold, R.H., 2003. Proteomics: the first decade and beyond. Nat. Genet. 33, 311–323.
Pereira, T.D., Tabris, N., Matsliah, A., Turner, D.M., Li, J., Ravindranath, S., Papadoyannis, E.S., Normand, E., Deutsch, D.S., Wang, Z.Y., McKenzie-Smith, G.C., Mitelut, C.C., Castro, M.D., D'Uva, J., Kislin, M., Sanes, D.H., Kocher, S.D., Wang, S. S.-H., Falkner, A.L., Shaevitz, J.W., Murthy, M., 2022. SLEAP: a deep learning system for multi-animal pose tracking. Nat. Methods 19, 628.
Perry, A.N., Grober, M.S., 2003. A model for social control of sex change: interactions of behavior, neuropeptides, glucocorticoids, and sex steroids. Horm. Behav. 43, 31–38.
Phoenix, C.H., Goy, R.W., Gerall, A.A., Young, W.C., 1959. Organizing action of prenatally administered testosterone propionate on the tissues mediating mating behavior in the female guinea pig. Endocrinology 65, 369–382.
Piersma, T., Drent, J., 2003. Phenotypic flexibility and the evolution of organismal design. Trends Ecol. Evol. 18, 228–233.
Policansky, D., 1982. Sex change in plants and animals. Annu. Rev. Ecol. Evol. Syst. 13, 471–495.
Portet, S., 2020. A primer on model selection using the Akaike information criterion. Infect. Dis. Model. 5, 111–128. https://doi.org/10.1016/j.idm.2019.12.010.
Pouchelon, G., Vergara, J., McMahon, J., Gorissen, B.L., Lin, J.D., Vormstein-Schneider, D., Niehaus, J.L., Burbridge, T.J., Wester, J.C., Sherer, M., Fernandez-Otero, M., Allaway, K.C., Pelkey, K., Chittajallu, R., McBain, C.J., Fan, M., Nasse, J. S., Wildenberg, G.A., Fishell, G., Dimidschstein, J., 2022. A versatile viral toolkit for functional discovery in the nervous system. Cell Rep Methods 2, 100225.
Poulat, F., 2021. Non-coding genome, transcription factors, and sex determination. Sex. Dev. 15, 295–307. https://doi.org/10.1159/000519725.
Pradhan, D.S., Newman, A.E.M., Wacker, D.W., Wingfield, J.C., Schlinger, B.A., Soma, K. K., 2010. Aggressive interactions rapidly increase androgen synthesis in the brain during the non-breeding season. Horm. Behav. 57, 381–389.
Pradhan, D.S., Connor, K.R., Pritchett, E.M., Grober, M.S., 2014a. Contextual modulation of androgen effects on agonistic interactions. Horm. Behav. 65, 47–56. https://doi.org/10.1016/j.yhbeh.2013.11.006.
Pradhan, D.S., Solomon-Lane, T.K., Willis, M.C., Grober, M.S., 2014b. A mechanism for rapid neurosteroidal regulation of parenting behaviour. Proc. Biol. Sci. 281, 20140239.
Pradhan, D.S., Solomon-Lane, T.K., Grober, M.S., 2015a. Contextual modulation of social and endocrine correlates of fitness: insights from the life history of a sex changing fish. Front. Neurosci. 9, 8.
Pradhan, D.S., Willis, M.C., Solomon-Lane, T.K., Thonkulpitak, K., Grober, M.S., 2015b. Simultaneous courtship and parenting in males and sex role reversal in females of the haremic bluebanded goby, *Lythrypnus dalli*. Behaviour 152, 917–940.
Prichard, M.R., Grogan, K.E., Merritt, J.R., Root, J., Maney, D.L., 2022. Allele-specific cis-regulatory methylation of the gene for vasoactive intestinal peptide in white-throated sparrows. Genes Brain Behav. 21, e12831.
Quintana, L., Jalabert, C., Fokidis, H.B., Soma, K.K., Zubizarreta, L., 2021. Neuroendocrine mechanisms underlying non-breeding aggression: common strategies between birds and fish. Front. Neural Circuits 15, 716605. https://doi.org/10.3389/fncir.2021.716605.
Remage-Healey, L., 2014. Frank Beach award winner: steroids as neuromodulators of brain circuits and behavior. Horm. Behav. 66, 552–560.
Remage-Healey, L., Bass, A.H., 2005. Rapid elevations in both steroid hormones and vocal signaling during playback challenge: a field experiment in gulf toadfish. Horm. Behav. 47, 297–305.
Reyer, H.U., Bättig, I., 2004. Identification of reproductive status in female frogs - a quantitative comparison of nine methods. Herpetologica 60 (3), 349–357. https://doi.org/10.1655/03-77.
Richardson, S., 2021. Sex contextualism. Philos. Theory Pract. Biol 13.
Richardson, S.S., 2010. Sexes, species, and genomes: why males and females are not like humans and chimpanzees. Biol. Philos. 25, 823–841.
Rich-Edwards, J.W., Kaiser, U.B., Chen, G.L., Manson, J.E., Goldstein, J.M., 2018. Sex and gender differences research design for basic, clinical, and population studies: essentials for investigators. Endocr. Rev. 39, 424–439. https://doi.org/10.1210/er.2017-00246.
Rodgers, E.W., Earley, R.L., Grober, M.S., 2007. Social status determines sexual phenotype in the bi-directional sex changing bluebanded goby *Lythrypnus dalli*. J. Fish Biol. 70, 1660–1668.
Ruiz-Ortiz, J., Tollkuhn, J., 2021. Specificity in sociogenomics: identifying causal relationships between genes and behavior. Horm. Behav. 127, 104882.
Schaeffer, C., Roos, J., Aron, C., 1990. Lordosis behavior in intact male rats: effects of hormonal treatment and/or manipulation of the olfactory system. Horm. Behav. 24, 50–61.
Schlinger, B.A., Barske, J., Day, L., Fusani, L., Fuxjager, M.J., 2013. Hormones and the neuromuscular control of courtship in the golden-collared manakin (*Manacus vitellinus*). Front. Neuroendocrinol. 34, 143–156.
Schmidt, K.L., Pradhan, D.S., Shah, A.H., Charlier, T.D., Chin, E.H., Soma, K.K., 2008. Neurosteroids, immunosteroids, and the balkanization of endocrinology. Gen. Comp. Endocrinol. 157, 266–274.
Schulz, K.M., Molenda-Figueira, H.A., Sisk, C.L., 2009. Back to the future: The organizational–activational hypothesis adapted to puberty and adolescence. Horm. Behav. 55, 597–604.
Schumer, M., Krishnakant, K., Renn, S.C.P., 2011. Comparative gene expression profiles for highly similar aggressive phenotypes in male and female cichlid fishes (*Julidochromis*). J. Exp. Biol. 214, 3269–3278.
Schuppe, E.R., Solomon-Lane, T.K., Pradhan, D.S., Thonkulpitak, K., Grober, M.S., 2016. Ancestral androgenic differentiation pathways are repurposed during the evolution of adult sexual plasticity. Evol. Dev. 18, 285–296.
Schwarz, J.M., Nugent, B.M., McCarthy, M.M., 2010. Developmental and hormone-induced epigenetic changes to estrogen and progesterone receptor genes in brain are dynamic across the life span. Endocrinology 151, 4871–4881.
Scott, B.B., Lois, C., 2005. Generation of tissue-specific transgenic birds with lentiviral vectors. Proc. Natl. Acad. Sci. U. S. A. 102, 16443–16447.
Scotti, M.A., Place, N.J., Demas, G.E., 2007. Short-day increases in aggression are independent of circulating gonadal steroids in female Siberian hamsters (*Phodopus sungorus*). Horm. Behav. 52, 183–190.
Shansky, R.M., 2019. Are hormones a "female problem" for animal research? Science 364, 825–826.
Shepard, R., Page, C.E., Coutellier, L., 2016. Sensitivity of the prefrontal GABAergic system to chronic stress in male and female mice: relevance for sex differences in stress-related disorders. Neuroscience 332, 1–12.
Shukla, S., 2014. A review ON K-means data clustering approach. Int. J. Inf. Comp. Technol. 4, 1847–1860.
Smiley, K.O., Lipshutz, S.E., Kimmitt, A.A., DeVries, M.S., Cain, K.E., George, E.M., Covino, K.M., 2022. Beyond a biased binary: a perspective on the misconceptions, challenges, and implications of studying females in avian behavioral endocrinology. Front. Physiol. 13, 970603.
Smith, K.S., Bucci, D.J., Luikart, B.W., Mahler, S.V., 2017. DREADDs: use and application in behavioral neuroscience. Behav. Neurosci. 130, 137–155.
Södersten, P., 1976. Lordosis behaviour in male, female and androgenized female rats. J. Endocrinol. 70, 409–420.
Södersten, P., De Jong, F.H., Vreeburg, J.T.M., Baum, M.J., 1974. Lordosis behavior in intact male rats: absence of correlation with mounting behavior or testicular secretion of estradiol-17β and testosterone. Physiol. Behav. 13, 803–808.
Solomon-Lane, T.K., Grober, M.S., 2012. Behavioral and physiological responses to central administration of corticotropin-releasing factor in the bluebanded goby (*Lythrypnus dalli*). Physiol. Behav. 106, 619–625.
Solomon-Lane, T.K., Grober, M.S., 2015. The effects of previous experience and current condition on status contests in the bluebanded goby (*Lythrypnus dalli*). Acta Etholog 19, 51–60.
Solomon-Lane, T.K., Crespi, E.J., Grober, M.S., 2013. Stress and serial adult metamorphosis: multiple roles for the stress axis in socially regulated sex change. Front. Neurosci. 7 https://doi.org/10.3389/fnins.2013.00210.
Solomon-Lane, T.K., Willis, M.C., Pradhan, D.S., Grober, M.S., 2014. Female, but not male, agonistic behaviour is associated with male reproductive success in stable bluebanded goby (*Lythrypnus dalli*) hierarchies. Behaviour 151, 1367–1387.
Solomon-Lane, T.K., Pradhan, D.S., Willis, M.C., Grober, M.S., 2015. Agonistic reciprocity is associated with reduced male reproductive success within haremic social networks. Proc. R. Soc. B Biol. Sci. 282, 10–20151914.
Solomon-Lane, T.K., Shvidkaya, P., Thomas, A., Williams, M.M., Rhyne, A., Rogers, L., Grober, M.S., 2016. Juvenile social status predicts primary sex allocation in a sex changing fish. Evol. Dev. 18, 245–253.
Spencer, K.A., Evans, N.P., Monaghan, P., 2009. Postnatal stress in birds: a novel model of glucocorticoid programming of the hypothalamic-pituitary-adrenal axis. Endocrinology 150, 1931–1934.
Spool, J.A., Macedo-Lima, M., Scarpa, G., Morohashi, Y., Yazaki-Sugiyama, Y., Remage-Healey, L., 2021. Genetically identified neurons in avian auditory pallium mirror core principles of their mammalian counterparts. Curr. Biol. 31, 2831–2843.
Steindal, I.A.F., Whitmore, D., 2019. Circadian clocks in fish—what have we learned so far? MDPI Biology 8, 17. https://doi.org/10.3390/biology8010017.
Subramoniam, T., 2017. Sexual Biology and Reproduction in Crustaceans. Academic Press.
Sudai, M., Borsa, A., Ichikawa, K., Shattuck-Heidorn, H., Zhao, H., Richardson, S.S., 2022. Law, policy, biology, and sex: critical issues for researchers. Science 376, 802–804.
Sun, S.D., Ashley, F., 2023. Anti-trans myths. OpenMind Magazine.
Sun, S.D., Tollkuhn, J., 2023. Regulation of gene expression in the brain by sex hormones. In: Legato, M.J. (Ed.), Principles of Gender-Specific Medicine (Fourth Edition). Academic Press, pp. 117–127.
Suzuki, R., Shimodaira, H., 2006. Pvclust: an R package for assessing the uncertainty in hierarchical clustering. Bioinformatics 22, 1540–1542. https://doi.org/10.1093/bioinformatics/btl117.
Syakur, M.A., Khotimah, B.K., Rochman, E.M.S., Satoro, B.D., 2018. Integration K-means clustering method and elbow method for identification of the best customer profile cluster. IOP Conf. Ser.: Mater. Sci. Eng. 336, 012017.
Tam, H., Schlinger, B.A., 2007. Activities of 3β-HSD and aromatase in slices of developing and adult zebra finch brain. Gen. Comp. Endocrinol. 150, 26–33.
Tambunan, H.B., Barus, D.H., Hartono, J., Alam, A.S., Nugraha, D.A., Usman, H.H.H., 2020. Electrical Peak Load Clustering Analysis Using K-Means Algorithm and Silhouette Coefficient, in: 020 International Conference on Technology and Policy in Energy and Electric Power (ICT-PEP), Bandung, Indonesia, 2020, pp. 258–262. https://doi.org/10.1109/ICT-PEP50916.2020.9249773.
Tata, J.R., 1993. Gene expression during metamorphosis: an ideal model for post-embryonic development, 15 (4), 239–248. https://doi.org/10.1002/bies.950150404.
Taves, M.D., Ma, C., Heimovics, S.A., Saldanha, C.J., Soma, K.K., 2011. Measurement of steroid concentrations in brain tissue: methodological considerations. Front. Endocrinol. 2, 39.
Taylor, J.H., Walton, J.C., McCann, K.E., Norvelle, A., Liu, Q., Vander Velden, J.W., Borland, J.M., Hart, M., Jin, C., Huhman, K.L., Cox, D.N., Albers, H.E., 2022. CRISPR-Cas9 editing of the arginine–vasopressin V1a receptor produces paradoxical changes in social behavior in Syrian hamsters. Proc. Natl. Acad. Sci. U. S. A. 119, e2121037119.

Tibshirani, R., Walther, G., Hastie, T., 2001. Estimating the number of clusters in a data set via the gap statistic. J. Roy. Stat. Soc. B. 63, 411–423.
Todd, E.V., Liu, H., Muncaster, S., Gemmell, N.J., 2016. Bending genders: The biology of natural sex change in fish. Sex. Dev. 10, 223–241.
Todd, E.V., Ortega-Recalde, O., Liu, H., Lamm, M.S., Rutherford, K.M., Cross, H., Black, M.A., Kardailsky, O., Graves, J.A.M., Hore, T.A., Godwin, J.R., Gemmell, N.J., 2019. Stress, novel sex genes, and epigenetic reprogramming orchestrate socially controlled sex change. Sci. Adv. 5, eaaw7006.
Trainor, B.C., 2011. Stress responses and the mesolimbic dopamine system: social contexts and sex differences. Horm. Behav. 60, 457–469.
Vaillant, S., Magre, S., Dorizzi, M., Pieau, C., Richard-Mercier, N., 2001. Expression of AMH, SF1, and SOX9 in gonads of genetic female chickens during sex reversal induced by an aromatase inhibitor. Dev. Dyn. 222, 228–237.
Vega-Fruits, R., Macías-Ordóñez, R., Guevara, R., FroTmhage, L., 2014. Sex change in plants and animals: a unified perspective. J. Evol. Biol. 27, 667–675. https://doi.org/10.1210/er.2008-0005.
Veldhuis, J.D., Keenan, D.M., Pincus, S.M., 2008. Motivations and methods for analyzing pulsatile hormone secretion. Endocr. Rev. 823–864.
Vijayakumar, N., Youssef, G., Allen, N.B., Anderson, V., Efron, D., Mundy, L., Patton, G., Simmons, J.G., Silk, T., Whittle, S., 2021. The effects of puberty and its hormones on subcortical brain development. Compr. Psychoneuroendocrinol. 7, 100074 https://doi.org/10.1016/j.cpnec.2021.100074.
Waddington, C.H., 1957. The Strategy of the Genes: A Discussion of some Aspects of Theoretical Biology. George Allen & Unwin, London.
Wallen, K., Baum, M.J., 2002. Masculinization and Defeminization in Altricial and Precocial Mammals. In: Hormones, Brain and Behavior. Elsevier, pp. 385–423. https://doi.org/10.1016/B978-012532104-4/50071-8.
Wallen, K., Winston, L.A., 1984. Social complexity and hormonal influences on sexual behavior in rhesus monkeys (*Macaca mulatta*). Physiol. Behav. 32, 629–637.
Warner, R.R., Swearer, S.E., 1991. Social control of sex change in the bluehead wrasse, *Thalassoma bifasciatum* (Pisces: Labridae). Biol. Bull. 181, 199–204.
Wellman, C.L., Bollinger, J.L., Moench, K.M., 2020. Effects of stress on the structure and function of the medial prefrontal cortex: insights from animal models. Int. Rev. Neurobiol. 150, 129–153.
West, G., 1990. Methods of assessing ovarian development in fishes: a review. Aus, J. Mar. Freshw. Res 41 (2), 199–222.
White, K.J., Rivas, M.G., Pradhan, D.S., 2023. Sex differences in aggressive intensities and brain steroids during status resolution in a sex changing fish, *Lythrypnus dalli*. Horm. Behav. 153, 105373.
Wilkins, A.S., 2008. Waddington's unfinished critique of neo-Darwinian genetics: then and now. Biol. Theory 3, 224–232.
Williams, C.L., Thalabard, J.C., O'Byrne, K.T., Grosser, P.M., Nishihara, M., Hotchkiss, J., Knobil, E., 1990. Duration of phasic electrical activity of the hypothalamic gonadotropin-releasing hormone pulse generator and dynamics of luteinizing hormone pulses in the rhesus monkey. Proc. Natl. Acad. Sci. U. S. A. 87, 8580–8582. https://doi.org/10.1073/pnas.87.21.8580.
Wingfield, J.C., Farner, D.S., 1978. The endocrinology of a natural breeding population of the white-crowned sparrow (*Zonotrichia leucophrys Pugetensis*). Physiol. Biochem. Zool. 51, 188–205.
Woodcock, M.E., Idoko-Akoh, A., McGrew, M.J., 2017. Gene editing in birds takes flight. Mamm. Genome 28, 315–323.
Wright, W.G., 1988. Sex change in the mollusca. Trends Ecol. Evol. 3, 137–140.
Wu, G.C., Chang, C.F., 2013. The switch of secondary sex determination in protandrous black porgy, *Acanthopagrus schlegeli*. Fish Physiol. Biochem. 39, 33–38.
Wu, M.V., Manoli, D.S., Fraser, E.J., Coats, J.K., Tollkuhn, J., Honda, S.-I., Harada, N., Shah, N.M., 2009. Estrogen masculinizes neural pathways and sex-specific behaviors. Cell 139, 61–72.
Yan, L., Silver, R., 2016. Neuroendocrine underpinnings of sex differences in circadian timing systems. J. Steroid Biochem. Mol. Biol. 160, 118–126.
Yan, L., Feng, H., Wang, F., Lu, B., Liu, X., Sun, L., Wang, D., 2019. Establishment of three estrogen receptors (esr1, esr2a, esr2b) knockout lines for functional study in Nile tilapia. J. Steroid Biochem. Mol. Biol. 191, 105379.
Ye, Z., Bishop, T., Wang, Y., Shahriari, R., Lynch, M., 2023. Evolution of sex determination in crustaceans. Mar. Life Sci. Technol. 5, 1–11.
Yokoi, S., Naruse, K., Kamei, Y., Ansai, S., Kinoshita, M., Mito, M., Iwasaki, S., Inoue, S., Okuyama, T., Nakagawa, S., Young, L.J., Takeuchi, H., 2020. Sexually dimorphic role of oxytocin in medaka mate choice. Proc. Natl. Acad. Sci. U. S. A. 117, 4802–4808.
Zilkha, N., Sofer, Y., Kashash, Y., Kimchi, T., 2021. The social network: neural control of sex differences in reproductive behaviors, motivation, and response to social isolation. Curr. Opin. Neurobiol. 68, 137–151.
Zilkha, N., Chuartzman, S.G., Sofer, Y., Pen, Y., Cum, M., Mayo, A., Alon, U., Kimchi, T., 2023. Sex-dependent control of pheromones on social organization within groups of wild house mice. Curr. Biol. 33, 1407–1420 e4. https://doi.org/10.1016/j.cub.2023.02.039.
Zuur, A.F., Ieno, E.N., Elphick, C.S., 2010. A protocol for data exploration to avoid common statistical problems. Methods Ecol. Evol. 1, 3–14. https://doi.org/10.1111/j.2041-210X.2009.00001.x.