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Vic Shao-Chih Chiang

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ADVANCING METHODS FOR STUDYING THE NEUROSCIENCE OF SEX
VARIABILITY AND DIVERSITY

A Dissertation Presented

by

VIC SHAO-CHIH CHIANG 蔣紹志

Submitted to the Office of Graduate Studies,
University of Massachusetts Boston,
in partial fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2024

Developmental and Brain Sciences Program

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ABSTRACT

ADVANCING METHODS FOR STUDYING THE NEUROSCIENCE OF SEX VARIABILITY AND DIVERSITY

August 2024

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Sex variability and diversity are pervasive across taxa and genera. Society is heavily structured around sex/gender conceptualizations, and neuroscience research actively shapes these constructs. This dissertation adopts frameworks that challenge ideologies perpetuating sex/gender injustices and developed neuroscience-related methodologies that could advance the conceptualisations of sex. First, I studied B6D2F1 mice which exhibit sexual behaviour months after castration. This challenged the dogma that sexual behaviour is dependent on gonadal steroid hormones. I enhanced the behavioural analysis of these mice using deep learning techniques. Second, applying similar deep learning

methods, I refined the behavioural analysis of *Astatotilapia burtoni* cichlids, which exhibit sexual behaviour in groups. Traditionally, neuroscience studies on sexual behaviour involve animal dyads singly isolated until experimentation. Third, recognising that laboratory cultivation alters cichlid behaviour and brain compared to the wild, I developed a framework utilising camera trap datasets to facilitate future neuroscience studies in wildlife settings. Lastly, in addition to sex at the behavioural level, numerous variables at cellular, molecular, and genetic levels are related to sex. Neuroscience currently lacks models to adequately construct human sex at these levels. To address this gap, I engineered a cell type relevant to human sex from stem cells. These efforts encompass various aspects of sex variability and diversity, emphasizing rigorous, precise, and transparent science to promote sex/gender justice and advance our construction of sex

DEDICATION

For my 奶奶 蔣沈敏, who inspired my pursuit of epistemological diversity especially for diverse communities fighting marginalisation with your spiritual contemplation and virtue

ACKNOWLEDGEMENTS

Getting to the US in 2019 is not possible without the support of many, including several past professors and my social impact organization, Savyn. Due to time constraints, my acknowledgements will focus on those directly related to my PhD and my life in Boston. I am enriched by many during this journey, which is my most treasured part of this PhD. My words simply cannot do justice to the deep gratitude I feel.

Doing my PhD in Boston, I want to first acknowledge the land and water Boston occupies. These are the unceded ancestral, traditional, and contemporary lands of the Massachusett, Pawtucket, Wampanoag, and Nipmuc tribal nations. I honour the cultures, contributions, and enduring presence of these Native peoples. At the same time, I call for us to be better stewards of the land and allies to Indigenous communities, committing to learning from and supporting Indigenous leaders. It is essential to move beyond acknowledgement towards action, working to dismantle oppressive systems and elevate Indigenous voices and stories in the community and beyond.

I extend my profound gratitude to my advisor, Jin Ho. I cannot imagine having anyone else guide me through my PhD. Your trust, unwavering support, openness and giving me the freedom to pursue ideas have been invaluable. I felt seen, heard, nurtured, and challenged to do better. Any strength in this research can be attributed to your influence. I am very appreciative of Lisa, my committee member, for all the warmth and support you've provided me. I also enjoyed participating in the many Brain Awareness Weeks hosted by you. I am very inspired by your vision of these initiatives to do more for underserved communities. To my other committee members, Vivian and Albert, I extend my heartfelt thanks for your support and for opening my eyes to the wonders of cognitive neuroscience and collective behaviour, which have profoundly influenced my thinking.

I want to thank the GSG, GEO, and RTF for the funding that helped make my research possible. Hosting the Boston Neuroscience Network to discuss with Boston guests, your recent publications have significantly shaped my research. Additionally, science events around Boston further influenced my research. One particularly notable event was the engaging debates between Nancy Kanwisher, physicist Alan Lightman, and

philosopher Kieran Setiya, which made me ruminate more on the spiritual, philosophical, and moral implications of my research.

My research direction was revolutionized by DeepLabCut. The AI residency with Alex and Mackenzie Mathis, the celebrity couple that developed DeepLabCut, was pivotal. I spent my 2022 summer in Geneva, learning from your pioneering spirit and commitment to justice. My heartfelt gratitude also extends to mentors especially Jessie, 牧, and Tim for your patience in always answering my questions and being generous with your time. The deep friendships built with fellow AI residents Sofi, Nesli, Sabri, Nirel, and Jonas have been immensely enriching, and I am grateful for everything each of you taught me.

My DeepLabCut projects benefited from the contributions of Park Lab members, particularly Sophie for the manual analysis and Danny for the labelling. My dissertation would not have been the same without Peter's generosity in sharing your cichlid data and willingness to collaborate on the project, including the labelling help from your lab members Amelia, Hope, and Lexi.

In 2023, I was in Kruger National Park during the Southern Hemisphere winter in South Africa, learning camera trap research from the Wild Ecology Lab. I extend special thanks to tutors and participants, especially Rob and Lucie, for your help with the extensive preparations required for this trip, managing the logistics during our stay, and patiently guiding me through this unfamiliar field. I also want to acknowledge that Kruger National Park is situated on the traditional lands and waters of the Tsonga, Shangaan, and other Indigenous communities who have lived in and cared for this land and water for generations.

Since 2022, I have been with the Church Lab at Harvard Medical School. I am grateful to George for this incredible opportunity, to Emma for all the administrative support, and to 瑜 for getting me into the lab. Lily has been indispensable during my time in the Church Lab, mentoring me in building the stem cell work from the ground up and pushing me to make progress. Additionally, this research would not have been possible without the teaching and assistance from many Church lab members, notably 松雷, Esther, and Zink in experiments, bioinformatics, and beyond.

A significant part of my PhD was the three semesters I served as an instructor of record. I value the lessons I learned from the diversity of students at this minority-serving institution, most notably Temi, Katie, Marietta, and Vitoria. My guest lecturers enriched these classes with insights from various fields, such as Kim on economic justice, Michelle on astronomy, and Dawn on Indigenous studies. My teaching would not have been the same without Laurel, who guided and supported me as I explored my pedagogy and inspired me with your teaching style.

Volunteering has been crucial in supporting me through my depression. Special thanks to 慈濟 for treating me like family, my fellow UCL alumni in Boston for making the US feel less foreign, and Gay4Good for fostering queer solidarity. Participating in activism such as in the anti-ELAB protests to support 香港人 and anti-genocide rallies to support Palestinians have been integral for me to think more about fostering a world I want to live in, and how science relates to this. I recognize the need to do more using and reflecting on my privilege. Boston has enriched my life by offering diverse events. One memorable event that continued to get me to think more about science and justice was a conference that discussed how elite universities accrued wealth and prestige from slavery. The conference included conversations with slave descendants and explored justice through various mediums like poetry, stories, and ceremonies.

Some people say it is not easy for introverts to make friends. I am grateful to those I met during the Introverts as Leaders course Savyn sent me to, for reminding me of our strengths as introverts. Although there are some stereotypes of introverts and socializing, the friendships I formed during my time in Boston are among my greatest blessings. Especially to Erinda and Sabrina, thank you for your kindness and for always providing a safe space to share my vulnerabilities. To Jamie and Javier, thank you for all the thought-provoking discussions that prompted me to reflect deeply. To Natasha and Kenny, thank you for all the times you reminded me why pursuing a PhD is worthwhile. To Huanran, living in Boston would have been much harder without you as the best roommate with 牛牛.

I express immense gratitude to my family for providing me with the privilege of completing a PhD. I think of the journey my parents made to the foreign land of New Zealand long ago when I was a small child, hoping to give our family a better future.

To my 寶寶 Stephen – my PhD is nurtured by your encouragement and all the help you provided such as with DeepLabCut, wildlife framework, and coding. I am profoundly grateful for the person you have helped shape me into, through our deep conversations and intellectual debates, as well as your curiosity and erudition. Together, we have explored love from the theory of forms, de Botton, and Fromm’s Art of Loving. Our discipline and devotion to each other have led me to this moment. I am humbled and filled with courage by our love. Thank you from the depth of my soul.

To my 奶奶, my most important person in the world – whenever I think about my beginnings, I think about you. I am shaped by your profound spirituality, exploring our relationship with the world and contemplating how we want to live this life. You are always inspiring me with your commitment to virtue. You are my most ardent supporter in everything I do and everything I am, including my sexuality. You passed away in 2023 before I got my PhD. I find it so difficult to truly express how much I miss you, but my dedication of this PhD to you is one way of trying. Just like what Charlotte Mary Mews wrote as part of her poem:

The forest road.

Of the black listening trees.

Against the lonely dark, if I could touch the fear

And leave it kissed away on quiet lids.

The scent of the unsilenced sea would linger on

In these dark waves, and round the silence that was you.

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LIST OF ABBREVIATIONS

2023 African Field School on Design and Analysis of Camera Trap Studies
(AFSCTS2023)

Animal Skeleton-Based Action Recognition (ASBAR)

artificial intelligence (AI)

Astatotilapia burtoni (*A burtoni*)

barcoded PBAN expression vector (pBAN-BC)

bovine growth hormone polyadenylation signal (bGH PolyA)

Contrastive Language Image Pretraining (CLIP)

differentially expressed genes (DEGs)

dominant cichlids with colourful egg spots on their anal fins (dominant CES cichlids)

dominant cichlids with dull-coloured egg spots (dominant DES cichlids)

elongation factor 1 alpha (EF-1 α)

endothelial cells (Endo)

fibroblasts (Fibro)

fluorescent-activated cell sorting (FACS)

forward scatter area/height/width(FSC-A/H/W)

Gene Ontology (GO)

glycine-serine-glycine linker (GSG)

Human endogenous retrovirus (HERV)

human induced pluripotent stem cells (hiPSCs)

inverted terminal repeats (ITRs)

Janelia Automatic Animal Behaviour Annotator (JAABA)

keratin-7 (KRT7)

keratinocytes (KC)

Kyoto Encyclopedia of Genes and Genomes (KEGG)

long-interspersed nuclear elements (LINE)

melanocortin-5 receptor (MC5R)

mice no visible scrotum and visible nipples (NP mice)

mice with no visible nipples and a visible scrotum (SC mice)

Mouse Action Recognition System (MARS)

Multi-Object Tracking in Heterogeneous environments (MOTHe)
Multiple Object Tracking for Fish (FishMOT)
One-Stop Interpretably Cell-type Annotation (TOSICA)
phosphoglycerate kinase (PGK)
phycoerythrin (PE)
polyadenylation signal (polyA)
principal component analysis (PCA)
protospacer adjacent motif (PAM)
puromycin resistance gene (PuroR)
puromycin resistance, thymidine kinase (PuroTK)
refinement, reduction, and replacement (3Rs)
region of interest (ROI)
RNA-sequencing (RNA-seq)
short-interspersed nuclear elements (SINE)
side scatter area/height/width (SSC-A/H/W)
Simple Behavioural Analysis (SimBA)
Single-cell RNA sequencing (scRNA-seq)
single-guide RNAs (sgRNAs)
Social LEAP Estimates Animal Poses (SLEAP)
spatiotemporal PoseNet (STPoseNet)
subordinate cichlids with large body size (subordinate LBS cichlids)
subordinate cichlids with smaller body size (subordinate SBS cichlids)
T cells (T)
tandem Tomato (tdTomato)
tetracycline-responsive element 3G (TRE3G)
transcription factor (TF)
Transposable elements (TEs)
Uniform Manifold Approximation and Projection (UMAP)
vascular smooth muscle cells (VSMC)
Where'd It Land (WILD)

CHAPTER 1

GENERAL INTRODUCTION

Studies that investigate sex/gender are needed in relation to medicine, education, sociocultural infrastructures and the ecosystem including those in non-human animals (McLaughlin *et al.*, 2023; Sharpe *et al.*, 2023; Ritz & Greaves, 2024). Scientific responsibility necessitates rigorous research. Scientists must carefully consider the implications of their research on setting standards that impact sex and gender-diverse individuals. This responsibility should not be placed on those who are already burdened and marginalised (Casto & Maney, 2024; Kennis *et al.*, 2024).

The objective of this dissertation was to adopt frameworks that recognize sex/gender diversity in scientific research and to challenge ideologies that perpetuate sex/gender injustices. Although sex and gender have been distinguished for clarity, they are deeply intertwined in both socio-cultural and physiological systems (including the brain), which shape and reshape each other (Massa *et al.*, 2023; Arraiza Zabalegui, 2024). Sex is a complex concept involving many factors, such as genes, molecules, body functions, organ geometry, behaviours, growth processes, physical traits, hormones, brain functions, reproductive cells, cell structures, body forms, face, microbiome and chromosomes (Maeng & Beumer, 2023; Massa *et al.*, 2023; Aghi, Anderson, *et al.*, 2024; Blackie *et al.*, 2024; Morina *et al.*, 2024; Smiley *et al.*, 2024). All these factors interact with each other in different ways. In terms of gender, scholars have referred to the various social norms and processes that shape identities and behaviours (Aghi, Anderson, *et al.*,

2024; Ashley *et al.*, 2024; Smiley *et al.*, 2024). These norms are influenced by psychology, relationships with others, and environment. They are deeply embedded in historical, societal, and cultural contexts, and they can change over time. Gender also involves performativity, meaning that gender identities and roles are constructed through social interactions to meet personal and social needs. While gender and sex are partially related to physical reality, it is important to acknowledge that there are aspects of material agency—how physical matter behaves—that exist beyond human control or influence (van Anders *et al.*, 2023). While gender is controversial in non-human entities, this has been discussed in the context of gender performativity by seeing the entanglement between human and non-human modes of life that help us imagine other ways of living and knowing (Dichman, 2024).

Throughout this dissertation, I used the term "sex" to align with my research focus. I do not treat sex as deterministic or immutable, acknowledging the contextual factors that influence sex characteristics over time and scale (Massa *et al.*, 2023; Donovan *et al.*, 2024; Smiley *et al.*, 2024). Sex is described as emergent, where multiple sex-related variables change over time, interacting to form a complex system that cannot be predicted by examining individual variables alone (Smiley *et al.*, 2024). Appreciating sex as emergent highlights the fluidity and variability of sex-related variables, which can lead to different outcomes depending on their interactions and timing. Many empirical studies have demonstrated support for this view of sex (Naqvi *et al.*, 2019; Eliot *et al.*, 2021; Fausto-Sterling, 2021; Blencowe *et al.*, 2022; Rocks *et al.*, 2022; Joel *et al.*, 2024). I focus on objectives within the more-than-human ecosystem, de-centring from humans to include non-human entities and environments (Nicenboim *et al.*, 2023; Silberzahn, 2024; Thorpe *et al.*, 2024; Wouters, 2024). Considering the breadth of sex/gender, it is not exclusively a scientific domain; science is one of many valuable ways to explore aspects of sex/gender.

Current Western society is heavily constructed around sex/gender conceptualizations, encompassing individual identity, interpersonal interactions, and structural/cultural arrangements such as patriarchy and cis-heteronormativity (Casto & Maney, 2024; Ridgeway & Saperstein, 2024; Ritz & Greaves, 2024). Gender biases have

also been noted in ChatGPT, a tool that is now an integral part of some people's everyday lives (Fulgu & Capraro, 2024). A significant number of sex/gender minorities face discrimination with repercussions in various areas, including the workplace, healthcare, domestic violence, mental health, and hate crimes (Aghi, Anderson, *et al.*, 2024; Galinsky *et al.*, 2024; Haupt *et al.*, 2024; Volpe *et al.*, 2024). Throughout history and across cultures, diverse gender identities have existed, such as the Fa'afafine in Samoa, sworn virgins in the Balkans, and Muxe in the Indigenous Zapotec community (Heyam, 2022). Despite this, recent years have seen intense and polarizing debates, along with vitriolic and violent reactions, regarding the existence and supposed novelty of these identities (Ridgeway & Saperstein, 2024; Ritz & Greaves, 2024).

The debate surrounding sex/gender diversity is frequently politicized, influencing legislation, medical practices, and regulations impacting diverse individuals, by weaponising science (Aghi, Goetz, *et al.*, 2024; Smiley *et al.*, 2024). This issue is partially rooted in challenges within scientific communication, where research and academic institutions also bear responsibility (Sharpe *et al.*, 2023). Historically, science has been dominated by white, cisgender men, who claim objectivity in their research (Sharpe *et al.*, 2023; Dunk *et al.*, 2024; Maeng *et al.*, 2024). Conforming to the standards set by this majority has traditionally been synonymous with 'success' in science (Aghi, Anderson, *et al.*, 2024). While these majorities may acknowledge their biases, mitigating them proves challenging, as they are often perceived as justified (van Anders *et al.*, 2023). These biases can result in the disregard or dismissal of differences in social location, identity, or worldview. Consequently, contemporary Western science still tends to view sex/gender diversity as rare, pathological, or unworthy of mention (Sharpe *et al.*, 2023; Dunk *et al.*, 2024; Velocci, 2024).

Studies examining sex often use it as a proxy for other variables (Massa *et al.*, 2023; Smiley *et al.*, 2024). These variables are generally multivariate and multidimensional (McLaughlin *et al.*, 2023). *A priori* categorization of sex can create the perceptual bias that members of different groups are assumed to be different. It can also create erroneous assumptions (Dubova & Goldstone, 2023) because it is fictional to think individuals fulfil the archetypes of all the variables that relate to sex. Sex is often treated

as a causal mechanism without considering other physiological and environmental variables (Grissom *et al.*, 2024; Pape *et al.*, 2024). Studying sex in non-human animals and their ecosystems offers valuable insights. However, generalisability between species is limited due to the many non-homology-related factors of sex (Pape *et al.*, 2024). This limitation is evident in studies showing different patterns in mental health illnesses between humans and rodents in relation to sex-related variables (Kokras & Dalla, 2014; Shansky, 2014; Becker *et al.*, 2017; Ma *et al.*, 2019). Homology has been referred to similarities in biological structures or sequences due to shared ancestry (Gouvêa, 2023), however, these similarities do not solely determine sex-related variables. Factors such as epigenetics, social interactions, and environmental adaptations also play significant roles.

Throughout this dissertation, I strived to use language that addresses sex/gender diversity (Massa *et al.*, 2023; McLaughlin *et al.*, 2023; Aghi, Anderson, *et al.*, 2024; Ridgeway & Saperstein, 2024; Smiley *et al.*, 2024). Considering the flaws in much of the research related to sex, it is crucial to continuously re-examine and reframe these studies to investigate how changes in interpretation can prompt re-evaluation (Smiley *et al.*, 2024). It is important to recognise that science is not solely about discovering phenomena but involves the active role of scientists as knowledge producers (van Anders *et al.*, 2023). For example, cognitive traits and gender/sex traits were observed to affect stances on contentious scientific issues and how a research question is approached (Sulik *et al.*, 2023; Picó-Pérez *et al.*, 2024). Observer bias in scientists has been widely recognised e.g. how the perceived sex/gender of the research subject affects the results collected (Fernández Pinto, 2023; Keaney *et al.*, 2024). Science actively shapes the world as it studies it; the way scientists conceptualise phenomena influences the evidence obtained (Dubova & Goldstone, 2023). This influence is reflected in the language of science, which can perpetuate biased notions. Therefore, I refrained from using terms like "understand," "discover," or "find" when discussing sex/gender, as these imply an essentialised phenomenon that is being uncovered.

The purpose of adopting these frameworks is to produce science that advances our conceptual construction of sex as well as addresses sex/gender justice for the more-than-human world. This dissertation aimed to achieve this by advancing neuroscience research

on sex variability and diversity in ways that integrate these frameworks. To begin, I investigated steroid-independent sexual behaviour (reviewed in Chiang & Park, 2020). To expedite this research in the rodent model B6D2F1 hybrid mice, I developed automated behavioural methods to analyse their sexual behaviour. However, the current approach relies on single dyads of mice that are singly isolated until experimentation, which is difficult to generalize to what occurs in nature (Clutton-Brock, 2021; Vogt *et al.*, 2024). Therefore, I also focused on research involving the East African cichlid *Astatotilapia burtoni*, a species that exhibits lekking, a collective behavioural manifestation of sexual behaviour (Maruska & Fernald, 2018; Rathore, Isvaran, *et al.*, 2023). To facilitate the study of this system, I aimed to develop automated behavioural methods to analyse not only behaviours traditionally considered sexual but also their interactions that contribute to emergent patterns of the collective. However, given that *Astatotilapia burtoni* studies were conducted in the lab, there could be limitations on external validity (Mathuru *et al.*, 2020). To address this, I examined images of animals in the wild captured with camera traps to offer a starting point for neuroscientists conducting neuroimaging studies on minimally captive and free-behaving wildlife animals. Lastly, I conducted a study focused on human sebocytes, a cell type that is relevant to both neuroscience and sex research.

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CHAPTER 2

AUTOMATION OF STEROID-INDEPENDENT SEXUAL BEHAVIOURAL ANALYSIS FOR NEUROSCIENCE STUDIES

Introduction

Sexual Behaviour

Current fossil records indicate the origins of sexual reproduction may point to 1-2 billion years ago (Shkliarevsky, 2021). Several hypotheses have been made on why sexual reproduction arose. One proposes that two DNA copies (diploid) may reduce the effects of DNA damage. Another proposes that integrating foreign DNA into the original genome (meiosis) may act as a type of stress-adaptation strategy (Hofstatter & Lahr, 2019). This antediluvian nature of sexual reproduction led to the diversity in eukaryotes, from plants to animals and this diversity subsequently underlies the diversity of sexual behaviour exhibited across the animal kingdom (Shkliarevsky, 2021; Fasel, Jeucken, Kravchenko, Fritze, Ruczynski, *et al.*, 2023; Fasel, Jeucken, Kravchenko, Fritze, Ruczyński, *et al.*, 2023; Würsig *et al.*, 2023; Yadav *et al.*, 2023; Larva & Rantala, 2024).

This diversity is seen in rodents, especially mice and rats which are the most commonly studied mammals by neuroscientists. Rodents labelled as male exhibit consummatory sexual behaviour in the forms of mounting, intromission and ejaculation (Karigo & Deutsch, 2022). These are preceded by appetitive sexual behaviour primarily in the form of anogenital sniffing. Following ejaculation, there is a refractory period of variable length. These three broad phases are also generally ascribed to rodents labelled as female (Yin & Lin, 2023). They begin with detecting, approaching and investigating

the conspecific. They then commonly exhibit lordosis, which is where they stay stationary and arch their back. Although neuroscientists often describe these behaviours as specific to males or females, they can occur in interactions with both the same or opposite sex (Gunst *et al.*, 2022). In addition, sex is usually assigned to the animal using a binary system through looking at their external genitalia. This is problematic because the sex of the external genitalia does not necessarily align with the sex at multiple dimensions including, but not limited to, the chromosomal, gonadal, hormonal and behavioural levels (Massa *et al.*, 2023). Therefore, I will refrain from assigning sexual behaviour as belonging to male or female but directly refer to the behaviour I am describing.

Variability in behaviour is a hallmark of individual differences, and many of these differences may be independent of hormonal fluctuations (Levy *et al.*, 2023). This individuality in sexual behaviour correlates with the multisensory nature of sexual interactions, incorporating senses such as hearing, touch, smell, and sight (Lenschow *et al.*, 2022). Scientists observed this in tungara frogs where their courtship occurs through vocalizations by inflating their vocal sacs (Stange *et al.*, 2017), and in stinkbugs with their long-range communication through airborne signals that integrate with plant-borne cues that are vibratory, visual etc. (Rodríguez, 2019). Multisensory perception is well-studied in humans including how audition and visions could work together (Chow *et al.*, 2020, 2023; Izen & Ciaramitaro, 2020), which could be germane to constructing multisensory perception in sexual behaviour. Neuroscientists commonly perceive sexual behaviour as primarily serving reproductive purposes, yet it fulfils numerous other functions. In animals, these range from bartering, social status, or aggression; for humans, revenge, spirituality, or altruism have been reported (Meston & Buss, 2007).

Ways to measure sexual behaviour

Scientific publications on sexual behaviour in English date back to 1888 in Saturniid moths (Soule, 1888). One of the most common model organisms that neuroscientists study is the nematode *Caenorhabditis elegans*. Their mating occurs with hermaphrodites and is examined on a dish of *Escherichia coli* (Liu & Sternberg, 1995). Measurements of time and distance to regions of interest, response to hermaphrodite contact, turning, contact with the vulva, insertion of spicules etc. are recorded to

determine levels of sexual behaviour. Other common animal models include *Drosophila* and zebrafish. Their sexual behaviour has been studied using video recording, followed by manual annotations of the sexual behaviour presented (Ryner *et al.*, 1996; Yabuki *et al.*, 2016). For zebrafish, these included courtship displays of chases, touches, and encircling (Yabuki *et al.*, 2016), and for *Drosophila*, these included courtship displays of tapping, orientation, following, and wing extension (Ryner *et al.*, 1996). Many studies on rodent sexual behaviour over the decades reflect changing approaches to observing behaviour. Written records were kept on hamster mating behaviours in the lab (Powers & Winans, 1975) and on how ground squirrels chased and rejected each other in the wild (Schwagmeyer & Parker, 1990). Subsequently, there was a transition towards adopting video recording as a standard methodology, exemplified by a study investigating the role of epigenetics in prairie vole sexual behaviour (Wang *et al.*, 2013).

In mice and rats, several stereotypical behaviours are recorded, including the latency and numbers of mounting, intromission and ejaculation (Park *et al.*, 2009; Bharadwaj *et al.*, 2013; McInnis *et al.*, 2016; David, Wyrosdic, & Park, 2022). Some studies also calculate the proportion of the animals exhibiting mounting, intromission and ejaculation. In partner preference tests, the time the animal spends investigating each conspecific is recorded. Common metrics used to assess partnering rodents include the frequency of lordosis responses, rated on a scale, as well as the occurrence of paracopulatory behaviours and sexual stimulations (Heijkoop *et al.*, 2018). Additionally, paced mating paradigms have been used that allow the smaller rodent to control the rate of mating by having compartments divided by a hole that the larger rodent cannot pass through (Ventura-Aquino & Paredes, 2023). This paradigm offers several advantages in terms of animal welfare and provides insight into the reward aspects for smaller rodents. Specific metrics are collected for paced mating, including the number of exits and the time it takes for the smaller rodent to re-enter the larger rodent compartment after each exit. Additionally, advancements have been made to streamline manual annotation by developing software that assists researchers in tracking and calculating various measurements (Inoue *et al.*, 2019; Chu & Ågmo, 2023).

Neurobiology of sexual behaviours in rodents

Much progress has been made in investigating the neurobiology of sexual behaviours in rodents. During the appetitive phase, when sexual stimuli increase arousal, kisspeptin neurons in the medial amygdala become active (Ågmo & Laan, 2022). Researchers also established that prolactin in the blood interacts with receptors in the brain, influencing sexual motivation. After encountering sexual stimuli, the rodent's cognitive processes determine whether to proceed to the consummatory phase of sexual behaviour (Kingsbury *et al.*, 2020). These processes may involve brain areas like the dorsal medial prefrontal cortex, which represents the sex, the infralimbic area involved in memory of familiarity with the individual, and the posterodorsal subdivision of the medial amygdala, which regulates the valence of sexual stimuli (Hu, Zuo, *et al.*, 2021; de León Reyes *et al.*, 2023).

Sexual stimuli trigger sensory responses from multiple brain systems (Lenschow *et al.*, 2022; Bentefour & Bakker, 2024). During the consummatory phase, rodent lordosis is centrally regulated by the ventrolateral part of the ventromedial hypothalamus (Yin & Lin, 2023). Mounting, intromission, and ejaculation involve oestrogen-responsive neurons in the bed nucleus of the stria terminalis and the medial preoptic area (Yu *et al.*, 2023). Recent findings also suggest the importance of the posterior amygdala in mounting and intromission. These brain areas collectively form what is known as the reproductive behaviour control column. They integrate various hormonal and metabolic cues to regulate sexual behaviour (Mei *et al.*, 2023).

Steroid-independent sexual behaviour

In general, sexual behaviour is regulated by gonadal steroid hormones (androgens, oestrogen, progesterone) binding to their cognate receptors; however, these receptors can be activated independently without binding to these hormones, as demonstrated in several *in vitro* studies (Hewitt & Korach, 2018; Srivastava *et al.*, 2022). On top of *in vitro* studies, there is also clinical evidence that human sexual behaviour occurs independent of gonadal steroid hormones. For example, sexual activity is preserved in a significant population after androgen deprivation (Duthie *et al.*, 2020).

Besides humans, other animal species similarly exhibit steroid-independent sexual behaviour, including some types of rats, bats, hamsters and primates (Park *et al.*, 2004; Carter, 2022; Sato *et al.*, 2023). The B6D2F1 hybrid mice is a rodent model that also demonstrates steroid-independent sexual behaviour in which 25-33% of the mice demonstrated steroid-independent sexual behaviour months after orchidectomy (McGill & Tucker, 1964; Park *et al.*, 2009; Bharadwaj *et al.*, 2013; McInnis *et al.*, 2016; David, Wyrosdic, & Park, 2022). These studies often label the mice as male, using this as a proxy for the presence of testicles. However, the presence of testicles should not be conflated with all other aspects presumed to relate to "maleness," which were not measured. The presence of testicles does not determine other sex-related variables (Smiley *et al.*, 2024).

Existing ways to measure sexual behaviours using automation

Technological advancements, including improvements in hardware and machine learning, have made it possible to automate behavioural analyses (Siegford *et al.*, 2023; Fazzari *et al.*, 2024). Yet, a key hurdle lies in agreeing on how to define behaviour for optimal computational task design. This proves challenging due to the diverse interpretations of behaviour across various disciplines that study it (Calhoun & Hady, 2023). For behavioural neuroscientists, a general rule of thumb is to consider behaviour in the context of how organisms interact with the environment, and sexual behaviour is no different (Monk *et al.*, 2019a; Calhoun & Hady, 2023).

In the last several years, a number of labs have begun to automate the annotation of a few behaviours. In *Drosophila*, courtship behaviour was automatically annotated by measuring the position and orientation of the wings and body over time (Dankert *et al.*, 2009). Automatically tracking *Drosophila* courtship was later improved through Janelia Automatic Animal Behaviour Annotator (JAABA), which can simultaneously capture a wider range of behaviours, and is easily accessible for scientists without computer science training (Kabra *et al.*, 2013). This was achieved by treating the *Drosophila* as elliptical trajectories and the data were fed into a machine learning algorithm to learn the behaviours that were labelled by humans.

Annotation of sexual behaviour has also been conducted in other model species. In *Caenorhabditis elegans*, automation was done by tracking the location of fluorescent neurons in the posterior brain to help determine which sexual behaviour was exhibited (Susoy *et al.*, 2021). In zebrafish, an unsupervised deep learning computer vision approach called ZeChat was used to capture their complex and multidimensional social behaviour (Geng *et al.*, 2023). The automation of mouse mounting and intromission behaviour has been implemented using top-view high-resolution video recordings with the Mouse Action Recognition System (MARS) and Simple Behavioural Analysis (SimBA)(Karigo *et al.*, 2020; Nilsson *et al.*, 2020; Segalin *et al.*, 2021). These systems use pose estimation and behavioural classification (Karigo *et al.*, 2020; Segalin *et al.*, 2021). MARS extract features from pose estimation outputs, which are then used to train ensemble-based classifiers to automatically identify mounting and sniffing behaviours in dyads categorised as opposite-sex and same-sex. Many studies have utilised front-view recordings to explore additional rodent sexual behaviours, such as ejaculation (e.g. Kukino *et al.*, 2022). Behavioural analysis also involves other senses like smell and sound. For instance, analysing bird behaviour, such as that of birds of paradise, has been automated using video and audio recordings (Ligon *et al.*, 2018).

Benefits of automating behaviour in neuroscience

Automating the analyses of behaviour offers numerous benefits to neuroscience research. Manual annotation of behaviour focuses on simple metrics that rely on consistent detection by human observers (Siegford *et al.*, 2023). However, many aspects of behaviour are beyond the detection limits of the human observer. They also become difficult to detect, when the human observer's attention shifts, especially during long annotation tasks.

Manual annotation is very labour- and time-intensive, especially considering many studies generate tens to hundreds of hours of video. Depending on the metrics that are being annotated, manual annotation can take 3-4 times longer than the video's duration (Kenkel, 2023). For studies investigating steroid-independent sexual behaviour in B6D2F1 mice in my lab, the video recordings exceed 500 hours (David, Wyrosdic, Wan, *et al.*, 2022). Some labs have simultaneously recorded multiple cages of dyads since

an extended duration of recordings over two hours may be required for a large number of rodents (David, Wyrosdic, Wan, *et al.*, 2022).

Despite the time and effort invested in any experiment in which manual annotation is used, experimenter error is a variable that cannot be excluded because individual annotations can vary significantly, often due to fluctuations in attention spans (Isik & Unal, 2023). Annotators within and across labs also show variation because human language is used to describe behaviour, and there are differences in how the behaviour is defined. Automating this process would be a significant step towards solving these issues.

Automation may help capture the richness of the behaviour. For instance, the velocity, tail curvature, and genital distances between the mating pair throughout different phases of sexual behaviour (Susoy *et al.*, 2021). To facilitate the neuroscience production of this phenomenon, such as the use of continuous neural recordings in free-behaving animals, scientists would require comparatively high resolution of behaviour tracking (Kennedy, 2022). Relying on simple metrics in neuroscience may be too coarse to capture fine details at the single neuron level or may not directly relate to the neural activity measured. Often, neuroscience averages results from a group, overlooking within-group differences that are essential for understanding individuality (de Bivort *et al.*, 2022). Individuality is crucial for sex diversity, as seen in the courtship calls of white rhinoceros (Cinková & Shrader, 2022). Variability in sexual behaviour post-castration has also been observed in B6D2F1 mice (Park *et al.*, 2010).

Specific Aim 1

Here, I developed a pose-estimation model using DeepLabCut pose estimation that could be applied to low-resolution videos cropped from recordings of multiple stacked cages. This was curated with our dataset from a previous experiment on steroid-independent rodent sexual behaviour in B6D2F1 hybrid mice. The pose estimation results were fed into SimBA to create two classifiers, one for both mice mounting and intromission, as well as another for mice ejaculation.

Methodology

Rationale for artificial categorisation of mice

Mice were group-housed at weaning based on artificial groupings commonly used in mice studies, which classify males and females by anogenital distance, visible nipples, and scrotum (Wolterink-Donselaar *et al.*, 2009; Deeney *et al.*, 2016). I did not measure the anogenital distance, so I artificially grouped mice into those with no visible nipples and a visible scrotum (SC mice) and those with no visible scrotum and visible nipples (NP mice). It is crucial to emphasize that these categories do not imply a binary limitation for the group, and discerning the presence of nipples and scrotum was occasionally challenging. I did not measure other sex-related variables, making it inaccurate to label these mice as male or female since this implies a range of sex-related traits not measured here (Aghi *et al.*, 2022; Haupt *et al.*, 2024; Kennis *et al.*, 2024). Additionally, I did not genotype these mice. However, even if they were genotyped, the chromosome combination cannot be assumed to inherit all traits from a binary system because sex-related traits are often dissociable from chromosomes, and many sex-related traits are continuous and overlapping (Zemenick *et al.*, 2022; Massa *et al.*, 2023).

Animals

I utilized C57BL/6J (*Mus musculus*; N = 10, RRID: IMSR_JAX:000664) and B6D2F1 hybrid mice (*Mus musculus*; N = 40, RRID: MGI:5651959). B6D2F1 hybrid mice were generated through the crossbreeding of C57BL/6J XX mice (RRID: IMSR_JAX:000664) with DBA/2J XY mice (RRID: IMSR_JAX:000671). All mice were bred and reared in the University of Massachusetts, Boston animal facility, weaned between 21 and 28 days, group-housed by SC or NP until the commencement of experiments (between 50 and 80 days of age), and SC mice were subsequently housed individually for the duration of the experiments.

Housing conditions were standardized, with cages equipped for negative airflow to prevent inter-cage transmission of olfactory cues. The mice were provided with *ad libitum* access to Teklad Global Rodent Diet 2016 (Harlan Laboratories Inc.) and water, and maintained under a 12:12 light: dark cycle, with lights off at 1200 h EST. All procedures were conducted in accordance with the University of Massachusetts, Boston

Institutional Animal Care and Use Committee guidelines (IACUC2020). Surgical and sacrifice procedures, including ovariectomies and orchidectomies, were performed under isoflurane anaesthesia, with utmost care taken to minimize discomfort during handling. Post-surgery analgesia was administered via meloxicam (LKT Labs; 0.5mg/mL in autoclaved distilled water) at a dosage of 5mg/kg.

Group-housed ovariectomised C57BL/6J mice served as stimulus animals for sexual experience and behaviour testing. Prior to sexual experience and behaviour testing, sex stimulus mice were hormonally primed with oestradiol benzoate (MilliporeSigma; 10µg in 0.1 mL sesame oil) and progesterone (Millipore Sigma; 400µg in 0.05 mL sesame oil).

Prior to orchidectomy, B6D2F1 hybrid mice were provided with four instances of sexual experience by cohabiting with a sex stimulus mouse overnight. In a few cases, our artificial categories at weaning were not consistent with later inspections of the mice as they grew older, or did not accurately predict the presence of testicles, possibly due to the difficulty in the categorisation procedure. These animals were excluded from the experiments. After orchidectomy, they were allowed a minimum of one week for recovery before sexual behaviour video recordings. C57BL/6J SC mice selected for sexual behaviour video recordings were also provided with a similar sexual experience paradigm but were not orchidectomized.

Sexual behaviour video recording

Orchiectomised B6D2F1 hybrid mice were subjected to sexual behaviour video recordings with sex stimulus on eleven occasions over a 62-week post-orchidectomy period, which was used as part of another study. After 37 weeks post-orchidectomy, sexual behaviour testing was also preceded by social exposure with social stimulus mice. Each mouse was exposed on three consecutive days before each test, to two social stimulus mice that were gonad-intact, one SC mouse, and another NP mouse. This was conducted each day by placing each social stimulus in the subject's home cage for two minutes. Prior to the introduction of the sex stimuli, all participants underwent a minimum 30-minute habituation period within a clear Plexiglas testing cage measuring 17.8 x 25.4 x 17.8 cm. The bedding within the cage, sourced from the participant's home

cage, remained unchanged for a period of at least 48 hours preceding the test. The procedure commenced with the presentation of a sex stimulus and was recorded via video for a duration exceeding 180 minutes. Video recordings were conducted under dim red light during the dark phase of the light/dark cycle with 9 cages stacked together to enable simultaneous recording (low-resolution videos).

In terms of the gonad-intact C57BL/6J SC mice, the habituation period, caging, and bedding were the same as for the orchietomised B6D2F1 mice. The differences were that the sexual behaviour video recording was made with a sex stimulus mouse under normal white light during the light phase of the light/dark cycle for 30 minutes, one cage at a time (high-resolution videos). I only used data from mice video recordings that displayed sexual behaviour.

Video processing and annotation

All video cropping and trimming were conducted using HD Video Converter Factory Pro (version 27.0). For the high-resolution videos, 15 – 20 second clips were trimmed from them for DeepLabCut training to include a range of behaviours: five clips of resting, seven clips of anogenital sniffing, seven clips of mounting and intromission, and nineteen clips of ejaculation. For the low-resolution videos, clips were cropped from the nine-cage setup, then trimmed to 10-20 seconds to include 40 clips of intromission and ejaculation, as well as 20 clips of mounting and intromission for SimBA classification. For SimBA validation, ten clips were cropped from the nine-cage setup, then trimmed to 4-5 minutes with sexual behaviour bouts that included ejaculation. These videos for SimBA validation were also annotated for the presence of each mounting, intromission and ejaculation in each second of the clip. Annotations were stored as csv files that indicated the time of the video, corresponding frames (29 frames per second), and a list of 1's or 0's for each of the behaviours annotated, if they were present, or absent, respectively.

Creating pose estimation models with DeepLabCut and image augmentation

A multi-animal DeepLabCut project was created with all of the high-resolution video clips according to

https://deeplabcut.github.io/DeepLabCut/docs/maDLC_UserGuide.html The project was configured with individuals (mouse1 and mouse2), identity setting as false since the mice were difficult to tell apart and a list of 31 body parts per mice: L_Eye, Btw_Eye, R_Eye, Nose, L_Ear, Btw_Ear, R_Ear, R_Paw, L_Paw, L_Foot, R_Foot, Tail_Base, Tail_Mid_Base, Tail_Mid, Tail_Mid_End, Tail_End, Spine_1, Spine_2, Spine_3, Spine_4, Spine_5, Spine_3_L1, Spine_3_L2, Spine_3_L3, Spine_3_L4, Spine_3_L5, Spine_3_R1, Spine_3_R2, Spine_3_R3, Spine_3_R4, Spine_3_R5.

Frames were extracted from all the videos through k-means clustering based on a wide range of visual appearance of the video frames, giving a total of 1139 frames. The frames were then consistently labelled for all the body parts when they were visibly present with assistance from Park Lab members, especially Danny Le. After all the labelling was completed, to enable the machine to learn to use this information on my low-resolution videos, Stephen Renjie Hu augmented all the labelled video frames using Photoshop (version 25.6) to visibly resemble those of low-resolution videos. This was done with the following settings: image size – 216 pixels height, with scale styles, with constrained proportions, automatic interpolations; hue/saturation – custom preset kind, without colourise, hue 0, saturation -70, lightness -35; colour balance – shadow levels(-5,0,-40), mid-tone levels(0, -15, -30), highlight levels(0, -10, -30) with preserve luminosity; add noise – uniform distribution, 2%, with monochromatic; smart blur – radius8, 3 threshold, quality low, mode normal; brightness/contrast – brightness -20, contrast 65 without use legacy.

The CSV files containing the labelled data for each video clip were then transformed to reflect the image augmentation using the python script: https://github.com/vchiang001/sexual_diversity_dissertation/blob/main/specificaim1/CSV_Transform_Image_Augment.ipynb The CSV files were then converted to H5 files with the DeepLabCut function, `convertcsv2h5`. The correct positioning of the body part labels was then checked using the DeepLabCut function, `check_label`.

To train the pose estimation model, a training dataset was created with `imgaug` data augmentation, 0.2 test-train split, and using the pre-trained network ResNet-50. The training of the network was then conducted on NVIDIA Quadro RTX 5000 (Driver v551.61, CUDA v12.4) for 200,000 iterations, and displaying loss every 500 iterations. Following training, the model was evaluated for mean average Euclidean error, between

DeepLabCut predictions and ground truth of the body part labels. To determine the success of my image augmentation to enable the use of pose labelling in high-resolution video clips to analyse my low-resolution video clips, I used DeepLabCut to analyse all these low-resolution video clips without performing animal assembly and tracking, by setting the `auto_track` argument as false. This generated pickle files which were used to visualise the pose estimation results using the DeepLabCut function, `create_video_with_all_detections`.

Creating behaviour classifiers with SimBA

Pickle files were converted to csv files that arbitrarily assign body parts to either `mouse1` or `mouse2`, since my strategy does not rely on individual identification, using the python script:

https://github.com/vchiang001/sexual_diversity_dissertation/blob/main/specificaim1/dlc_pickle_to_simba_csv.ipynb A SimBA project was created following instructions at <https://github.com/sgoldenlab/simba/blob/master/docs/Scenario1.md> A project config was created with two predictive classifiers, 1) `mount_intromit` and 2) `ejaculate`, classic tracking settings, and a 62 body part body plan. The low-resolution clips that were used for training the behaviour classifiers were imported. Following that, DeepLabCut tracking data were imported with “Body-parts: Quadratic” interpolation, which would interpolate every occurrence of a missing body part using a quadratic function, as well as Gaussian smoothing to reduce unrealistic body part jumps between sequential frames, with a 100ms time window to identify preceding and proceeding frames.

Frames were then extracted for each clip, and video parameters were set as 254mm, specified at the edges of the Plexiglas arena, which represented its length to enable conversion of pixels to Euclidean distance. Outlier correction was skipped because SimBA uses body-length distance for this process, which was irrelevant to my videos, since I obtained a front-view, instead of a top-view. Default SimBA settings were used to extract features from pose estimation data, which calculates a larger set of features to be used for behaviour classification including these listed:

https://github.com/sgoldenlab/simba/blob/master/misc/features_user_defined_pose_config.csv

To create annotations for predictive classifiers, I used the SimBA behavioural annotator graphical user interface, to annotate in every single frame if mounting or intromission were present for the mount_intromit classifier, and if ejaculation was present for the ejaculate classifier. The definition of mounting began when Mouse1 swiftly pursued Mouse2, followed by Mouse1 assuming a copulatory stance. This posture involved Mouse1 positioning its front paws on the back of Mouse2, accompanied by frantic pelvic movements. Intromission was characterized by Mouse1 firmly grasping the back of Mouse2 and exhibiting deep, rhythmic pelvic thrusts. Ejaculation was recognized when the tail of Mouse1 twitched while Mouse1 held onto the back of Mouse2, followed by a period of approximately 10-30 seconds during which Mouse1 remained motionless. This phase often concluded with Mouse1 falling off to the side. The annotation of ejaculation continued until Mouse1 and Mouse2 were physically separated.

The mount_intromit classifier was trained using the random forest algorithm with the following hyperparameters: 2000 decision trees (estimators), square root of total features used as maximum number of features when evaluating a split (max features), quality of the split was measured with entropy (criterion), 80% of frames were used for training and 20% of frames for testing (train-split, train test split type), and minimally one sample required to be a leaf node (minimum sample). The ejaculate classifier was also trained using the random forest algorithm with the following hyperparameters: 1000 decision trees (estimators), square root of total features used as maximum number of features when evaluating a split (max features), quality of the split was measured with Gini (criterion), 80% of frames were used for training and 20% of frames for testing (train-split, train test split type), and minimally one sample required to be a leaf node (minimum sample). To evaluate the performance of these classifiers, a classification report was generated in comparison to the ground truth displaying precision, recall, f1, and support values.

Video analyses with SimBA classifiers

A new SimBA project was created with the out-of-domain low-resolution videos used for validation, following the same steps in “*Creating behaviour classifiers with SimBA*” except, no behaviour was annotated and no classifiers were trained. The

classifiers were run on these video clips with 0ms minimum bout, with the following threshold settings for different parameter combinations: mount_intromit 1)0.463, 2)0.720, 3)0.895, 4)0.728; ejaculate 1)0.4, 2)0.758, 3)0.57, 4)0.85. This generated CSV files with classifier probabilities for each behaviour, and the machine annotations of 1 for the presence and 0 for the absence of each behaviour, based on the threshold set.

These machine annotations required smoothing so that the presence or absence of a behaviour in the preceding frame would be treated as the same for the current frame unless enough specified percentage of frames in the specified window of succeeding frames indicate the likely presence or absence of that behaviour. This smoothing process was performed with the python script

https://github.com/vchiang001/sexual_diversity_dissertation/blob/main/specificalm1/simba_smoothing.ipynb with different parameter combinations (format: rolling window of frames, percentage of frames): mount_intromit 1)60 frames, 40%; 2)60 frames, 25.1%; 3)100 frames, 79.5%; 4)100 frames, 25.1% and ejaculate 1)100 frames, 30%; 2)150 frames, 50%; 3)270 frames, 69.7%; 4)150 frames, 30%.

Following smoothing, classifier performance was assessed by comparing ground truth (manually annotated behaviour) with the machine predictions of the behaviour presence. The ground truth files were created by concatenating the mount_intromit annotations of all videos into one file, and ejaculate annotations of all videos into another file. From these files, the classifier performance including precision, recall, differences of behaviour latency and differences in behaviour duration were obtained with the python script:

https://github.com/vchiang001/sexual_diversity_dissertation/blob/main/specificalm1/simba_classifiers_performance.ipynb

Results were visualised with the python script:

https://github.com/vchiang001/sexual_diversity_dissertation/blob/main/specificalm1/specificalm1_graphs.ipynb

Results

Curating a DeepLabCut dataset that could be augmented for low-resolution videos.

I automated analysis of frontal-view sexual behaviour recordings under red light between orchietomised B6D2F1 hybrid mice and hormonally primed ovariectomised C57/BL mice in a nine-cage stacked setup. However, the low-resolution of the mice in these videos made it extremely difficult to label body parts consistently across video frames for DeepLabCut, which was a major barrier to obtaining a satisfactory pose estimation model. To circumvent this, I obtained high-resolution video recordings and then augmented the properties of the images for computer vision to derive deep features that could accurately predict specific body parts in the low-resolution video using the DeepLabCut feature detector architecture (Mathis *et al.*, 2018). In other words, I would label body parts in high-resolution video recordings and augment the image features, so they resemble those from low-resolution recordings.

For the high-resolution recordings, I obtained 30-minute front-view footage between a dyad of gonad-intact C57/BL SC mice (n=10) and hormonally primed ovariectomised C57/BL mice (n=10). Short 20-second clips were trimmed from these videos to capture a range of behaviours (anogenital sniffing, mounting, intromission, and ejaculation). Following that, a total of 1139 video frames were extracted with DeepLabCut using k-means clustering. Using trimmed clips that included behaviours of interest ensures that body part labelling would be prioritised on these behaviours. We labelled 31 body parts for each of the two mice for all 1139 video frames (Figure 1A). Following that, I augmented all the video frames, to visually resemble the low-resolution videos we collected for a previous study (Chiang, Lipkin, *et al.*, 2022), in terms of image size, hue/saturation, colour balance, noise, blur and brightness/contrast (Figure 1B). After augmentation, the csv files containing the xy coordinates of the labelling data were transformed to reflect the changes in the image dimensions. Using the “check labels” function in DeepLabCut, I confirmed that the modifications I made corresponded to the correct pose location in the annotated frames that were augmented (Figure 1C).

DeepLabCut model could be generalised to out-of-domain low-resolution videos.

I trained the DeepLabCut model for 200,000 iterations, with ResNet-50 pre-trained network architecture, and imgaug data augmentation. After 200,000 iterations of training, all of the loss functions have converged (Figure 2A), including score map loss of the predicted key point heatmaps, location refinement loss that refines precise locations of key points during downsampling, limb loss that targets the connection between key points and the total loss that combines all loss functions (Lauer *et al.*, 2022). The model was then evaluated to compute the mean average Euclidean error between the manual labels and DeepLabCut predictions for each body part. The highest mean average Euclidean errors were observed for L_Foot (train error 2.435, test error 2.652) and the lowest were observed for Spine_3_R2 (train error 1.687, test error 1.733) (Figure 2B). These were anticipated given that the left foot of the mice was frequently not seen clearly in the video frames, and the right side of the mouse's body from the middle of the spine was usually labelled as a near equidistant series of points. These errors are considered low, on the account that the images were generally 288×216 pixels. Manual and predicted labels on test frames were plotted, showing almost all manual labels (labelled as '+') concorded with positions of the DeepLabCut's predictions (labelled as '.') (examples in Figure 2C).

To determine how my DeepLabCut model performed on out-of-domain videos, which were the 9-cage setup B6D2F1 hybrid mice video recordings, I obtained crops of videos to encompass only one cage. I acquired clips that were trimmed to ~10-20 seconds and exhibited mounting, intromission, or ejaculation: 40 clips containing intromission and ejaculation, as well as 20 clips containing mounting and intromission. Using DeepLabCut, I analysed these clips by pose estimation and created videos that could visualise all the detected poses. I found my augmentation strategy enabled consistent detection of various mice body parts on these low-resolution videos despite the machine never seeing these videos in the training dataset (examples in Figure 2D). I did not do the downstream steps in multi-animal DeepLabCut for individual identification by animal assembly and tracking, as my behaviour classification strategy did not rely on individual identification.

SimBA classification of rodent sexual behaviour performed well for within-domain videos.

After pose estimation was completed, I fed DeepLabCut pose estimation output into SimBA to create behavioural classifiers for rodent sexual behaviours (Nilsson *et al.*, 2020). Given that I did not do animal assembly and tracking in DeepLabCut, the output file generated could not be directly fed into SimBA. It generated pickle files which contained pose estimation information of different body parts, however, the individuals to which these body parts were assigned were arbitrary. On the basis that my strategy does not depend on individuals to be identified but on the overall pose relationships of the entire dyad, this was not a problem. I converted the pickle files from all the low-resolution clips into csv files that could be imported into SimBA. Using SimBA, frames were extracted from all the clips, and then a larger set of features from the pose estimation results were extracted using SimBA's default setting.

From these features, I performed supervised classification on two sets of behaviours, where I told the machine when the behaviours were present: one including both mounting and intromission, and the second being ejaculation. I combined both mounting and intromission because they shared sufficient similarities in features that would enable a robust classifier to be created, and they do not need to be differentiated for my current purposes. I annotated each video frame for the presence or absence of 1) mounting or intromission (mount_intromit classifier), and 2) ejaculation (ejaculate classifier). Mounting was defined when Mouse1 swiftly pursued Mouse2, and if it was succeeded by Mouse1 adopting a copulatory stance. This posture was characterized by Mouse1 positioning its front paws on the back of Mouse2, accompanied by frantic pelvic movements (examples in Figure 3A). Intromission was defined when Mouse1 firmly grasped the back of Mouse2, demonstrating deep, rhythmic pelvic thrusts (examples in Figure 3A). Ejaculation was defined when the tail of Mouse1 twitched as Mouse1 grasped the back of Mouse2, followed by Mouse1 remaining motionless for approximately 10-30 seconds, often resulting in Mouse1 falling off to the side (examples in Figure 3B). Ejaculation was annotated until Mouse1 and Mouse2 were separated.

The mount_intromit classifier was trained using the random forest algorithm with specified hyperparameters (see methods). A classification report was generated with the metrics: precision, recall and F1, all out of 1.0 (Figure 3C). The classifier achieved a

precision of 0.859, indicating that 85.9% of all video frames annotated with the behaviour were correctly identified as true positives, relative to all video frames labelled as positive by the classifier (account for false positives). Furthermore, the classifier attained a recall of 0.846, implying that 84.6% of all video frames that should have been labelled as positive were correctly identified (account for false negatives). The F1 score was 0.852, which represents the harmonic mean of precision and recall. The ejaculate classifier was trained with the random forest algorithm with specified hyperparameters (see methods) (Figure 3C). The classifier achieved a precision of 0.916, a recall of 0.844, and an F1 score, of 0.878.

SimBA classification of rodent sexual behaviour could be tuned for out-of-domain videos.

In order to validate the performance of the classifiers, it needed to be evaluated in videos that were not used for training and testing i.e. out-of-domain data. For validation, I obtained ten video clips of single cage crops from the 9-cage recording setup of orchietomised B6D2F1 hybrid mice, that were trimmed to ~5 minutes to encompass mounting, intromission and ejaculation. All ten videos were manually annotated for the behaviours of interest at each frame which were used as ground truth to evaluate the performance of the classifiers. Video analysis in SimBA required defining the presence of behaviour based on a discrimination threshold (0.00 – 1.00) in each frame. There was also an option to smooth the data in SimBA by setting minimum bout length (ms), where interruptions in behaviour bouts less than the specified length were re-classified as the behaviour being present. However, I also wanted to smooth the data by reducing the noises from the classifiers thinking a behaviour was present when it was actually absent. Therefore, after SimBA analysis, the results were smoothed by setting a rolling window (number of frames) and setting the percentage of frames in the window where the behaviour was detected. This enabled the correction of machine detections depending on whether the machine consistently detected the behaviour in subsequent frames. Setting these parameters depended on the typical duration of the behaviours of interest and the amount of noise that may be present in the machine detections.

The mount_intromit classifier was run on all ten videos using four different parameter settings for the discrimination threshold, rolling window, and percentage of

frames. The resulting csv files for each video were then analysed to calculate the true positives, false positives, and false negatives to the ground truth data. This was used to calculate precision (fraction of true positives out of all frames detected as positive, which account for false positives), recall (fraction of true positives detected out of all true positives, which account for false negatives) and F1 (harmonic mean between precision and recall) for each video. The highest recall was obtained for Parameter #1 (0.832 ± 0.019) which used a discrimination threshold of 0.463, a rolling window of 60, and 40% frames (Figure 4A). The highest precision was obtained for Parameter #2 (0.720 ± 0.071) which used a discrimination threshold of 0.895, a rolling window of 60, and 25.1% frames (Figure 4A).

The latency (when the behaviour first occurs) and duration (total frames of the behaviour occurring) of the behaviour were also calculated for both the manual annotations and the machine results for each video. The absolute differences were then calculated and then divided by the total number of frames of the video, and represented as the % of error to the ground truth. The lowest % error to ground truth for mount_intromit duration was obtained for Parameter #3 ($3.199\pm 0.901\%$) which used a discrimination threshold of 0.463, a rolling window of 100, and 79.5% frames (Figure 4B). The lowest % error to ground truth for the mount_intromit latency was obtained for Parameter #4 ($8.557\pm 1.724\%$) which used a discrimination threshold of 0.728, a rolling window of 100, and 25.1% frames (Figure 4B). With regards to how to determine which parameters to adopt depends on the research question. For instance, a study that examines how a gene knockout could affect the duration of steroid-independent mounting and intromission would benefit most from Parameter #3, which quantifies the mount_intromit duration most accurately. SimBA provides an interface of an analysed video and behaviour probabilities in each frame, so users could use that to tune parameters suitable for their purpose.

In terms of the ejaculate classifier, the highest recall was obtained for Parameter #1 (0.837 ± 0.041) which used a discrimination threshold of 0.4, a rolling window of 100, and 30% frames (Figure 4C). The highest precision was obtained for Parameter #2 (0.758 ± 0.097) which used a discrimination threshold of 0.85, a rolling window of 150, and 50% frames (Figure 4D). With regards to time-related metrics, Parameter#3 and Parameter#4 obtained the lowest % error to ground truth for ejaculation duration

($1.920 \pm 0.604\%$, discrimination threshold of 0.57, rolling window of 270, and 69.7% frames) and ejaculation latency ($29.553 \pm 9.280\%$, discrimination threshold of 0.85, rolling window of 150, and 30% frames), respectively.

Discussions

Here, I developed an automated method to identify mounting, intromission and ejaculation in a data set that included orchietomised B6D2F1 hybrid mice demonstrating steroid-independent sexual behaviour using a frontal-view, multiple-cage stacked setup under red light night vision with low-resolution. A DeepLabCut pose estimation model was created that worked on these low-resolution videos, which were then fed into SimBA to generate two behavioural classifiers: mount_intromit (mounting and intromission) and ejaculate (ejaculation).

Several studies have previously attempted to automate the analysis of rodent sexual behaviour (Matsumoto *et al.*, 2013; Hong *et al.*, 2015; Nilsson *et al.*, 2020; Segalin *et al.*, 2021). Some of these required special equipment that could be expensive to obtain for a lot of academic labs (Matsumoto *et al.*, 2013; Hong *et al.*, 2015). In one prior study that utilised SimBA, DeepLabCut pose estimation results were obtained that were based on 16 body parts labelled for each of two mice in 8315 frames extracted in top-view recordings (Nilsson *et al.*, 2020). The poses were then added to SimBA to be annotated for the presence of mounting or intromission, without differentiating the two. MARS also relied on a pose estimation system where 9 anatomical key points were labelled in top-view videos across 15,000 frames. Following that, a classifier that detected mounting and intromission, without differentiating the two, was trained on 14 hours of behaviour video and reached above 0.9 performance for both precision and recall (Segalin *et al.*, 2021). My approach was distinct to these, primarily because I focused on front-view recordings, that could provide additional angles to capture rodent sexual behaviour. Furthermore, my classifiers were viable for low-resolution recordings that are cropped from multiple stacked cage setups.

This approach holds promise for advancing studies on rodent sexual behaviour, offering potential time savings by automating the analysis of sexual behaviour in video recordings. The dataset used encompassed steroid-independent sexual behaviour, where many diverse research inquiries remain to be answered. For instance, researchers could investigate neuron subtypes involved in regulating steroid-independent mounting or probe signalling pathways underlying motivation in these mice.

One limitation of my approach is the lack of individual identification, as pose estimation cannot distinguish between the two mice. Incorporating individual identification could enhance classifier performance by attributing pose relationships to specific mouse identities rather than poses themselves. Additionally, several rodent sexual behaviours necessitate individual identification for accurate assessment. For example, being able to characterise lordosis and paracopulatory behaviours, that rely on the actions of one mouse, is challenging without individual identification. Another limitation of my approach is its reliance on pose estimation, which requires defining relevant poses beforehand, though new poses can be added later. Pose estimation might oversimplify animal behaviour, as it may not fully capture the behaviours of interest. These limitations highlight the need for future improvements and complementary methods to better capture the complexities of rodent sexual behaviour.

Automating the analysis of rodent sexual behaviour has significant implications for investigating sex variability and diversity. Automated techniques enable the exploration of a broader range of rodent species commonly used in neuroscience studies, such as rats, hamsters, guinea pigs, squirrels, gerbils, degus, mole rats, voles, and shrews, some of which similarly exhibit steroid-independent sexual behaviour (Krubitzer *et al.*, 2011; Atrooz *et al.*, 2021). Furthermore, framing these neuroscience studies beyond binary classifications of sex allows for investigation of how various continuous sex-related variables could be mapped to specific sexual behaviours (Massa *et al.*, 2023). This type of research not only advances the study of rodents but also impacts broader societal contexts by debunking stereotypes and informing policy-making decisions.

Summary and Conclusions

In conclusion, I automated the analysis of rodent sexual behaviour using DeepLabCut pose estimation to create SimBA behavioural classifiers. This approach was applied to a dataset investigating steroid-independent sexual behaviour in B6D2F1 hybrid mice, using front-view recordings and multiple stacked cages. By automating sexual behaviour analysis in these mice, I aim to facilitate future studies on steroid-independent sexual behaviour by reducing the labour-intensive process of manual annotation. More broadly, automated analysis of sexual behaviour can be utilised to study various aspects of sex variability and diversity, moving beyond binary frameworks.

Recommendations

Significant progress is being made to streamline the automation of behavioural analysis, including the use of spatiotemporal data across frames to improve tracking robustness with methods like spatiotemporal PoseNet (STPoseNet) (Lv *et al.*, 2024). Current approaches to automation mostly rely on human annotations, requiring substantial initial training time for machines. There are emerging advancements that use pose estimation results to automatically label behaviours that are present including SaLSa (a combination of semi-automatic labelling and long short-term memory-based classification) and LISBET (seLf-supervIsed Social Behavioural Transformer) (Chindemi *et al.*, 2023; Sakata, 2023). Despite this, pose estimation algorithms still rely heavily on time-consuming human labelling of specific body parts. Consequently, efforts are being made to develop solutions like B-KinD for automatic pose labelling (Behavioural Keypoint Discovery) (Sun *et al.*, 2022).

Moving forward, the immediate focus will be on developing individual identification capabilities in my pose estimation model. DeepLabCut offers the potential for individual identification through pose estimation, allowing specific individuals to be tracked over time (Lauer *et al.*, 2022). To enable this, refinements are needed to address unusual animal assemblies and correct ID swaps in tracklets. Recent advances in individual identification could be utilised (Zhang, Zhao, *et al.*, 2024). Additionally, exploring other approaches for automated behavioural analysis could provide

comprehensive representations of sexual behaviour. For example, LabGym uses motion patterns and spatiotemporal details, which could complement pose-based methods by identifying sexual behaviours that may be challenging to detect using poses alone (Hu *et al.*, 2023).

Numerous labs and institutions independently invest significant time and resources in annotation processes and model tuning, resulting in redundant efforts. Collaborative resource sharing could alleviate this duplication, benefiting labs worldwide and promoting more efficient advancements in the field. Scholars advocate for the curation of multiple datasets to develop robust deep-learning-based behavioural models, which can be readily applied to new data from any laboratory (Siegford *et al.*, 2023). Although data sharing poses challenges, initiatives like Neurodata without Borders aim to improve standardization in this process. (Pierré *et al.*, 2023).

Currently, most automation methods in behavioural analysis demand a level of computer science proficiency, particularly in areas like computer vision and machine learning. This can pose a significant barrier for researchers and labs unfamiliar with programming and computing terminology. To address these barriers, some groups are working to make behavioural analysis tools more accessible to a broader audience, including the general public. For instance, natural language processing paired with large language models allows users to simply instruct the desired behaviour output through a user interface, eliminating the need for coding, scripting, or deep learning familiarity (Guo *et al.*, 2023; Ye *et al.*, 2023).

Automating the study of sexual behaviour could yield higher volumes of empirical data for a greater number of research questions, particularly those areas that have been overlooked, like steroid-independent sexual behaviour. There are recent technological advancements that could be leveraged to control gonadal hormone secretion (Rosenfeld *et al.*, 2020; Maeng *et al.*, 2022). Just as scholars are reevaluating the prevailing notion that sexual behaviour is solely influenced by gonadal steroid hormones, it is imperative to explore other neglected aspects within sexual behaviour research, which have been overshadowed by cisnormative, heteronormative, and patriarchal ideologies. For example,

in avian behavioural neuroendocrinology, researchers are recognizing the limitations of binary sex classifications, given the diverse range of androgen levels and behavioural expressions observed in individuals categorized under these binaries (Smiley *et al.*, 2022). Shifting away from such classifications in animal studies has implications for human societies, as people often draw on observations in non-human animals to reinforce certain gender stereotypes within society (Massa *et al.*, 2023).

A multivariate conceptualization of sex across the animal kingdom has been suggested and backed by evidence, as many traits commonly associated with sex by scientists often fail to neatly align (McLaughlin *et al.*, 2023). This perspective is particularly relevant to individuals with transgender, nonbinary, and gender-nonconforming identities. Automating behaviour analysis could help identify these behavioural traits that defy the sex binary, often overlooked by simplistic metrics that aim to represent behaviour and average out individual variations. Constructing the neuroscience relating to these individuals should avoid seeking neurobiological markers of these identities or viewing the brain as exclusively causal (Arraiza Zabalegui, 2024). Scholars argue that translating identities into brain types reinforces pathologising views in scientific authority. Instead, neuroscience could engage with how the brain contributes to the plastic experiences of sex/gender-diverse individuals, moving away from pathologisation and binary frameworks and supporting social change.

Some argue that adopting a multivariate perspective on sex forces scientists to conform to those that deviate from the norm. However, even if variations in sexual phenotype are less common in certain species, it doesn't justify dismissing sex diversity as abnormal or insignificant to the ecosystem (Massa *et al.*, 2023). In human societies, a small number of individuals can have significant impacts that alter the course of history (Chenoweth & Belgioioso, 2019). In other words, a small number of minorities should not be dismissed as insignificant. The research topics exploring sex variability and diversity in non-human animals hold immense importance, especially considering how they have been used to discriminate against marginalised communities (Massa *et al.*, 2023).

Figures & Legends

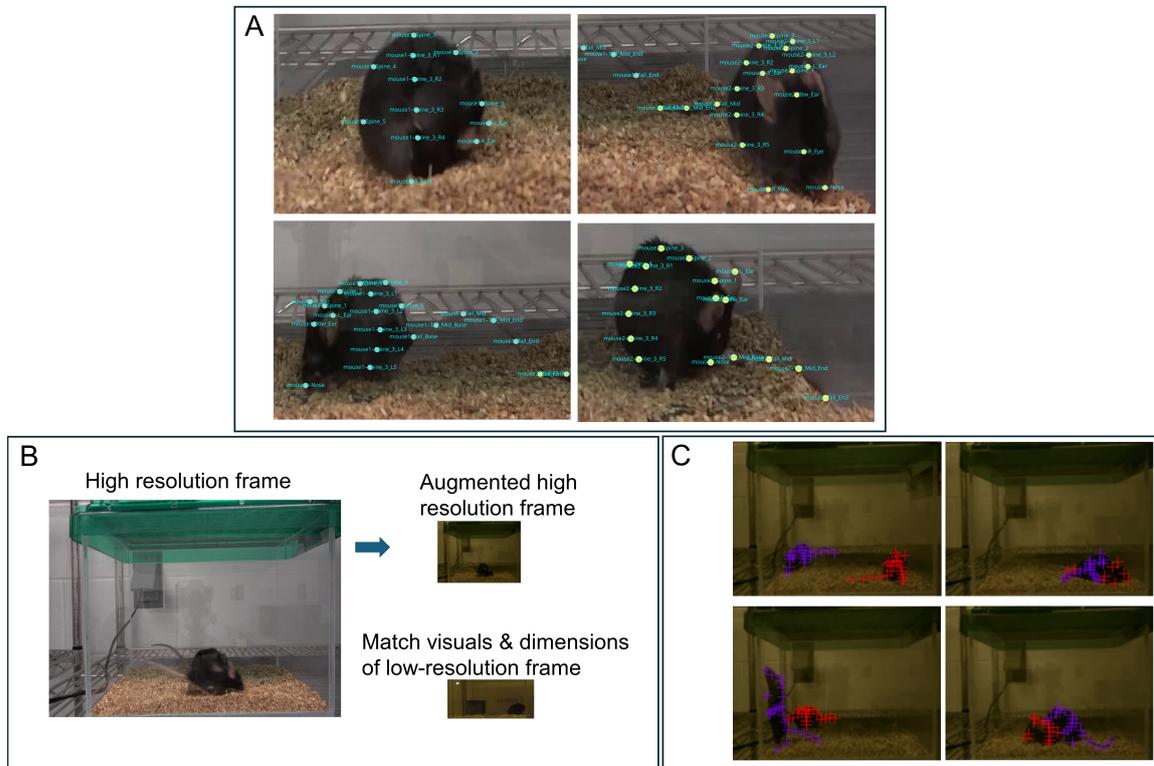


Figure 1 Curating a DeepLabCut dataset that could be augmented for low-resolution videos.

A) Examples of DeepLabCut labelling with mouse body parts. B) A schematic of image augmentation process where high-resolution frames were augmented to visibly resemble that of low-resolution frames. C) Using the DeepLabCut function, `check_labels`, to check if labels were correctly transformed in the augmented high-resolution frames.

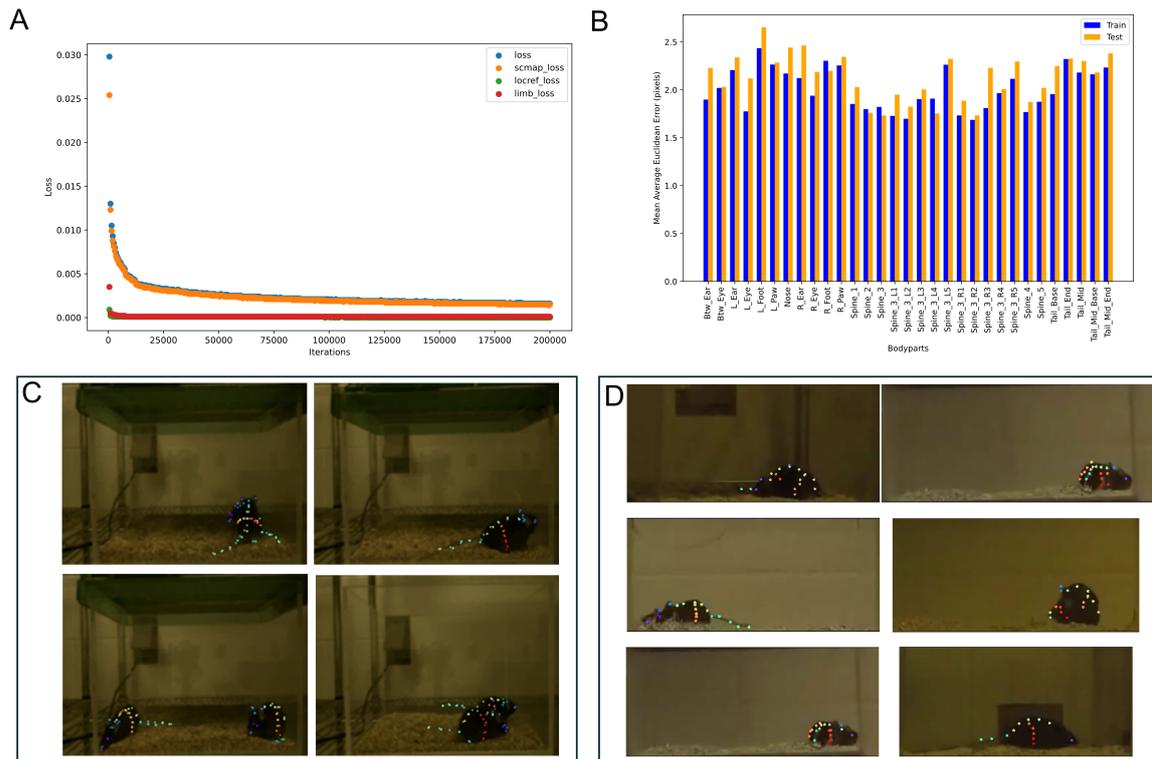


Figure 2 DeepLabCut model could be generalised to out-of-domain low-resolution videos.

A) A scatterplot showing changes in different types of losses across 200,000 iterations of neural network training. The types of losses displayed included total loss (loss), scoremap loss (scmap_loss), location refinement loss (locref_loss), and limb loss (limb_loss). B) A bar plot showing the mean average Euclidean error (pixels) between DeepLabCut predictions and ground truth of mice body parts. The plot showed both training and testing data after 200,000 iterations of training. C) Examples of both DeepLabCut predictions and ground truth plotted during evaluating the trained network. Ground truth labels by humans were plotted as plus ('+') and DeepLabCut predictions were as "." D) Examples of using the trained network to analyse out-of-domain low-resolution videos from my nine-cage setup.

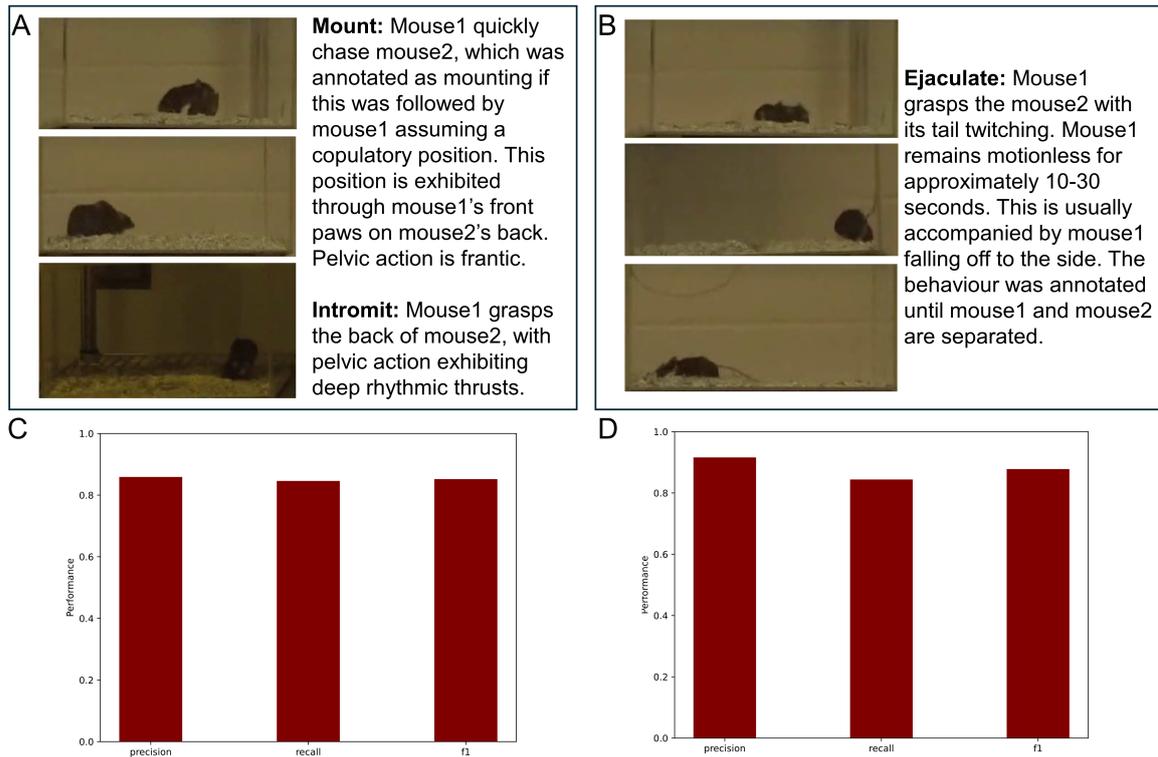


Figure 3 SimBA classifiers for rodent sexual behaviour performed well for within-domain videos.

A) Descriptions on how mounting and intromission were defined to be annotated as the presence of those behaviours. Example frames that were annotated as mounting or intromission present are also shown. B) A bar plot showing metrics of classifier performance (out of 1.0) for the mount_intromit classifier including precision, recall and F1 during SimBA training. C) Descriptions of how ejaculation was defined to be annotated as the presence of that behaviour. Example frames that were annotated as ejaculation present are also shown. D) A bar plot showing metrics of classifier performance (out of 1.0) for the ejaculate classifier including precision, recall and F1 during SimBA training.

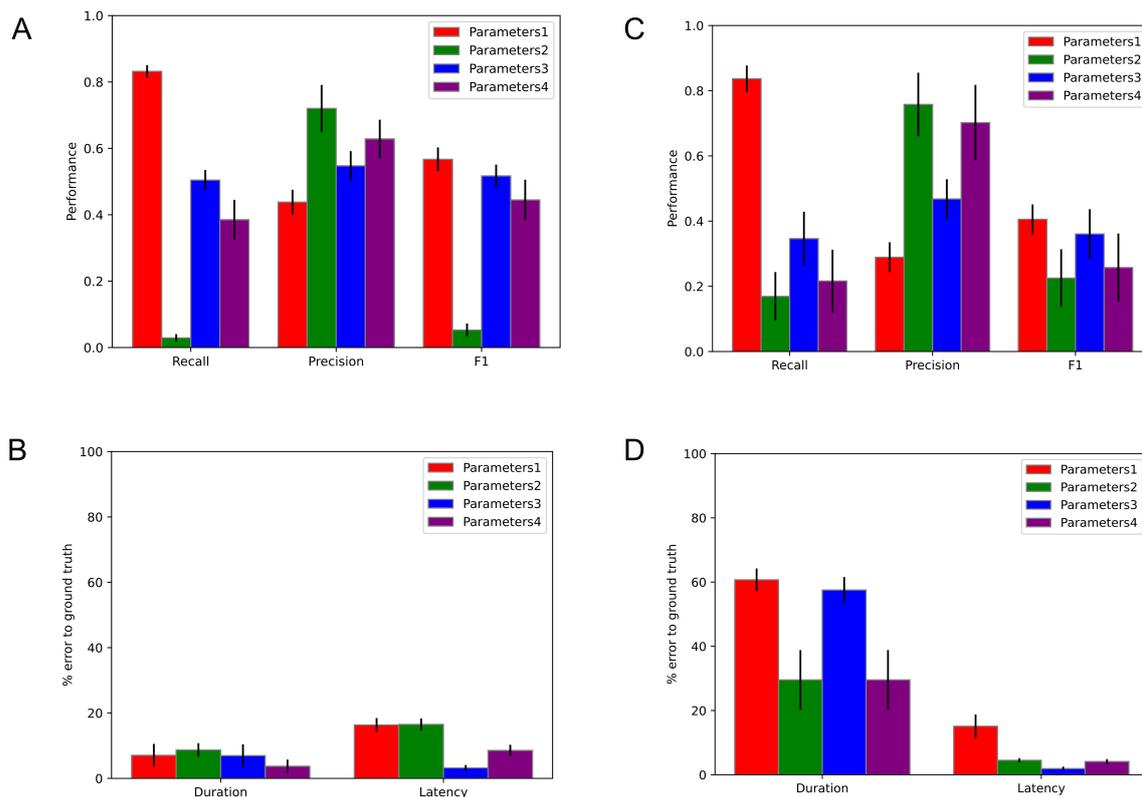


Figure 4 SimBA classification of rodent sexual behaviour could be tuned for out-of-domain videos

A) A bar plot showing metrics of classifier performance (out of 1.0) for the mount_intromit classifier including precision, recall and F1, as well as their standard error for ten out of domain videos. Results for four different parameter settings are shown, including differences in threshold, smoothing window and % of frames with behaviour present. B) A bar plot showing % error to ground truth (out of 100%) of behaviour duration and latency for the mount_intromit classifier, as well as their standard error for ten out of domain videos. Results for four different parameter settings are shown, including differences in threshold, smoothing window and % of frames with behaviour present. C) A bar plot showing metrics of classifier performance (out of 1.0) for the ejaculate classifier including precision, recall and F1, as well as their standard error for ten out of domain videos. Results for four different parameter settings are shown, including differences in threshold, smoothing window and % of frames with behaviour present. D) A bar plot showing % error to ground truth (out of 100%) of behaviour duration and latency for the ejaculate classifier, as well as their standard error for ten out

of domain videos. Results for four different parameter settings are shown, including differences in threshold, smoothing window and % of frames with behaviour present.

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CHAPTER 3

AUTOMATION OF SEXUAL BEHAVIOUR ANALYSIS IN COLLECTIVE CONTEXTS FOR NEUROSCIENCE STUDIES

Introduction

Mating systems across the animal kingdom

Across the animal kingdom, there are solitary species such as Bramble Cay melomys, polar bears, and snow leopards (Olivier *et al.*, 2022). Animals also display diverse social systems that vary in size, composition, social units, reproductive skew, cooperation, and competition (Clutton-Brock, 2021). This diversity in social structures shows that sexual behaviour can occur in a collective context, observable in various mating systems (Kempnaers, 2022). Studies on mating systems have traditionally relied on binary classifications of male and female, and it is important to consider how these categories were assigned. I will reframe these categories as individuals with various sex-related attributes.

An example of sexual behaviour in a collective context is the Damaraland and common mole-rat, with only one breeding pair per colony while all other members, usually their offspring, remain subordinate and do not breed (Wallace *et al.*, 2023). Another example is the ostrich, which displays cooperative breeding, a mating system where multiple individuals cooperate to raise the offspring (Melgar *et al.*, 2022). Ostriches with various sex-related attributes incubate eggs and protect them, with groups differing in sex ratio but remaining similar in size. Pectoral sandpipers travel long distances to visit

multiple breeding sites, face intense competition, and perform elaborate aerial displays (Santema *et al.*, 2023).

Mating systems are currently determined through various approaches, typically involving behavioural observations related to mate choice and displays both in the field and in captivity (Wittman & Cox, 2021). The resulting dynamics, resource distribution and population density are used to determine the mating system. Furthermore, physiological measures such as hormones, genetics, and anatomy are examined to investigate parentage, mating periods, and mating strategies (Wittman & Cox, 2021). However, there are limitations to assigning mating systems due to intra-species variation. For example, in socially monogamous species, sometimes the genetics of the multiple offspring were attributed to multiple sperm producers rather than just the social partner in the monogamous pair (Søraker & Dunning, 2023). Mating system paradigms may also shift according to changes in geography and resource availability (Candolin, 2019).

Understanding the mating system of a species helps scientists piece together its care systems, social organization, and social structure (Kappeler, 2019). This is also relevant to collective behaviour, defined as the coordinated actions of large groups of animals pursuing various goals and adapting to past experiences (Galesic *et al.*, 2023). This type of sociality requires a delicate balance of resource competition related to behaviour (Kao, Hund, *et al.*, 2023). Mating systems also need to consider inter-individual variation, alternative reproductive tactics, and the statistical relationship between mating success and reproductive success (i.e. Bateman gradient) (Kappeler, 2019). However, current sexual behaviour paradigms in neuroscience studies often neglect this collective context and are conducted primarily in dyads. For instance, rodents are typically socially isolated until sexual behaviour testing, whereas in their natural environments, they interact with multiple conspecifics and copulate with multiple partners (Heijkoop *et al.*, 2018; Vogt *et al.*, 2022, 2024; Lambert, 2023; Zippel *et al.*, 2023). Conventionally, neuroscience measures would be taken from only one focal individual, treating the other as a stimulus.

Lekking

One prominent example where sexual behaviour is collective is lekking, where animals cluster with some distance between them, each defending a small territory (Isvaran, 2020). Within their territories, they perform courtship displays that entice potential mating partners that move between territories. Lekking has been observed across the taxa including insects, fish, lizards, birds and mammals. One example of lekking behaviour is observed in fallow deer. Within the lek, some animals adopt various mate selection mechanisms depending on their age and the distance of the lek from their home range (Imperio *et al.*, 2020). These mechanisms differ in terms of the time spent on the lek and whether they select territories with numerous copulations or existing animals present. Another instance of lekking behaviour was established in the great snipe, where animals show competitive displays and courtship rituals within the leks (Witkowska *et al.*, 2023). Additionally, the study established that lekking sites of the great snipe remain stable in space and time due to strong site fidelity among members.

Lekking is considered a collective behaviour because individual interactions, such as the spatial movement of animals and their interactions with neighbours, collectively result in group-level patterns and functions (Rathore, Isvaran, *et al.*, 2023). Multiple animals are drawn to a site to establish territories, leading to clustering, while repulsion between animals maintains small distances between territories. Various movements occur within or near the lek, including mate sampling, courtship, consummatory sexual behaviours, competition, and aggression. Coordinated behaviour can emerge, such as groups departing the lek to forage together or local cooperation to repel intruders. Additionally, as potential mates move across the lek, animals defending territories may display synchronized behaviour unintentionally.

Although not yet investigated in the context of lekking, courtship displays may also display collective synchrony, as seen in marine ostracods through their visually synchronised bioluminescence displays (Hensley *et al.*, 2023). Social behaviours during time away from the lek, may also hold significance, as evidenced by studies on umbrellabirds showing their influence on sexual selection (Anderson *et al.*, 2023). One example where lekking has been studied as a collective behaviour is in blackbucks. Analysis of aerial videos of lekking behaviour in this species has provided insights into movement patterns, territory establishment, and local interactions (Rathore, Vadavalli, *et*

al., 2023). Lekking may also shed some light on sexual selection as seen in birds-of-paradise (Blom *et al.*, 2024).

Brain studies should be contextualized within collective dynamics

Neuroscientists aim to construct neural phenomena and how they correlate with observable social behaviours seen throughout the animal kingdom. Current efforts are starting to establish that studying individual brains in isolation may limit scientists' understanding of neural processes related to social behaviour, highlighting the need to study brains within a group context (Sliwa, 2021). This approach makes sense because social interactions involve highly complex and dynamic negotiations, where each individual's actions are influenced by the past, present, and anticipated actions of others (TenHouten *et al.*, 2023; Shemesh *et al.*, 2024; Yu *et al.*, 2024).

In human studies, researchers use hyperscanning experiments to simultaneously image multiple brains (Hakim *et al.*, 2023). This approach has been applied to investigate sexual behaviour, such as in a study using functional near-infrared spectroscopy during heterosexual speed-dating (Yuan *et al.*, 2022). The interpersonal neural synchronization at the dorsolateral prefrontal cortex predicted the outcome of mate choice. However, it is still unclear which specific sensory information during social interactions drives this synchronization.

This synchronization, also known as coupling, correlation, or connectivity in different studies, refers to the coordinated and reciprocal changes in neural activity (Pfaus *et al.*, 2023). Critics argue that the term "synchrony" implies a self-sustained neural oscillation that must be present in all individuals (Pérez & Davis, 2023). This definition excludes other neural similarities beyond phase consistency that could offer valuable insights, such as the complementary and continuous cooperation and adaptation of neural activity that may not align in terms of oscillation. As a result, some scholars propose adopting the term "inter-brain relationships" instead. While further research is needed to explore inter-brain relationships during sexual behaviour in multiple participants simultaneously, scholars have begun to speculate on the possibilities in the context of attraction, courtship, and consummatory sexual behaviours (Pfaus *et al.*, 2023).

New approaches are emerging in the study of non-human animals, allowing for the simultaneous examination of neural activity in interacting individuals (Kelly, 2023). However, this area remains largely unexplored, particularly in constructing how such neural processes contribute to collective behaviour. While some studies are starting to investigate grouping behaviour in birds, fish, and insects, many still focus solely on individual animals rather than their interactions with multiple conspecifics.

In one notable study that explored collective brain activity, researchers recorded local field potentials in the dorsal medial prefrontal cortex of four mice (Lee *et al.*, 2022). They established that the social context, mainly huddling in this case, and the locomotor states of the mice reciprocally influenced the structure of the local field potential. Unique signatures were observed when the mice were in groups compared to when they were individually isolated. In another study on collective brain activity, researchers used wireless neurophysiological recording devices to investigate sleep behaviour and brain activity in freely moving mice under different social conditions (Sotelo *et al.*, 2024). The social context significantly influenced sleep patterns and brain oscillations, with sibling mice showing strong synchronisation in brain activity during both wakefulness and non-rapid eye movement sleep, unlike non-sibling mice.

Besides mice, scientists have examined collective spatial behaviour in bats, a highly complex behaviour that involves tracking their own positions as well as those of numerous group members, along with signals broadcasted by group members (Forli & Yartsev, 2023). Using electrophysiological recordings, the study established that hippocampal neurons were tuned to different group dynamics, including conspecifics and their identities, landing sites, and various sensory signals.

Astatotilapia burtoni as a model for studying lekking

One suitable species to study lekking is the East African cichlid *Astatotilapia burtoni* (*A. burtoni*), which belongs to the Actinopterygii class (ray-finned fish), teleost infraclass and cichlid family. They display lekking where certain cichlids breed in small, clustered territories, while others near spawning visit these leks (Fernald & Hirata, 1977; Fernö, 1987). *A. burtoni* evolved from the rapid parallel adaptive radiations of repeating the process of hybridization and specialisation that gave rise to diverse cichlid species

from one of the African rift valley lakes, Lake Tanganyika in East Africa (Meier *et al.*, 2023).

Scholars have categorised *A burtoni* into male and female, dominant or subordinate, based on body colouration, body size, and egg spot characteristics on the anal fin (Henning & Meyer, 2012; Böhne *et al.*, 2016; Woltering *et al.*, 2018; Alward *et al.*, 2020). However, many studies do not explicitly state in the publication their criteria for categorising males and females, so it is not clear whether different studies used varying standards for categorisation of males and females. Different to this, these studies provide detailed descriptions for dominant and subordinate distinctions and consider this a continuum (Butler *et al.*, 2021, 2021; Maruska *et al.*, 2022). Dominant cichlids are brightly coloured (typically bright blue or yellow) with black bars across the eye and body, and black spots and red patches on or near the gill cover (Insel & Fernald, 2004). In contrast, subordinate individuals lack these colourful attributes and are usually pale blue or yellow. To avoid treating sex as a binary and deterministic trait, I will denote dominant cichlids with colourful egg spots on their anal fins as dominant CES cichlids; dominant cichlids with dull-coloured egg spots as dominant DES cichlids; subordinate cichlids with large body size as subordinate LBS cichlids; and subordinate cichlids with smaller body size as subordinate SBS cichlids.

Dominant cichlids are primarily observed defending territories and soliciting subordinate SBS cichlids for mating opportunities (King *et al.*, 2022). To court subordinate SBS cichlids, dominant individuals show visual displays such as body quivering (curving the body in a C-shape and vibrating while displaying egg spots along the anal fin), tail wagging to present the tail fin and egg spots, dorsal fin flaring, and leading movements into the territory in a zig-zag pattern, accompanied by low-frequency pulsed sounds. Courtship in this species also involves chemosensory, mechanosensory, and tactile signals, often directed toward gravid cichlids with extended abdomens full of eggs (Maruska & Butler, 2021).

As spawning approaches, cichlids develop large yolky eggs and ovulate. Upon selecting a mate with a territory, the spawning cichlid follows the mate into their territory, and the mate nips at the spawning cichlid's urogenital opening. Upon stimulation, the spawning cichlid deposits eggs on the substrate (bottom floor) and then picks up the eggs in their mouth (Butler *et al.*, 2021). Subsequently, the spawning cichlid nips at the egg

spot on the selected mate's anal fin, prompting the mate to release sperm that fertilises the eggs. Sometimes subordinate LBS cichlids may display sneaky spawning by interrupting the spawning pair. The eggs are mouthbrooded by the spawning cichlid for two weeks until the fry (young fish) is released.

The description of the various cichlid types suggests that groups of *A burtoni* may exhibit a social hierarchy through competition for spawning territories to monopolise them (Fialkowski *et al.*, 2021). Behavioural displays related to this hierarchy include chases, lateral displays, border displays, and flees. Chases involve aggressive swimming after another fish, sometimes including biting or forcibly moving other fish. Conversely, fleeing is demonstrated as a rapid retreat away from aggressors. Border threats occur when fish charge head-on with extended pectoral fins and open mouths. In lateral displays, fish present their bodies sideways to others, with fins erected and opercula flared. Additional aggression-related behaviours include lunges, and nips, as well as coping behaviours such as freezing (remaining stationary) and searching (swimming perpendicular to the wall). Beyond the dominant-subordinate binary, there are also differences in aggression among dominant cichlids, which correlate with their colour patterns. Generally, yellow individuals tend to be more aggressive than blue individuals, although their colours may change (Dijkstra *et al.*, 2017).

The hierarchy is non-permanent and reversible, so social ascent could occur when the subordinate fish becomes dominant (Wayne *et al.*, 2023). Repeated social defeat experienced by subordinate *A burtoni* impacts their likelihood of achieving social ascent. In communities consisting solely of subordinate SBS cichlids, some individuals may ascend to dominance (dominant DES cichlids) and display characteristics typical of dominant CES cichlids. These changes in characteristics include aggression, territory defence, physical appearance, and levels of androgen hormones (Funnell *et al.*, 2022). Research has explored the social network dynamics within *A burtoni* groups, which is particularly pertinent to constructing lekking behaviour. One such study examined rank transitions by identifying behaviours such as chases, displays, and territory ownership (Dijkstra *et al.*, 2022). The dynamics and characteristics of these groups are highly complex, involving individual behaviours toward the group that depends on how the group responds in a closed-loop manner (Maguire *et al.*, 2021).

Studying the neuroscience of sexual behaviour in fish

Physiological aspects of sexual behaviour have been investigated in fish. For instance, research dating back to the 1970s demonstrated the involvement of gonadotropin-releasing hormones from the hypothalamus in fish reproduction, leading to gonadotropin release (Kalarani *et al.*, 2021). In teleost fish, sexual behaviour is governed by neural networks involved in social behaviour and decision-making, resulting in diverse behavioural phenotypes across species and genera (Ogawa & Parhar, 2022). Teleost fish are notable among vertebrates for their ability to transition across the sex-related phenotype spectrum, a process driven by the actions of hormones and pheromones on the brain (Okubo *et al.*, 2022; Ahamed & Tokumoto, 2023).

Numerous fish species have served as valuable models for studying the neuroscience of sexual behaviour. Among these, the zebrafish (*Danio rerio*) stands out as a prominent organism in neuroscience research, particularly in the investigation of sexual behaviour (Ahamed & Tokumoto, 2023; Gerlai, 2023). Other fish species, such as the guppy (*Poecilia reticulata*) and sailfin molly fish (*Poecilia latipinna*), have been subjects of brain transcriptome studies related to mate choice and preference, respectively (Bloch *et al.*, 2021; Young *et al.*, 2023). Additionally, studies on mating calls in Plainfin midshipman (*Porichthys notatus*) and courtship behaviour in brown ghost knifefish (*Apteronotus leptorhynchus*) have explored the involvement of brain aromatase in these processes (Kalarani *et al.*, 2021). Investigations into courtship behaviour in Bluehead wrasses (*Thalassoma bifasciatum*) have established the regulatory role of the telencephalic-habenular-interpeduncular pathway (Luong *et al.*, 2023). In one notable study on medaka fish (*Oryzias latipes*), the decision of medaka to mate or fight was influenced by two distinct androgen receptor signalling pathways (Nishiike & Okubo, 2024). Medaka deficient in androgen receptor subtype A were less aggressive and instead courted other medaka that were categorised as the same-sex, while those deficient in androgen receptor subtype B were less inclined to mate with medaka categorised as opposite-sex and more likely to attack them.

In addition to investigating appetitive sexual behaviour, researchers have also investigated the neural mechanisms underlying consummatory sexual behaviour in fish. Studies in species like goldfish (*Carassius auratus*) and salmon (*Oncorhynchus nerka*) have identified several brain regions crucial for sexual behaviour (Ogawa & Parhar,

2022). Furthermore, research on sexual receptivity in medaka has established correlations with the activity of gonadal steroid-responsive peptidergic neurons (Fleming *et al.*, 2022).

Astatotilapia burtoni as a model to study the neuroscience of sexual behaviour in a collective context

A burtoni has been extensively studied in neuroscience experiments, particularly regarding its social hierarchy and related physiology. Studies have established that dominant CES cichlids exhibit increased oxidative damage and higher levels of testosterone compared to subordinate LBS cichlids (Border, DeOliveira, *et al.*, 2019; Border *et al.*, 2021; Maguire *et al.*, 2021). Although these studies focused on blood, plasma, liver, and gonads rather than nervous system tissues, they provide crucial insights for future neuroscience investigations. Given the increasing interest in oxidative stress and antioxidant defence, it is anticipated that future neuroscience studies will explore this area more extensively (Ryan *et al.*, 2023). Changes in social status through social ascent led to temporal changes in the antioxidative system and oxidative stress levels (Fialkowski *et al.*, 2021). Variations in the propensity for social ascent among subordinate fish have been linked to cortisol and testosterone levels (Wallace *et al.*, 2022). Additionally, differences in social status correlated with distinct relationships between DNA damage, cortisol, and reactive oxygen species (Culbert *et al.*, 2023). Notably, oxidative regulation appeared to be more coordinated in dominant cichlids compared to subordinate ones (Fialkowski *et al.*, 2022).

In communities comprising only subordinate SBS cichlids, some individuals also become dominant (dominant DES cichlids). However, unlike dominant CES cichlids, they did not exhibit higher levels of oxidative damage compared to their subordinate SBS cichlids (Funnell *et al.*, 2022). Moreover, stability in the social environment influences oxidative stress levels, with effects mediated by cichlid social status (Border *et al.*, 2021). Similarly, cortisol and testosterone levels were impacted by social network stability (Maguire *et al.*, 2021). Notably, social hierarchies were also observable in juvenile *A burtoni*, regulated by the vasopressin system in the brain, which was associated with changes in nonapeptide signalling and the neuroendocrine stress axis (Solomon-Lane *et al.*, 2022). While group living may introduce additional stress, it also offers benefits such

as enhanced learning and decision-making compared to non-social contexts, reflected in differences in neural activity across various brain regions (Rodriguez-Santiago *et al.*, 2022).

Aggression plays a crucial role in establishing social status in *A burtoni* (Jackson, Dumitrascu, *et al.*, 2023). While both dominant CES and DES cichlids exhibit similar aggressive tendencies, there were distinctive patterns. These differences in aggression were also reflected in neural activity patterns, as shown in studies utilising phosphorylated ribosome marker phospho-S6 ribosomal protein. These patterns varied between the types of cichlids, particularly in regions like the anterior tuberal nucleus (equivalent to the ventromedial hypothalamus in mammals) and the central part of the ventral telencephalon (equivalent to the lateral septum in mammals). Aggressive behaviour in *A burtoni* was modulated by gonadal steroid hormones, regulated in part by aromatase-expressing cells and androgen receptor alpha in the anterior tuberal nucleus (Lopez & Alward, 2023).

Oxidative systems have also been investigated in the context of maternal care in *A burtoni*, with an equilibrium observed in antioxidant levels and oxidative DNA damage (Sawecki & Dijkstra, 2022). This equilibrium was adjusted through filial cannibalism, which is when the mouthbrooding cichlid consumes some of the young (Sawecki *et al.*, 2019). Filial cannibalism impacted oxidative stress levels throughout the reproductive cycle. Fluctuations in the brain transcriptome across the reproductive cycle have also been noted, with changes in neurotensin gene expression associated with other genes related to oxygen transport and neuroprotection (Faber-Hammond & Renn, 2023). Neural activation patterns, measured through phospho-S6 ribosomal protein levels, also fluctuated across the reproductive cycle, including during instances of filial cannibalism (Butler *et al.*, 2020; Maruska *et al.*, 2020; Ray *et al.*, 2023).

Body colouration in *A burtoni* plays a crucial role in communication during social interactions and is regulated by the melanocortin system, impacting behaviour depending on the cichlid's colouration (Border, Piefke, *et al.*, 2019). This colouration is generated through changes in xanthophores, which are regulated by the neuroendocrine centre – the melanocortin system, through alpha-melanocyte stimulating hormone (Dijkstra *et al.*, 2017). This was seen with the increase in aggression by administering alpha-melanocyte-stimulating hormone, but only in dominant CES cichlids that were blue, and not those that

were yellow. These may have relevance across taxa because the pro-opiomelanocortin gene family, which is part of the melanocortin system, is highly conserved across cichlids, teleosts and mammals (Harris *et al.*, 2014).

Sensory plasticity in body colouration is mirrored in the visual system, with increased visual sensitivity during ovulation correlating with higher retinal neural activation and greater opsin expression (Maruska & Butler, 2021). This heightened sensitivity facilitates mate choice, with prostaglandin-E2 and $17\alpha,20\beta$ -progesterone signalling implicated (Kidd *et al.*, 2013; Li, Lawrence, *et al.*, 2023). The courtship of *A burtoni* is multimodal activating several socially relevant brain nuclei, as evidenced by c-Fos expression patterns (King *et al.*, 2022; Howard *et al.*, 2024).

Neuroscience research in *A burtoni* is expanding, partly due to its emergence as a non-traditional model organism, facilitated by advancements in gene-editing techniques (Jackson, Lopez, *et al.*, 2023). For instance, researchers have utilized gene editing to render androgen receptor genes non-functional, aiming to elucidate their roles in aggressive and mating behaviours. Similarly, prostaglandin receptors have been targeted for gene editing in *A burtoni*, with knockout studies shedding light on their significance in initiating sexual behaviour.

Automated tracking of collective behaviour

The automation of tracking multiple animals has become an essential endeavour in behavioural research. With individual identification being a cornerstone, significant progress has been made in computer science to achieve this feat (Vidal *et al.*, 2021). Typically, this process involves capturing images of individuals using sensors like cameras, extracting pertinent features from these images, and then utilizing decision-making modules to assign identities based on a reference database. Deep learning algorithms, such as deep metric learning, automate the comparison of features across different individuals, aiding in efficient identification (Vidal *et al.*, 2021).

Several tools have emerged to track and identify individuals in group settings. AlphaTracker, for instance, employs pose estimation techniques to predict key points on animals, enabling individual identification based on hierarchical visual information (Chen, Zhang, *et al.*, 2023). Similarly, Multi-Object Tracking in Heterogeneous

environments (MOTHe) has proven effective in detecting and tracking unmarked animals, even in environments with dynamic lighting conditions (Rathore *et al.*, 2020). MOTHe has been used successfully in the contexts of wasp nests, and antelope herds, both of which have heterogeneous backgrounds and dynamic light conditions. However, challenges persist, particularly in environments where animals can be occluded or undergo size discrepancies. Researchers have addressed this by developing sophisticated techniques such as those used in the Where'd It Land (WILD) project, which utilizes convolutional neural network-based segmentation to detect and track animals in three-dimensional spaces (Xiao *et al.*, 2023). There are also self-supervised approaches to multi-animal detection and tracking, where the machine generates its own training data from the limited data available by setting up internal challenges to itself to learn the structures and patterns in the data (Moosa *et al.*, 2024).

A method called Multiple Object Tracking for Fish (FishMOT) has emerged to automate the analysis of fish behaviour (Liu, Han, *et al.*, 2023). FishMOT tackles common challenges in tracking fish collectives, such as morphological changes, occlusions, and complex environments, using detection boxes and individual identification. To maintain consistency in fish identity across successive frames, FishMOT employs a metric called the intersection over the union of adjacent frames, which measures the overlap between them. Another widely utilized tool for multi-animal behavioural analysis is DeepLabCut, which boasts a substantial user base of over 1000 labs and institutions worldwide (Madhusoodanan, 2024). With more than 5000 citations and over 700,000 downloads, DeepLabCut has garnered popularity among researchers. It has been extensively employed to study various collectives, including desert termites and leafcutter ants (Plum *et al.*, 2023), human crowds (Zhou, Stoffl, *et al.*, 2023), and schools of fish (Lauer *et al.*, 2022).

The operation of multi-animal DeepLabCut begins with pose estimation, where the likelihood of a pose occurring at a specific location is calculated. These estimated poses are then associated with specific individuals through a process known as assembly, facilitated by part affinity fields that establish connections between different body parts. Individual identification is further refined by learning visual features using metric learning techniques. Subsequently, each individual is tracked over time across a predetermined number of frames, and these tracks are seamlessly combined to create a

cohesive trajectory spanning the entire video duration, utilizing spatiotemporal pose information (Lauer *et al.*, 2022). Various recent endeavours have been undertaken to track animal collectives in diverse contexts (Ipek *et al.*, 2023; Sakamoto *et al.*, 2023; Su, Wang, *et al.*, 2023).

Specific Aim 2

Professor Peter Dijkstra's lab has extensively researched *A burtoni* in various setups, including different numbers and types of cichlids (Sawecki *et al.*, 2019; Border *et al.*, 2021; Fialkowski *et al.*, 2021, 2022; Piefke *et al.*, 2021; Dijkstra *et al.*, 2022; Funnell *et al.*, 2022; Culbert *et al.*, 2023). While tank environments may not fully replicate natural leks, studying individual interactions in tanks remains valuable in investigating lekking. Studying these interactions could be facilitated with automated behavioural analysis. This has not been done for *A burtoni*, but has been done on a closely related species, the Lake Malawi cichlids (Johnson *et al.*, 2020; Long *et al.*, 2020), suggesting this could be feasible. In this study, I trained a DeepLabCut pose estimation model using video datasets from the Dijkstra lab. Due to challenges in individual identification within the DeepLabCut model, I focused on a two-separated-cichlids setup. This setup allowed us to attribute poses to each cichlid and analyse their kinematics.

Methodology

Astatotilapia burtoni Video Dataset

The video datasets were generously provided by Professor Peter Dijkstra, with some previously published by his lab. These datasets included up to 24 cichlids in a single video, showcasing different combinations of dominant CES cichlid, dominant DES cichlid, subordinate LBS cichlids and subordinate SBS cichlids (Sawecki *et al.*, 2019; Border *et al.*, 2021; Fialkowski *et al.*, 2021, 2022; Piefke *et al.*, 2021; Dijkstra *et al.*, 2022; Funnell *et al.*, 2022; Culbert *et al.*, 2023). Each video was accompanied by manual annotation files detailing the behaviours of interest, including the time points and the specific fish performing each behaviour, identified by the colour beads most cichlids were tagged with underneath their dorsal fins. Using these annotations, clips were trimmed to approximately 10 seconds using HD Video Converter Factory Pro v25.6, capturing a

range of behaviours as annotated in the videos (Table 1). A total of 100 clips were generated, encompassing the various setups in the video datasets and the diverse behaviours exhibited.

Training a DeepLabCut multi-animal pose estimation model

A standard DeepLabCut multi-animal project was created according to https://deeplabcut.github.io/DeepLabCut/docs/maDLC_UserGuide.html. Given that most of the fish could be identified by their coloured beads, the project was configured with the setting “identity: True”. Due to the system requirements of DeepLabCut, individuals were labelled as ID1 to ID38 instead of by bead colour (e.g., black, hot pink) and sex, which were assigned by the Dijkstra lab. Some cichlids were untagged in setups where a barrier divided the left and right tanks, and these were also set as different individuals.

The unique body parts were labelled to distinguish the left and right sides of the coloured beads as UL1-UL26 (unique labels left) and UR1-UR26 (unique labels right). This labelling conformed to the DeepLabCut system, rather than using descriptive information including bead colours, types of cichlids, and bead side. Each number in the UL and UR labels corresponded to each other, so UL1 referred to the left side of the same bead as UR1.

Two frames were then extracted from each of the 100 video clips using k-means clustering, resulting in a total of 200 frames. Labelling was completed with the assistance of Dijkstra lab: Amelia Beswick, Lexi Balasa, and Hope Phillips. After all the labelling was completed, the labels were checked using the “check_labels” function in DeepLabCut. A training dataset was then created with default settings, using a 0.2 test-train split, imgaug, ADAM optimization, DLCRNet, and batch training with the pre-trained network ResNet-50. The network was trained on an NVIDIA Quadro RTX 5000 (Driver v551.61, CUDA v12.4) for 100,000 iterations, as suggested by DeepLabCut, to prevent overfitting, with loss displayed every 500 iterations. The trained network was then evaluated for the mean average Euclidean error per body part.

All video clips were then analysed without animal assembly and tracking to generate pose estimation pickle files. Animal assembly and tracking were also attempted by first assembling the poses to individuals using the “convert_detections2tracklets”

function with “identity_only = True,” as the cichlid identities could be determined by the coloured beads. The tracklets were then used for tracking with the “stitch_tracklets” function, and pose-labelled videos were created, with poses coloured by the individual to which they were assigned.

Extracting pose estimation result from DeepLabCut pickle file for individual identification

After videos were analysed by DeepLabCut without animal assembly and tracking, it generated a pickle file with all the pose estimation data. While individual identification was not successful for my current model, I could manually do the individual identification for the two-separated-cichlids setup, as each individual could be identified by the side of the tank they occupy. Firstly, a polygon region of interest (ROI) was defined, from which if the pose was present in that location, this information was extracted to assign to an individual. The output file then becomes a single-animal project to facilitate downstream analysis. This was done with the code provided here https://github.com/vchiang001/sexual_diversity_dissertation/blob/main/specificaim2/Dijkstra_lab_African_cichlids_behavioural_analysis.ipynb in the section “Transform pose estimation output for downstream analysis of two-cichlids separated setup by a process called “unpickling” ”.

Calculating the total distance travelled and visualising the paths of travel

The total distance was calculated for each body part when the body part at a specific frame was above the set machine confidence. It also considered the maximum distance the body part can travel before being treated as erroneous and omitted from the calculation. This was done with the code provided here https://github.com/vchiang001/sexual_diversity_dissertation/blob/main/specificaim2/Dijkstra_lab_African_cichlids_behavioural_analysis.ipynb in the section “Calculating total distances”. The total path that was travelled by each part was also visualised using the code provided here https://github.com/vchiang001/sexual_diversity_dissertation/blob/main/specificaim2/Dijk

stra_lab_African_cichlids_behavioural_analysis.ipynb in the section “Plotting xy coordinates occupied”.

Kinematics

The kinematics of the poses were calculated using DLC2Kinematics for each body part. Velocity in pixels/second, acceleration in pixels per second per second, and speed is the magnitude of the velocity, which were all calculated for each frame the body part exceeded a set machine confidence threshold. This was done with the code provided here

https://github.com/vchiang001/sexual_diversity_dissertation/blob/main/specificaim2/Dijkstra_lab_African_cichlids_behavioural_analysis.ipynb in the section “Calculating velocity, acceleration, speed using DLC2kinematics”.

Automating butts behaviour recognition

I attempted to automate the recognition of butts behaviour using a few handcrafted features in body parts in frames that were above a set machine confidence threshold. First, the body part had to be in a defined ROI, where the butts behaviour typically occurs at the barrier dividing the two sides of the tank. Second, the cichlid had to be moving toward the divider, which was determined by the change in the x-coordinate towards the barrier. This change in the x-coordinate was screened through a list of previous x-values within a set velocity window, representing the number of previous frames to be considered. Third, the velocity within this window had to exceed a defined level in pixels per second. These criteria allowed us to detect the presence of butts behaviour in each frame.

However, since these features could qualify for butts behaviour in consecutive frames, the data were adjusted for the frame rate, which was 30 frames per second for all videos. If any butts behaviour was detected within a 30-frame chunk, then that second was marked as containing butts behaviour. This adjustment facilitated comparisons to the manual annotations, where each second was annotated if butts behaviour was present. To combine information from all body parts, the data were transformed such that if any body part predicted the presence of butts behaviour, then that second was predicted to contain butts behaviour. These were done with the code provided here

https://github.com/vchiang001/sexual_diversity_dissertation/blob/main/specificaim2/Dijkstra_lab_African_cichlids_behavioural_analysis.ipynb in the section “Calculating the number of butts”.

Assessing butts behaviour algorithm performance

Ten videos were manually annotated for the presence of butts behaviour by the second, by three observers from Dijkstra lab independently: Amelia Beswick, Lexi Balasa, and Hope Phillips. For each second, only if at least two observers annotated the presence of butts behaviour, then that second would be assigned to having the butts behaviour present. This was then used for subsequent analysis as the ground truth. In terms of my algorithm, different parameter combinations were used to generate output csv files of the algorithm predicted results for each video: parameter1(velocity threshold: 1pixel/second, rolling window:40 frames), parameter2(velocity threshold: 15pixel/second, rolling window: 40 frames), and parameter3(velocity threshold: 35pixel/second, rolling window: 40 frames). These automated predictions were then compared with manual annotations in terms of the recall, and precision for each video using the following code:

https://github.com/vchiang001/sexual_diversity_dissertation/blob/main/specificaim2/Assessing_butts_behaviour_algorithm_performance_.ipynb

The F1 was then calculated using the formula $(2 * Precision * Recall) / (Precision + Recall)$.

Visualization of results

The following results were visualised: model training curve for the different types of losses across training iterations, mean average Euclidean error for each body part, total distance travelled for each body part, speed across the video frame, average speed per body part and the performance of the butts behaviour recognition. These graphs were plotted using this code:

https://github.com/vchiang001/sexual_diversity_dissertation/blob/main/specificaim2/specificaim2_graphs.ipynb

Results

An Astatotilapia burtoni dataset for multi-animal pose estimation was curated.

Professor Dijkstra generously provided a range of videos from his lab, some of which were used in the lab's previous studies (Sawecki *et al.*, 2019; Border *et al.*, 2021; Fialkowski *et al.*, 2021, 2022; Piefke *et al.*, 2021; Dijkstra *et al.*, 2022; Funnell *et al.*, 2022; Culbert *et al.*, 2023). My goal was to develop a pose estimation model that could generalize across all these settings (Figure 5A). These videos were accompanied by annotation files indicating the time and specific behaviours performed by each fish, identified by coloured beads tagged below their dorsal fins. The behaviours included in these annotations had been previously defined (Table 1). From these videos, I extracted 100 clips, each approximately 10 seconds long, capturing a range of behaviours to train the pose estimation model (Figure 5B). A DeepLabCut multi-animal project was created using these clips, and two frames were extracted from each clip, resulting in a total of 200 frames. These frames were labelled to identify the coloured beads and specific body parts associated with each individual fish (Figure 5C). The “check_label” function was used to visualize all the labelling before training the model (Figure 5D).

Multiple Astatotilapia burtoni can be pose estimated across different setups.

To train the DeepLabCut model, I created a training dataset using transfer learning with ResNet_50 and data augmentation with imgaug. The model was trained for 100,000 iterations, during which various loss functions converged (Figure 6A). I then evaluated the model by calculating the mean average Euclidean error between the ground truth of each body part and the model's predictions (Figure 6B). The training data showed relatively small pixel errors, all below approximately 4 pixels, considering each video frame was 1920 x 1080 pixels. However, the test data exhibited higher errors than the training data, indicating potential overfitting and suggesting that the model could not generalize to all the data. Future improvements could involve labelling more video frames to increase training data, which would help the model generalize better. The test error was especially high for the pectoral fin bases (left and right), exceeding 18 pixels errors, likely due to the difficulty in visually discerning the pectoral fins in the cichlids.

Subsequently, I ran the assembly and tracking pipeline on DeepLabCut with supervised identity tracking, utilizing the coloured beads to discriminate between individuals. The results were used to create labelled videos where the colour scheme indicated the individual identities. However, individual identification performed poorly on the cichlid videos, with frequent identity swaps across frames (examples shown in Figure 6C). Improving individual identification would require more labelling of the cichlids, particularly focusing on the coloured beads to help the model learn to identify individuals based on these markers.

Given the difficulties with individual identification in the current videos, I shifted my focus to the setup where there were two cichlids which were separated by a barrier in the middle of the tank (two-separated-cichlids setup). This setup allowed me to ascribe poses to individuals based on their location in the video. Videos were created for this setup focusing solely on pose estimation without individual identification. The poses were robustly predicted in these videos (examples shown in Figure 6D).

*Individual identification of two *Astatotilapia burtoni* divided by barriers.*

To utilise the DeepLabCut pose estimation results for manually setting individual identification based on location, it is necessary to manipulate the file structure to extract the required information. During the pose estimation step, DeepLabCut generates a pickle file containing keys that refer to video frames and values comprising the pose estimation data (Figure 7A). This pose estimation data is organized as a dictionary, where each body part serves as a key, and the corresponding pose estimation data is the value. This data is structured as an array that indexes an assigned individual, with the array containing the x and y coordinates of the respective poses. I unpickled this file by extracting body part information if it was present within an ROI, which is a polygon area I defined for each video. The pose estimation information for each individual was then stored in .csv and .h5 files. These files were separated by the individual's position on the left or right side of the tank and structured to be compatible with typical single-animal DeepLabCut analyses, treating them as a single-animal DeepLabCut project.

To verify the correctness of my code, I mapped the pose outputs to the respective frames in the video, ensuring that the poses were accurately superimposed onto the

cichlids on the correct side of the tank (Figure 7B). For an example video, I plotted the x and y locations of each body part as they appeared in a selected video frame (Figure 7C). Additionally, for the same video, I calculated the total distance travelled by each body part across the frames, only including those with a machine confidence score greater than 0.4 and accounting for major jumps in body part location that were deemed erroneous (Figure 7D). There was variability in the total distance calculated because not every body part was visible in each frame, depending on the angle of the cichlid.

*Kinematics can be analysed for two *Astatotilapia burtoni* divided by barriers.*

In addition to measuring distance, pose estimation data can be used to conduct kinematic analyses. For the example video analysed for total distance, I also calculated the speed (pixels/second) of selected body parts in frames that exceeded the defined machine confidence (>0.4) (Figure 8A). The body parts were chosen based on those that were most frequently detected above the machine confidence threshold across all frames. I then calculated the average speed for each body part across all video frames (Figure 8B). Similar to the total distance, the average speed varied for each body part since they might not all be visible at the same time.

Speed data can also help identify specific behaviours. For instance, "butts" behaviour involves cichlids performing aggressive displays by quickly moving toward another cichlid but hitting a barrier instead (Figure 8C). I attempted to automate the detection of this behaviour using pose estimation and speed data. By analysing pose and speed information, I aimed to determine whether a butts behaviour occurred at a specific second in the video. The handcrafted features I used included whether the cichlid was moving toward the divider based on information from a selected number of previous frames (rolling window), whether the velocity was above a defined level (velocity threshold), and whether the cichlid was in an ROI close to the divider. This data was obtained for each body part, and I combined it to indicate a butts behaviour at a particular second if any body part detected it.

I tested different parameters for the velocity threshold and rolling window to identify butts behaviour using these handcrafted features. Concurrently, we manually annotated 10 videos (approximately 5 minutes each) for the presence of butts behaviour at

each second of the video. My automated analysis was compared to this ground truth in terms of precision (the fraction of true positives out of all frames detected as positive, accounting for false positives), recall (the fraction of true positives detected out of all true positives, accounting for false negatives), and F1 score (the harmonic mean between precision and recall). This was done for three different parameter sets: parameter1 (velocity threshold: 1 pixel/second, rolling window: 40 frames), parameter2 (velocity threshold: 15 pixels/second, rolling window: 40 frames), and parameter3 (velocity threshold: 35 pixels/second, rolling window: 40 frames) (Figure 8D). The only satisfactory result was the recall for parameter 1, which achieved a score of 0.78 ± 0.25 , where values closer to 1.0 are better. This suggests that either my parameter settings have not been optimised or that the handcrafted features I considered were not appropriate. Further optimisation and feature refinement are necessary to improve the performance of this automated behaviour detection system.

Discussions

In this study, I curated an *Astatotilapia burtoni* dataset for multi-animal pose estimation using videos provided by the Dijkstra lab. This dataset included a diverse range of behaviours and experimental setups. I trained a DeepLabCut model which demonstrated adequate pose estimation performance but exhibited poor multi-animal assembly and tracking. Consequently, I focused on setups where two cichlids were separated by a barrier within a tank. Poses were extracted for each individual based on their location, which were then used to calculate distance. Additionally, kinematic data from these poses informed my algorithm to predict "butts" behaviours.

To my knowledge, there are no published studies on automated tracking of *A burtoni*. However, a recent poster tracked individual cichlids using Social LEAP Estimates Animal Poses (SLEAP) (Edwards *et al.*, 2024). SLEAP uses temporal-based identification via optical flow to predict movement between frames and appearance-based cues for bottom-up identification using landmarks or a top-down approach using an animal-centred sub-image and pose (Pereira *et al.*, 2022). The poster compared different parameters of these methods by measuring the proportion of frames that had an individual

swap error in the video. However, it was not clear how many cichlids were present in the videos they used, and their videos were top-view which differed from our front-view videos. Furthermore, since their error measurements were done based on the proportion of frames out of all the frames that one model produces, it was not clear how frequent identification swaps occurred based on their results. In contrast, DeepLabCut uses part affinity fields to relate body parts for identity and reidentification, as well as temporal tracklets for interrupted events due to occlusion, stitching them together based on predicted trajectories (Lauer *et al.*, 2022). While there is no clear data that shows which approach is better, my study is the first to perform pose estimation on up to 24 fish from a frontal view.

My work represents an important step toward automated tracking of *A burtoni*, a key species for neuroscience research, including studies on collective behaviour in lekking. Despite limited knowledge about how the brain facilitates collective behaviours, automated collective tracking in species like zebrafish has shown promise (Tang *et al.*, 2020; Messina *et al.*, 2022). Automated tracking of African cichlids can enhance research on sexual behaviour in a collective context, and enable long-term studies of multiple interacting animals.

There are several limitations to my approach. First, my approach relied on pose estimation which may not capture some behavioural nuances that key point relationships cannot address. Another limitation was that due to the frequent occlusion and overcrowding in the cichlid setups, this may necessitate extensive pose labelling of frames in the thousands or even tens of thousands to enable accurate individual identification. Additionally, interpreting behaviours solely from poses is challenging. Therefore, it remains difficult to show a clip to artificial intelligence (AI) with pose estimation capabilities for the AI to interpret the clip such as how the objects in the clip interact and contextualise these interactions.

Automating behavioural analysis in *A burtoni* has broader implications for animal ethics. For example, it could enable home-tank monitoring without needing to transfer animals to alternative tanks during experiments (Klein *et al.*, 2022; Hölter *et al.*, 2023).

This reduces the need for manually handling the fish and priming the fish for observation, thereby allowing for a more naturalistic study environment with lesser human interference (Benedict & Cudmore, 2023; Kahnau *et al.*, 2023). Furthermore, this could facilitate more longitudinal types of data collection. This type of high-throughput data allows for more hypotheses to be tested on animals that are sacrificed and could reduce the number of animals needed for statistically significant results (Anderson & Perona, 2014).

Summary and Conclusions

In conclusion, I developed an automated tracking system for communities of *A burtoni* using a DeepLabCut pose estimation model. This system allowed me to track two cichlids separated by a barrier and obtain kinematic features from each of them. My work facilitates more comprehensive neuroscience studies on this species, particularly focusing on individual interactions and lekking behaviour. Improved animal ethics can be achieved through these automated tracking efforts.

Recommendations

The next immediate steps include enabling individual identification of *A burtoni* communities. A recent virtual marker approach significantly reduces the annotation burden, which could aid in individual identification in crowded scenes (Azechi & Takahashi, 2024). Additionally, alternative methods like instance segmentation could be employed to identify behaviours by considering the whole body instead of poses (Kaul *et al.*, 2024). Finally, pose estimation could be integrated with AI which could gain a semantic understanding of the pose trajectories, whereby the AI could comprehend and describe the nature and context of those movements (Li *et al.*, 2024). The AI could then generate outputs in natural language that allow researchers to easily comprehend what is occurring in the scene of the input. In relation to sex diversity, some examples of this type of AI could be, “the bird is performing a courtship dance”, “the frog is selecting a mate based on their visual display”, and “the hyenas is asserting dominance in a competition for mates”. These could be combined with existing approaches to study collective behaviour with computational modelling (Pan & Lauder, 2024).

Sexual behaviour is crucial for many species, and constructing the neural pathways linked to these behaviours can serve as welfare indicators and advance animal ethics including for teleost fish like *A burtoni* (Coria-Avila *et al.*, 2022; Cavallino *et al.*, 2023). Studies on sexual behaviour including in the collective context could benefit from the automation of behavioural analysis. Additionally, robotics and *in silico* models built from the vast amount of behaviour data generated through automation could help eventually replace some of the substantial numbers of animals used for experimentation (Connor *et al.*, 2023; Zhou, Liu, *et al.*, 2023; Aldarondo *et al.*, 2024; Turab *et al.*, 2024). *In silico* models could be in the form of digital twins, whereby the model could simulate, analyse, and predict the behaviour and performance of an animal. This could potentially replace some model organisms in certain research areas (Chen, Yu, *et al.*, 2023; van Beuningen *et al.*, 2023). Apart from ethics, behaviour often serves as a primary indicator in health interventions and environmental changes and is often salient that draws attention from scientists and the public (Patricelli, 2023).

Besides centring the research on sexual behaviour, emphasis could also be placed on the collective aspects of it to construct broader implications for collective behaviour. This could explore how individual interactions create larger patterns and structures, influencing and being influenced by each other in complex systems (Bak-Coleman *et al.*, 2021; Zeng & Fu, n.d.). These systems could experience sudden, irreversible changes in collective behaviour when disturbed by technological and social changes. In the scale and structure of modern human social networks, this could be relevant to predicting and managing emergent social dynamics in information flow and collective action.

Figures & Legends

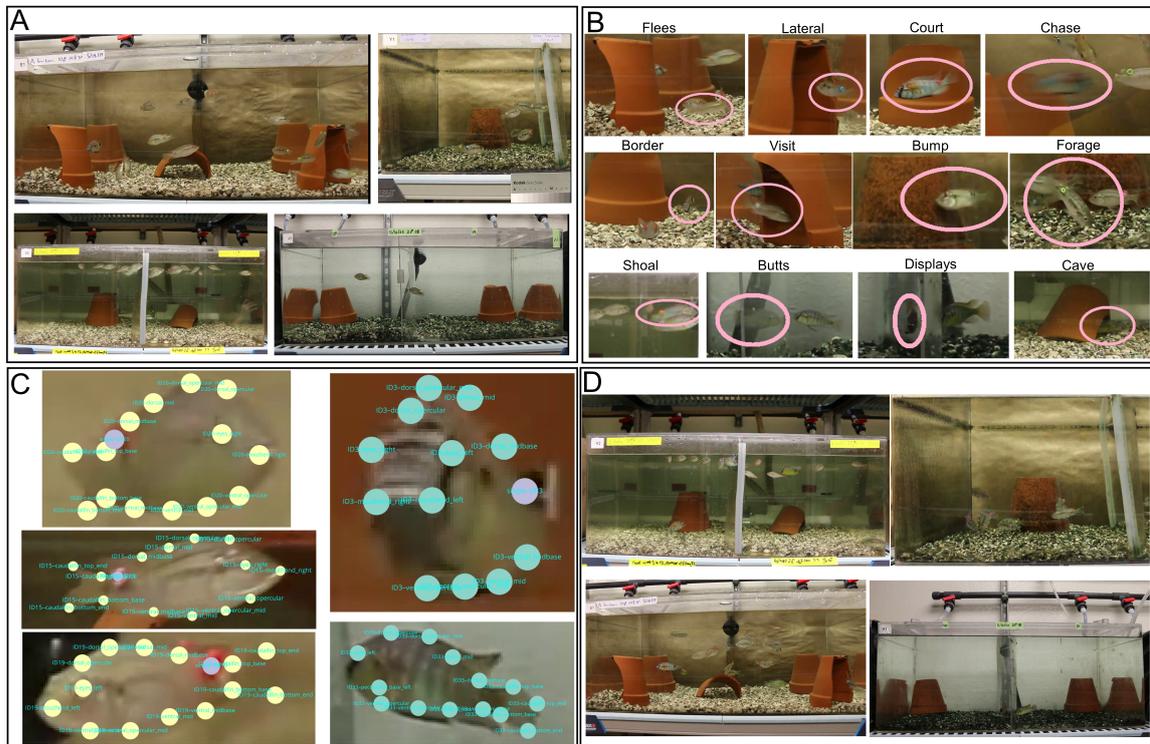


Figure 5 An *Astatotilapia burtoni* dataset for multi-animal pose estimation was curated.

A) Examples of video datasets generously provided by Professor Peter Dijkstra, some from previous publications from his lab (Sawecki *et al.*, 2019; Border *et al.*, 2021; Fialkowski *et al.*, 2021, 2022; Piefke *et al.*, 2021; Dijkstra *et al.*, 2022; Funnell *et al.*, 2022; Culbert *et al.*, 2023). B) Zoomed-in images of behaviours of interests performed by the cichlid circled in pink. C) Examples of poses that were labelled at different angles of the cichlids including individual identity, body part and the coloured beads. D) Examples of the output from the DeepLabCut “check_label” function to check all the labelling before moving on to training.

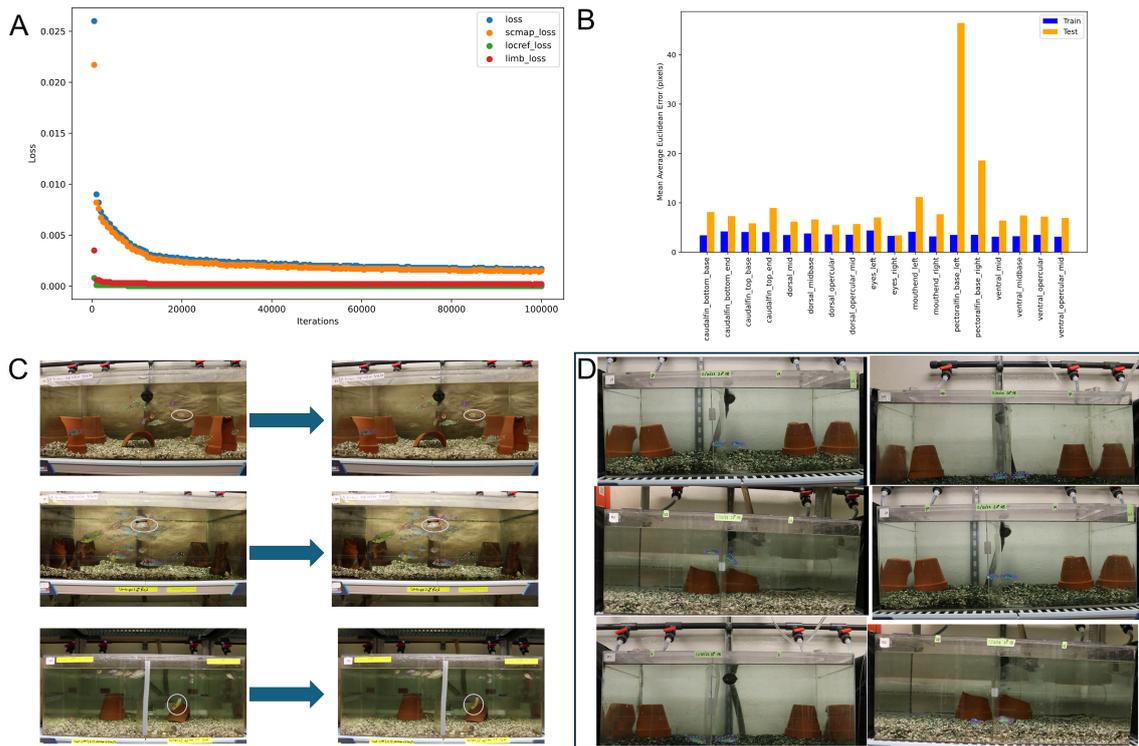


Figure 6 Multiple *Astatotilapia burtoni* can be pose estimated across different setups

A) A scatterplot showing changes in different types of losses across 100,000 iterations of neural network training. The types of losses displayed included total loss (loss), scoremap loss (scmap_loss), location refinement loss (locref_loss), and limb loss (limb_loss). B) A bar plot showing the mean average Euclidean error (pixels) between DeepLabCut predictions and ground truth of cichlid body parts. The plot shows both training and testing data after 100,000 iterations of training. C) Examples of individual identification performance, where identity swaps were evident in subsequent video frames. D) Examples of pose estimation results for the two-separated-cichlids setup.

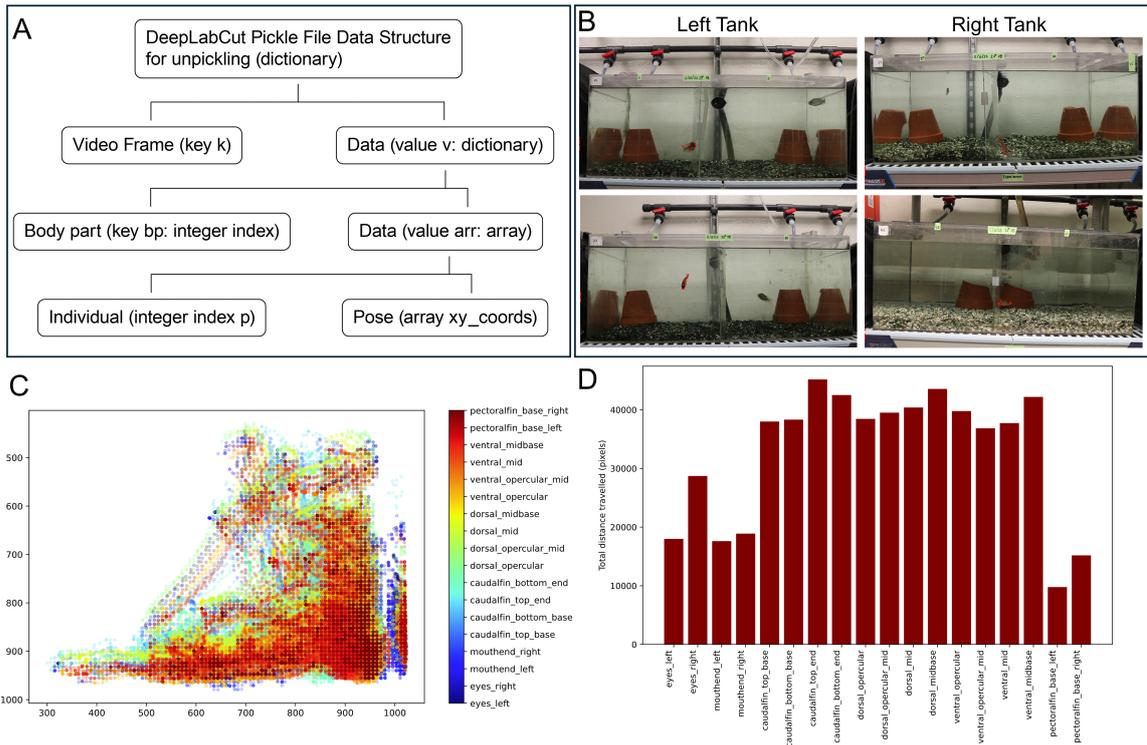


Figure 7 Individual identification of two *Astatotilapia burtoni* divided by barriers

A) Data structure of DeepLabCut pickle file relevant to my objectives to unpickle to assign pose data to specific individuals. It includes a dictionary of video frames (key k) referring to the pose estimation data (value v). This pose estimation data is a dictionary of body part (key bp) which is an integer index that refers to its respective pose estimation data of that body part (value arr). This pose estimation data is in the form of an array including an individual as an integer index (p) that points to the pose coordinates as an array of xy coordinates and the machine confidence. This figure was created by Stephen Renjie Hu. B) Examples of superimposing the poses extracted from the pickle files for individuals at different sides of the tank. C) Map of the path travelled in the x and y axis for each body part in an example 5-minute video, that represents the side of the respective tank in the dimensions. Each body part is shown as a different colour as specified in the colour map. D) A bar plot showing the total distance travelled in pixels, for each body part in an example 5-minute video.

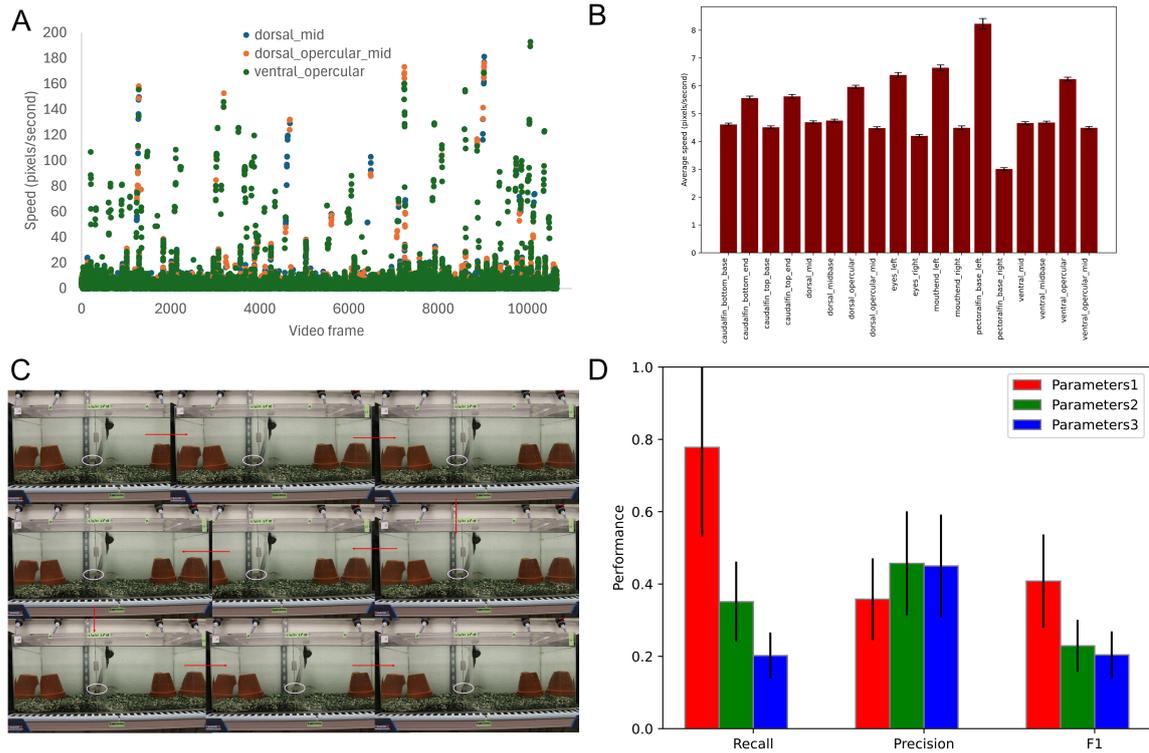


Figure 8 Kinematics can be analysed for two *Astatotilapia burtoni* divided by barriers

A) A scatterplot showing speed in pixels / second across video frames of an example 5-minute video for three selected body parts: dorsal_mid, dorsal_opercular_mid, ventral_opercular. B) A bar plot showing the average speed (pixels per second) with standard error in an example 5-minute video for all body parts. C) Consecutive frames of an example butts behaviour with sequence indicated by the red arrow. D) Bar plots showing recall, precision and F1 (all out of 1.0) for ten algorithm-predicted videos when compared to manual annotations. Three parameter settings of velocity window and velocity threshold are shown.

Tables

Table 1 Astatotilapia burtoni behaviours of interest in Dijkstra lab's datasets

Behaviour	Description
chases	Aggressively swimming after other cichlids, biting, and forcibly moving other fish
border displays	Interactions between two cichlids where both charges head-on towards each other with extended pectoral fins and an open mouth
lateral displays	A cichlid presents the side of their body to another cichlid with erect fins and flared opercula, making themselves look as large as possible.
courtship displays	A combination of behaviours involved in a courtship display: approach and lead swim. In a courtship display, a cichlid will swim towards another cichlid before directing towards the flowerpot in a zig-zag pattern, flaring their dorsal fin. This is followed by a rapid back-and-forth motion of the tail, presenting their tail fin and egg spots towards the cichlid.
flees	Rapidly retreating away from a chasing cichlid at matching intensity
butts	A cichlid could also initiate a chase towards another cichlid in a neighbouring compartment by aggressively swimming up against the clear divider.
forage	Obtaining food resources from the floor to forage
visit	Cichlid enters the flowerpot to visit
cave	Seeking shelter and refuge in the terracotta pot
bump	Physical contact or collision with an object
shoal	Grouping together with other cichlids

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CHAPTER 4

USING CAMERA TRAPS TO STUDY SEX DIVERSITY IN WILDLIFE

Introduction

The effects of labestication and captivity on sexual behaviour

The Anthropocene era has significantly impacted Earth's biodiversity and genetic composition through deliberate technological and social innovations partly through Western science (Guerra *et al.*, 2020; Alberti, 2023; Richerson *et al.*, 2023; Cano *et al.*, 2024; Zeller *et al.*, 2024). In neuroscience, one example is the use of domesticated model organisms for laboratory research (Mathuru *et al.*, 2020). Neuroscience today relies predominantly on a few model organisms: rats (*Rattus rattus*), mice (*Mus musculus*), zebrafish (*Danio rerio*), fruit flies (*Drosophila melanogaster*), and worms (*Caenorhabditis elegans*) (Mathuru *et al.*, 2020; Lambert, 2023). In the US, an estimated 100 million rats and mice are used for research each year. Globally, they make up over 93% of animal model studies. Their dominance is due to their easy maintenance in labs and the ability to manipulate their genomes. The extensive use of rats and mice creates a self-fulfilling prophecy, where new researchers find it easier to standardise them in their labs, and accumulating genetic data facilitates further genetic experimentation.

One significant limitation of these model organisms is the artificial selection of traits due to their long history in the lab, a process recently termed “labestication” (Mathuru *et al.*, 2020). This has notably altered rodent sexual behaviour, as lab rodents are often selected for rapid breeding (Zala *et al.*, 2020). Changes include prevalent

auditory defects and a routine oestrous cycle, unlike wild rodents, which exhibit poly and spontaneous oestrous cycles (Wölfl *et al.*, 2023). Additionally, the complexity and levels of vocalisation are similarly distorted (Zala *et al.*, 2020). A key reason for behavioural differences between lab and wild environments is that natural environments offer more behaviour affordances, where there are more opportunities for different behaviours to be exhibited and allow animals to adapt based on their experiences (Lambert, 2023).

Captivity often affects the sexual behaviour of animals. Examples include mate choice in coho salmon (Auld *et al.*, 2021), assortative mating in honeyeaters (Appleby *et al.*, 2023), and sexual behaviour in blue-and-yellow macaws (Miglioli & Vasconcellos, 2021). This misrepresentation of animal behaviour can have significant societal consequences. For example, male-centred investigations of animal behaviour have led to an androcentric view of sexual behaviour in textbooks affecting societal views on sex diversity (Ah-King, 2022; Pollo & Kasumovic, 2022; Spaulding & Fuselier, 2023). To address these issues, scientists must capture the diverse range of animal sexual behaviours including those that reflect their ancestral experiences (Clark *et al.*, 2023). This approach also benefits conservation and translational efforts, when non-human animal behaviours are observed that are not heavily confounded by human activity (Brown *et al.*, 2023).

Neuroscience studies require research on diverse species

Contrary to the current reliance on a few model organisms, neuroscience once encompassed a diverse range of animals. Ramon y Cajal's work included species such as crabs, grasshoppers, newts, chameleons, eagles, and horses (Garcia-Lopez *et al.*, 2010). Other neuroscientists extensively studied squids, gastropods, and barn owls (Gallant & O'Connell, 2020). However, this diversity has diminished due to the discouragement of curiosity-driven research, fewer publications on diverse species, and reduced funding for such research (Farris, 2020; Zupanc & Rössler, 2022).

Studying diverse species in neuroscience provides evolutionary insights, as the nervous system is a product of evolution (Cisek & Hayden, 2022). Evolutionary changes in the nervous system depend on ancestral structures and functions (Miller *et al.*, 2019). When assigning brain functions, such as behaviour, this dependency on ancestral conditions offers interpretative power to support specific claims (Miller *et al.*, 2019).

These can be explored by examining clades, which are groups of species sharing a common ancestor (Beans, 2020; Jourjine & Hoekstra, 2021; Tateishi *et al.*, 2024). Furthermore, these can be explored through convergent evolution, where similar phenotypic features develop independently in different species over time (Gallant & O'Connell, 2020). Many examples show how these approaches can help construct the mechanisms and overarching principles of brain organisation that drive behaviour across taxa and genera (Roberts *et al.*, 2022).

Evolutionary perspectives can challenge assumptions in neuroscience regarding neural regulation of behaviour (Westlin *et al.*, 2023). One assumption is that behaviours are localised to dedicated neural networks, but recent evidence suggests that behaviour is distributed across the entire brain (Gonzalez-Castillo *et al.*, 2012, 2015; Aimon *et al.*, 2019; Stringer *et al.*, 2019). Another assumption is a one-to-one relationship between behaviour and neural networks. However, evidence indicates a degeneracy relationship, where multiple mechanisms can generate the same behaviour (Feinstein *et al.*, 2011; Wilson-Mendenhall *et al.*, 2015; Singh *et al.*, 2021; Doyle *et al.*, 2022). Additionally, the assumption that neural networks function independently of other phenomena within the body and environment is being questioned. A closed-loop view of neural circuits, where the input and output are influenced by each other, other systems and the environment, is gaining traction. Evolutionary insights may help move beyond these dogmatic assumptions in neuroscience (Cisek & Hayden, 2022).

Studying sexual behaviour provides a way to gain evolutionary insights. For instance, in *Drosophila*, courtship wing vibrations differ across species (Jourjine & Hoekstra, 2021). These flies taste the abdomen of potential partners to evaluate chemical cues crucial for mating (Roberts *et al.*, 2022). This feature allows comparative studies to investigate sensory and motor circuits concerning the conservation and divergence of components and how slight modifications can lead to significant behavioural changes. This approach was applied to two frog species to study how their courtship songs were generated from different neural properties (Roberts *et al.*, 2022). Courtship songs have also been studied in Hawaiian crickets, a model clade that diversified ~4 million years ago (Jourjine & Hoekstra, 2021). Comparative studies with mammalian species have similarly been conducted, such as different vole species exhibiting various mating systems. Differences in mating systems were linked to vasopressin receptor gene

expression patterns across the brain (Beans, 2020). A major line of investigation in sexual behaviour and evolution is sexual selection which involves mate preference for certain characteristics (Fuss, 2021; Ryan, 2021; Hoops *et al.*, 2022; Kruger *et al.*, 2023). Additionally, studying evolution can help address brain-related issues across animal species, including humans (Lambert, 2023). Evolution-derived solutions incorporate knowledge from the immense challenges faced by over a trillion species over billions of years.

Using camera traps to study sexual behaviour in diverse species of animals

To study a diverse range of species in the wild, camera traps are widely used (Bassing *et al.*, 2023; Vélez *et al.*, 2023). These automated cameras capture images or videos of animals in their natural habitat without human presence for extended periods. They use motion or infrared sensors to detect an animal's presence. This method is especially beneficial for species where direct observation is challenging in remote areas. Recent sexual behaviour examples include camera trap studies in small-toothed palm civets in Cambodian forests (Barca *et al.*, 2015), Pacific martens in northern California (Delheimer *et al.*, 2023), jaguars in Mexico (Cassaigne *et al.*, 2023), stump-tailed macaques in a Thailand forest park (Toyoda *et al.*, 2023), porcupines in Gunung Merapi National Park, Indonesia (Sulaksono *et al.*, 2023), and panthers in Pantanal, Brazil (Fragoso *et al.*, 2023).

Camera traps provide valuable data for wildlife research. They can determine the times of day animals are active (Delisle *et al.*, 2021) and measure species diversity within a community (Kays *et al.*, 2020). They also help estimate abundance and density, as well as identify species occupancy, which is the probability of a species being present at a site (Green, Chynoweth, *et al.*, 2020; Kays *et al.*, 2020; Delisle *et al.*, 2021). Camera traps can similarly provide information on home range size, habitat use, animal interactions, and periodic events relevant to different species (phenology). They allow access to a wide range of habitats, including subnivean (Kalhor *et al.*, 2021) and arboreal environments (Agostini *et al.*, 2022). Conducting camera trap studies requires extensive review and involvement of all parties, such as Indigenous communities (Fisher *et al.*, 2021), local knowledge (Alempijevic *et al.*, 2022), and citizen science (Green, Rees, *et al.*, 2020).

The ethics of these studies need to be carefully and comprehensively assessed (Sharma *et al.*, 2020).

The implications derived from camera trap data are immense, especially when combined with other data sources. For instance, this type of research could highlight the importance of specific geographical regions in sustaining mammalian diversity (Chen *et al.*, 2022), monitoring the effects of human disturbances (Lovell *et al.*, 2022), climate change (Buchholz *et al.*, 2021), and human-mediated predation risks (Burton *et al.*, 2022). Camera traps also track social interactions (Tichon *et al.*, 2020), identify their drivers (Smith *et al.*, 2020), and provide insights into archaeological bone accumulations (Wadley, 2020). With the acceleration of industrialisation and urbanisation, non-human animals are increasingly deprived of natural habitats, an effect often investigated through camera trap studies (Vélez *et al.*, 2023). Humans face a similar situation, as nature is vital for various aspects of human health (Kolokotsa *et al.*, 2020; Marselle *et al.*, 2021). Natural environments are also crucial for sex-related variables and sexual health (Mailloux *et al.*, 2021; Sillman *et al.*, 2022).

Existing wildlife studies can guide neuroscientists in prioritising species, behaviours, and contexts, but these studies may harbour biases (Chaudhury & Colla, 2021; Christie *et al.*, 2021; Hughes *et al.*, 2021; Maas *et al.*, 2021; Poulin *et al.*, 2022; Freeberg *et al.*, 2024). Neuroscience should avoid perpetuating these biases and also consider advancements in the field that may alter interpretations of earlier studies, benefiting from a contemporary perspective (Kao, Banerjee, *et al.*, 2023).

Processing camera trap data with artificial intelligence

Camera trap studies often cover multiple sites within a selected geographical area and span several years, generating millions of images. This volume makes manual management and analysis challenging (Nazir & Kaleem, 2024). Consequently, researchers are turning to AI to reduce the time, effort, and errors associated with manual methods (Bohnett *et al.*, 2023). AI assists in object detection, identifying non-human animals and classifying them into broader categories such as birds, bobcats, bears, and coyotes (Nazir & Kaleem, 2024). While species classification is more complex, efforts

like BatNet, which identifies thirteen European bat species, demonstrate progress (Krivek *et al.*, 2023). AI can now also identify individual animals in some species, such as jaguars in the Eastern Amazon and snow leopards in Europe, the US, and Afghanistan (Bohnett *et al.*, 2023; Wiig *et al.*, 2023).

Large AI models, such as those used by ChatGPT, can manage multiple tasks, including image analysis. Contrastive Language Image Pretraining (CLIP) can describe objects and actions in images (Vogg *et al.*, 2024). This technology has been adapted for camera trap data as WildCLIP, which assists in action recognition in camera trap images by identifying behaviours like vigilant standing, grooming, and foraging (Gabeff *et al.*, 2024; Schindler *et al.*, 2024). Another method, Animal Skeleton-Based Action Recognition (ASBAR), uses DeepLabCut for pose estimation to identify key points in images. Following pose estimation, ASBAR leverages the PanAf dataset to help recognise behaviours based on skeletal motion data. ASBAR has been shown to detect great ape behaviours such as walking, standing, and climbing in camera trap images (Fuchs *et al.*, 2023). Behaviour recognition in other data types, such as drone videos, may also facilitate advancements in camera trap data analysis. For instance, the BaboonLand Dataset enabled behavioural recognition in drone videos of primates (Duporge *et al.*, 2024).

Neuroscience studies conducted in the wild

Studies outside the lab are needed as these settings can introduce confounding variables (Ross & Leinwand, 2020). Several studies on re-wilding have investigated animals formerly held in captivity (Clark *et al.*, 2023; Zippel *et al.*, 2023). However, many of these re-wilded animals have spent their entire lives in captivity, which hampers the development of behaviours suited for natural environments. Neuroscience experiments on live wild animals are becoming more common. For example, blood volume and haemoglobin oxygenation in the brain were measured using near-infrared spectroscopy during diving in temporarily captive harbour seals (McKnight *et al.*, 2019). Neurologgers are portable devices that record neural activity such as EEG and local field potentials, which have been used in freely moving animals (Piot *et al.*, 2024; van Hasselt *et al.*, 2024; Zaid *et al.*, 2024). They have been utilised to obtain EEG recordings during

various behaviours in temporarily captive octopuses (Gutnick *et al.*, 2023). However, captivity can impact these findings, as even short-term captivity can impair certain animal phenotypes (Crates *et al.*, 2023), social networks (Pacheco & Madden, 2021) and gene expression (Duque *et al.*, 2023).

Other studies were carried out postmortem, including diffusion tensor brain imaging on California sea lions and coyotes (Cook & Berns, 2022), and a histology study on raccoons (Lambert, 2023). Postmortem samples alone are insufficient to elucidate the temporal dynamics, functionality, network connectivity, behavioural relevance, and long-term changes of the nervous system (Terstege *et al.*, 2022; Krassner *et al.*, 2023; Koehler *et al.*, 2024). Studying animals in captivity, re-wilded ones and postmortem samples has its advantages. Yet, valuable insights also arise from studies on wild animals that have lived their entire lives in natural habitats (Hawkes *et al.*, 2021a).

Studies examining the physiology of organisms in relation to their physical and biological environments fall under the field of ecophysiology (Beaulieu, 2024). Physiological measurements of the nervous system have been limited by the need for miniature electronic devices carried by the animal. Advances in wearable technologies in medicine and sports have led to improved neurologging devices that are capable of capturing data over long distances and in various environments as they are smaller, portable, and more cost-effective (Hawkes *et al.*, 2021b). For wild animals, it is now feasible to obtain encephalogram data and information on blood flow and tissue oxygenation using near-infrared spectroscopy technology.

One challenge of field studies with wild animals is determining causality, mainly due to numerous potential confounding variables. However, laboratory studies on model organisms, which often emphasize high homogeneity in sex, age, housing, climate, diet, and ancestry, also face issues of external validity (Lambert, 2023). The heterogeneity of research subjects is common in human research, and many existing paradigms could be applied to wild animals to improve causal inference (Noel *et al.*, 2023; Salim *et al.*, 2023). Additionally, ecology has made substantial advances in this area that could benefit interdisciplinary research on the neuroscience of wild animals (Couzin & Heins, 2022; Runge, 2023).

To date, there are only three published studies that have neuroimaged live, minimally captive wild animals in their natural environment, and they are all related to

sleep. The first study used EEG to identify neural signatures of sleep in frigate birds in Galápagos National Park (Rattenborg *et al.*, 2016). Researchers had to gain proficiency with EEG of the species relevant to their behaviour, prepare for the safe capture of birds, and establish infrastructure to care for the birds' offspring temporarily. Safe and efficient surgical procedures were necessary for implanting and later removing the neurologger. To enhance data collection and measure behaviour, accelerometers and other tracking devices may also be required during capture. Another study obtained sleep EEG data from Northern elephant seals during dives in Monterey Bay, California (Kendall-Bar *et al.*, 2023). This study required regulatory approval and careful assessment of the behaviour details needed. They captured eye movements, nostril movements, facial expressions, social interactions, postures, activity levels, and vocalisations. The third study involved chinstrap penguins near King George Island, Antarctica, assessing EEG during microsleep while at sea, nesting, and when exposed to predators (Libourel *et al.*, 2023). Researchers implemented standard assessments, including computed tomography to monitor neurologger implants. A strong research design was essential, requiring thorough sampling methods beyond opportunistic sampling and direct observations to capture qualitative behavioural data effectively.

Significant advancements are needed to facilitate studies exploring the physiology of nervous systems in live wild animals. First, it is important to develop biodegradable neurologging devices capable of transmitting large travel ranges (Williams *et al.*, 2021). A major limitation is the requirement for invasive surgery to implant neural recording devices. Advances in human biomedicine have produced non-invasive versions of these devices that could be adapted for different animal species to avoid affecting behaviour. However, expanding this research faces financial constraints, particularly due to limited funding for non-traditional research models (Lambert, 2023). Increasing awareness of the value of this research in this field is crucial. This call to action is not meant to diminish the value rodent research in the lab has provided but to include other neglected species in neuroscience. Diversifying the animals studied in neuroscience could enhance the validity of results, representation of related species, and, where appropriate, translatability to human applications. For instance, despite extensive lab rodent sexual behaviour research, treatments for human sexual dysfunction remain unsatisfactory (Leeners *et al.*, 2023).

Ethics related to neuroscience studies with wild animals

The ethical considerations for conducting neuroscience studies with wild animals are extensive and require thorough review. Here, I only attempted to briefly summarise existing work. Constructing animal welfare is crucial, that has been guided by a tripartite model of welfare that includes biological function, mental state, and natural living (Colditz, 2023). This model encompasses positive health, beyond the absence of illness, and positive social interactions, beyond freedom from isolation. Additionally, animal agency refers to the capacity of animals to make choices and act both independently and in collectives, affecting their environment and well-being (Winklmayr *et al.*, 2020, 2023; Edelblutte *et al.*, 2023; Sueur *et al.*, 2023). This concept recognises animal autonomy, considering their sentience, decision-making, individuality, culture, and relationships with humans. Recent efforts aim to centre both human and non-human individuals within wildlife ethics (Orrick *et al.*, 2024)

In wild animal research, current best practices focus more on practicality than on optimizing animal welfare (Soulsbury *et al.*, 2020). To shift towards ethical research endpoints, recommendations based on refinement, reduction, and replacement (3Rs) should be implemented from the design stage to publication. An ethical assessment framework has been developed specifically for novel tools and technologies to study wild animal sociality, emphasising minimal disturbance and invasion (Gruber, 2022). Additionally, normative ethics arguments suggest focusing on beneficence, non-maleficence, autonomy, and justice (Katz, 2007). Another framework combines consequentialist moral theory, principle and rule-based approaches, and virtue ethics (Smith *et al.*, 2024). Historically, these frameworks have focused on ecological collectives and resisted anthropomorphism. However, considering animals as individuals and attributing them with behaviours such as mourning and personality traits may benefit ethical considerations (Ferraro *et al.*, 2023).

A crucial question in animal ethics is how to measure the direct and indirect effects of research on animal species. Affective states and related behaviours are typically monitored before, during, and after studies (Beaulieu, 2024). Affective states are emotional experiences that animals undergo, such as happiness, fear, or stress, and can range from short-lived emotions to longer-lasting moods. Recently, there has been a shift towards physiological measures, which can be more objective and comparable across

diverse taxa than solely measuring affective states. Genomics has been proposed as an important resource, but it requires substantial costs and resources and carries the risk of misinterpretation and overemphasis on genetic data at the expense of ecological, environmental, and demographic factors (Schmidt, Thia, *et al.*, 2024).

Adopting an ethical framework and gathering the empirical data needed to assess compliance require relevant committees to work alongside regulatory bodies and laws (Stucki, 2023; Smith *et al.*, 2024; Palmer & Greenhough, n.d.). This process involves thorough consideration of public opinion, field site stewards, and other relevant communities (Palmer & Greenhough, n.d.). It is equally important to engage with indigenous and local communities and to decolonise current approaches (John & John, 2023; Thomsen *et al.*, 2023). While these processes generally focus on the final output, they should not replace the ongoing thinking necessary to navigate ethical tensions that will arise during the research (Turnbull & Patter, 2022). A continuous dialogue with all individuals directly or indirectly involved in the research is essential.

Specific Aim 3

Sex diversity can be studied using machine learning for action recognition in camera trap datasets. Deep learning models can identify behaviours (Norouzzadeh *et al.*, 2018) and use flow-guided feature aggregation to capture temporal information in videos (Schindler & Steinhage, 2021). However, these methods require substantial training data and computing resources, with models often tailored to specific purposes. Pose estimation offers an alternative requiring less training data and is more adaptable across species and settings (Monosov *et al.*, 2024). This strategy has been used in studies employing a triple-stream deep metric learning system for behavioural recognition in great apes (Brookes *et al.*, 2023), and other endangered animals (Liu, Hou, *et al.*, 2023). Despite progress in behaviour prediction and camera trap analysis, these studies are limited to the species they were trained on and did not directly investigate sex diversity. In this study, I curated a quadruped camera trap dataset and labelled it to allow fine-tuning DeepLabCut's Model Zoo Super Animal Quadruped. This would help facilitate screening behaviours related to sex diversity in camera trap datasets. I also developed a framework for designing

neuroimaging studies of minimally captive free-behaving wildlife, ensuring rigorous and ethical research practices.

Methodology

Pose estimation of camera trap images containing sexual behaviour

Camera trap images containing sexual behaviour were searched on Google Scholar using the search terms: (“camera trap”) AND (“mating” OR “sexual behaviour” OR “copulation” OR “courtship”). Articles published after 2020 were selected so it could be easier to reach corresponding authors if the images of interest were absent. I focused on articles containing camera trap images of quadrupeds because I am using DeepLabCut’s Model Zoo Super Animal Quadruped. I identified 16 relevant images from these articles and added four more from Snapshot Kruger, found during the manual inspection of the dataset as detailed later. I found 18 additional studies mentioning relevant images, but further correspondence with authors yielded no response. Pose estimation on the images was performed using the DeepLabCut’s Model Zoo Super Animal Quadruped, hosted on the Hugging Face platform: <https://huggingface.co/spaces/DeepLabCut/DeepLabCutModelZoo-SuperAnimals> First, MegaDetector was deployed to detect the animals, followed by pose estimation. MegaDetector models md_v5a or md_v5b were used, depending on which performed better for animal detection in the image. Pose estimation results were downloaded as a JSON file, and visualisations were saved as image files.

Filtering images from camera trap datasets

I obtained MegaDetector results for the Snapshot Kruger and Wellington Camera Trap datasets from LILA BC, focusing on identifying sexual behaviours, which often involve physical interactions between animals. These interactions can be detected as overlapping MegaDetector bounding boxes in camera trap images. However, it is important to clarify that not all sexual behaviours involve physical interaction. Some behaviours can be performed individually or without physical contact, such as some forms of courtship (Roth *et al.*, 2022; Alam *et al.*, 2024; Gomes *et al.*, 2024; Lamar *et al.*, 2024; Lillvis *et al.*, 2024). To refine my dataset, I identified images containing more

than two animals with a MegaDetector threshold above 0.01 and overlapping bounding boxes. This subset of images was downloaded and re-analysed using MegaDetector md_v5b to facilitate subsequent steps in my analysis. These were completed using this code:

https://github.com/vchiang001/sexual_diversity_dissertation/blob/main/specificaim3/camera_trap_deeplabcut_quadraped.ipynb

Curating quadruped camera trap dataset

I also collected camera trap data during the 2023 African Field School on Design and Analysis of Camera Trap Studies (AFSCTS2023). Given the small dataset collected over a week, all images were processed with MegaDetector. The resulting images from all three datasets (Snapshot Kruger, AFSCTS2023, and Wellington Camera Traps) were manually inspected using the Timelapse image analysis system, an open-source tool for annotating camera trap images (Schneider *et al.*, 2020). In Timelapse, MegaDetector bounding boxes could be directly visualised using the MegaDetector JSON file. I selected images to include a range of quadrupeds: 111 images from Snapshot Kruger, 110 images from Wellington Camera Traps, and all images from AFSCTS2023. The number of images selected needed to balance a high enough number that could make meaningful contributions but not too high that would require excessive labour to process, jeopardising the deadline of this dissertation.

From the selected images, MegaDetector bounding boxes were cropped using the “Cropping bounding boxes” section of the code:

https://github.com/vchiang001/sexual_diversity_dissertation/blob/main/specificaim3/camera_trap_deeplabcut_quadraped.ipynb Crops were further selected resulting in 468 total crops: 276 crops from Snapshot Kruger, 165 from Wellington Camera Traps, and 27 from AFSCTS2023. A single-animal DeepLabCut project was created

(https://deeplabcut.github.io/DeepLabCut/docs/standardDeepLabCut_UserGuide.html)

following the format of the quadruped model that included 39 body parts: nose, upper_jaw, lower_jaw, mouth_end_right, mouth_end_left, right_eye, right_earbase, right_earend, right_antler_base, right_antler_end, left_eye, left_earbase, left_earend, left_antler_base, left_antler_end, neck_base, neck_end, throat_base, throat_end,

back_base, back_end, back_middle, tail_base, tail_end, front_left_thai, front_left_knee, front_left_paw, front_right_thai, front_right_knee, front_right_paw, back_left_paw, back_left_thai, back_right_thai, back_left_knee, back_right_knee, back_right_paw, belly_bottom, body_middle_right, and body_middle_left. Labelling was completed with the assistance of Stephen Renjie Hu. The body parts were then labelled and checked using the DeepLabCut “check labels” function to ensure labels were processed correctly.

Camera trap occupancy analysis

I obtained the Snapshot Kruger data from LILA BC. For impala occupancy, I estimated the presence or absence of impalas at each camera trap site. I calculated the effort as the number of days the camera trap was active (from the first capture date to the final capture date). I then identified the days on which impalas were captured at least once. The occupancy probability (out of 1.0) was calculated by dividing the number of days impalas were captured by the camera trap effort. In my analysis, the detection probability equals the occupancy probability because I only used one site for this calculation. For an overall occupancy estimate across all Snapshot Kruger sites, occupancy models would be needed to account for imperfect detection, using detection/non-detection records, site-specific covariates (e.g., habitat variables), and detection covariates (e.g., trap effort).

Camera trap activity analysis

I also analysed impala activity, referring to movement patterns captured by camera traps. Using the “capture_time_local” data, I organised the information into hourly temporal units. I grouped the number of impala captures for each hour and calculated the detection density by dividing this number by the total number of impala captures. I used kernel density estimation with a Gaussian function and a bandwidth smoothing of 0.5 to represent the shape of the probability density function.

Camera trap community analysis

I obtained the Snapshot Kruger information from LILA BC. To quantify the richness and evenness of species in a given area, I calculated the Shannon diversity index

for each site. This involved determining the proportion of captures for each species by dividing the number of camera trap captures of each species by the total captures across all species. The Shannon diversity index was then calculated by applying the entropy function with base-2 logarithms to these proportions. The resulting index reflects the complexity and heterogeneity of the wildlife community, ranging from 0 to between 1.5 and 4.5. Higher values indicate greater species diversity, with more species and a more even distribution of individuals. Values closer to 0 indicate low diversity, 1.5–3 moderate diversity, and above 3 high diversity.

Camera trap abundance analysis

To measure the abundance of wildlife species, I computed the relative abundance index, a standardised measure considering the number of independent captures relative to the camera trap effort. For each site, I calculated effort as the number of days the camera trap was active (from the first to the last capture date). I then recorded the number of independent capture events for impalas, spotted hyenas, and humans. The relative abundance index was calculated by dividing the number of independent captures of a species by the total camera trap effort, then multiplying by 100. Higher numbers indicate greater species abundance relative to the camera trap effort. To assess the abundance relationship between impalas, humans, and spotted hyenas, each camera trap site was treated as one data point. This allowed us to analyse how the abundance of impalas correlated with the abundance of humans and spotted hyenas across all sites.

Camera trap analysis plots

The results were visualised using the following code:

```
https://github.com/vchiang001/sexual\_diversity\_dissertation/blob/main/specificalm3/camera\_trap\_analysis\_visualisation.ipynb
```

Developing a framework to assess the feasibility and ethics for conducting sex diversity neuroimaging studies in minimally captive free-behaving wildlife species

Recent scholarly articles on human-wildlife interaction, environmental ethics, animal welfare, traditional ecological knowledge, sex variability, and sex diversity were shortlisted for detailed reading (Soulsbury *et al.*, 2020; Gruber, 2022; Colditz, 2023; Edelblutte *et al.*, 2023; Ferraro *et al.*, 2023; John & John, 2023; Massa *et al.*, 2023; Sharpe *et al.*, 2023; Sueur *et al.*, 2023; van Anders *et al.*, 2023; ASAB Ethical Committee/ABS Animal Care Committee, 2024; Beaulieu, 2024; Pape *et al.*, 2024; Smiley *et al.*, 2024; Smith *et al.*, 2024). Additional literature from these readings was reviewed to brainstorm an extensive list of factors for assessing the feasibility and ethics of conducting sex diversity neuroimaging studies on minimally captive free-behaving wildlife species. This process was undertaken in collaboration with Stephen Renjie Hu. The points for consideration we came up with were divided into ten major branches, each point presented in the form of a question to encourage additional research from the readers. My efforts to structure them as different branches and points were mainly for the purpose of easy visualisation, but many of these branches and points were actually interconnected. Each point was accompanied by a scholarly article to provide a starting point for further exploration of the topic. Additionally, the intention, characteristics, impact, and limitations were outlined for the entire framework as guidelines for how to approach this framework.

Results

DeepLabCut Model Zoo Super Animal Quadruped could perform pose estimation on camera trap images of quadrupeds, identifying behaviours related to sex diversity.

I conducted a literature search for camera trap images capturing sexual behaviours in quadrupeds to attempt pose estimation with DeepLabCut's Model Zoo Super Animal Quadruped without further training the model (zero-shot learning) (Ye *et al.*, 2024)(Figure 9A). My search yielded 35 studies meeting these criteria, but only 16 included accessible images. I reached out to researchers from the remaining studies but received no responses. Additionally, I explored Snapshot Kruger (Pardo *et al.*, 2021) and Wellington Camera Traps (Anton *et al.*, 2018) for images that may relate to sex diversity.

In images I found that may relate to sex diversity, I first used MegaDetector for animal detection and displayed results with a machine likelihood threshold above 0.1 (Figure 9B). Within the detected MegaDetector bounding boxes, I applied pose estimation and displayed poses with a confidence threshold over 0.1 (Figure 9B). The results were stored in JSON files that could be used for downstream analysis. However, pose estimation performed poorly in most cases, limiting its utility in its current form. An exception was a few images from Snapshot Kruger, where social interactions between impalas were adequately captured (Figure 9C).

Additional quadruped camera trap images labelled for future memory replay fine-tuning of quadruped model specific for camera trap purposes.

To address the suboptimal performance of DeepLabCut's Model Zoo Super Animal Quadruped on camera trap images, I explored methods to enhance its performance on camera trap images with a small number of additional training data (few-shot learning). This may be achieved through DeepLabCut's Super Animal approach, which compiles data from various datasets by first unifying the key point space between all datasets and any datasets that are later added (Ye *et al.*, 2024). One major challenge with later adding training datasets without the presence of all the data the model was previously trained on is that it loses knowledge of these previous tasks, an issue scholars termed as "catastrophically forgetting". It is generally not feasible to include all the previously trained datasets to re-train the model because it requires a tremendous amount of computational resources. To prevent catastrophic forgetting, gradients from the Quadruped Model must be masked to avoid penalising missing ground-truth data from the previous Quadruped data. In this way, the Quadruped Model's knowledge could be preserved and used as a hybrid to learn new data through a process called "memory replay fine-tuning".

This Super Animal feature was incorporated into version 3.0 of DeepLabCut released in June 2024 that enables users to directly fine-tune the Quadruped Model. Due to this release coinciding with the deadline for this dissertation, this was not included as a scope of this dissertation. In preparation for future work to use this feature, we labelled key points from three camera trap datasets: Snapshot Kruger (Pardo *et al.*, 2021), Wellington Camera Traps (Anton *et al.*, 2018), and images collected during the

AFSCTS2023. For Snapshot Kruger and Wellington Camera Traps, I used MegaDetector to filter images containing at least one animal detected above a 0.01 likelihood threshold. To identify physical interactions between animals, I further filtered for images with at least two overlapping MegaDetector bounding boxes used for animal detection. This process yielded approximately 2400 images per dataset, which I manually inspected using the Timelapse image analysis system to visualise the MegaDetector bounding boxes (Schneider *et al.*, 2020). For AFSCTS2023, I visualised all 145 images due to the smaller dataset size.

I selected images representing a diverse range of animals and cropped them to their respective MegaDetector bounding boxes above the 0.01 likelihood threshold. This resulted in 276 crops for Snapshot Kruger, 165 for Wellington Camera Traps, and 27 for AFSCTS2023, totalling 468 images. A single-animal DeepLabCut project was created using the Quadruped Model's pose-config.yaml file, and we labelled the 39 body parts accordingly (Figure 10A, B, C). This dataset can now be used for memory-replay fine-tuning the Quadruped Model for better performance on quadruped camera trap images once that feature becomes available to users shortly in the upcoming version 3.0 of DeepLabCut.

Camera trap analyses provide valuable information on species and ecosystems of interest relevant to neuroscience studies on wildlife animals.

In the Snapshot Kruger dataset, I observed potential behaviour relevant to sex diversity in images labelled “impala” (Figure 9C). To assess the feasibility of further neuroscience studies on this species, I reviewed the literature on impala sexual behaviour. During the impala rutting season, which peaks in May, impalas chase each other, emit vocalisations, defend breeding territories, and copulate (Dasmann & Mossman, 1962; Hanks *et al.*, 1976; Frey *et al.*, 2020). Camera trap datasets focused on specific species can establish behavioural patterns and inform research decisions. If no datasets are available for a species, neuroscientists can collaborate with camera trap experts to design suitable studies.

Camera trap datasets also provide insights into species occupancy, the likelihood of a species being present in a given area (Kays *et al.*, 2020) My analysis of the Snapshot Kruger dataset established five sites where impala occupancy probability exceeded 0.3:

site 7, site 13, site 16, site 41, and site 49 (Figure 11A). For neuroimaging studies at Kruger National Park, these sites could be ideal for observing impala behaviour. Time of day is another crucial variable; camera trap datasets can identify when animals are most active (Delisle *et al.*, 2021). In the Snapshot Kruger dataset, the data showed that impalas were most active between 12 p.m. and 4 p.m. (Figure 11B).

Conducting wildlife studies necessitates a comprehensive understanding of the ecosystem, including the community containing the target species (Kays *et al.*, 2020). The Shannon diversity index measures species abundance and evenness. In the Snapshot Kruger dataset, five sites had an index below 1.5: site 8, site 10, site 14, site 16, and site 45 (Figure 11C). Removing impalas from these sites could significantly impact biodiversity, so researchers should evaluate this risk carefully. A thorough literature review can help identify species dependent on impalas. For example, spotted hyenas rely on impalas as a primary food source (Vissia *et al.*, 2021; Braczkowski *et al.*, 2022). However, the data showed no correlation between impala and spotted hyena abundance in the Snapshot Kruger dataset ($R^2 = 0.00000$, Figure 11D). Additionally, there was a weak negative correlation between impala abundance and human presence ($R^2 = 0.00550$, Figure 11D), suggesting a modest reduction in impala numbers with increased human activity.

Framework for assessing the feasibility and ethics of conducting sex diversity neuroimaging studies on minimally captive free-behaving wildlife animals.

When planning neuroimaging studies on wildlife species, it is essential to consider species with behaviours relevant to sex diversity and obtain comprehensive information about these species. A detailed framework addressing feasibility and ethical considerations is crucial. To formalise this framework, I reviewed recent relevant articles as a starting point to synthesise a broad framework (Soulsbury *et al.*, 2020; Gruber, 2022; Colditz, 2023; Edelblutte *et al.*, 2023; Ferraro *et al.*, 2023; John & John, 2023; Massa *et al.*, 2023; Sharpe *et al.*, 2023; Sueur *et al.*, 2023; van Anders *et al.*, 2023; ASAB Ethical Committee/ABS Animal Care Committee, 2024; Beaulieu, 2024; Pape *et al.*, 2024; Smiley *et al.*, 2024; Smith *et al.*, 2024).

I identified ten major thematic branches (Figure 12). These themes encompassed overlapping factors, highlighting the intricate connections between various ethical and

practical aspects. The figure provided a high-level overview of my framework that was zoomed in to the more elaborate framework that may not be clear enough to see in the format embedded in this dissertation. For the high-resolution version, I refer readers to this link:

https://github.com/vchiang001/sexual_diversity_dissertation/blob/main/specificaim3/wildlife_neuroscience_framework.png This resource is intended to be dynamic and regularly updated by the community, recognising that there is no universal or static pipeline in conducting neuroimaging studies in wildlife animals. The framework aims to catalyse constructive discussions, ensuring researchers consider complex ethical, scientific, and logistical implications before starting these studies.

Considerations include strategies to encourage voluntary participation from wildlife subjects while ensuring ethically sound and scientifically robust study designs (Sueur *et al.*, 2023). These studies benefit from inclusive principles rooted in moral philosophy, emphasising research ethics, conservation discourse from Buddhist perspectives, and integrating diverse knowledge systems beyond Western science (Cairns, 2024). Such perspectives advocate for the well-being of wildlife subjects and challenge traditional ideologies of human dominion over animals, transforming them from objects to research partners and fostering a collaborative approach (Nustad & Swanson, 2022). This reframing requires responsible study design that prioritises transparency, open science principles, and animal-centred decision-making (Pichler & Hartig, 2023). The key to achieving these aims is implementing biosecurity measures to prevent illness transmission among animals, researchers, and local communities (Reaser *et al.*, 2024). Additionally, the adaptive agency of wildlife, informed by lived experience and social learning, must be considered in terms of how their agency shape shared human cultures and spaces (Galesic *et al.*, 2023). Researchers must also ensure that data collection, analysis, and modelling are conducted using explainable, responsible AI that adheres to reproducibility and ethical research standards.

Interacting measures of sex across phenotypic, sociocultural, and ecological levels need thorough consideration (Massa *et al.*, 2023). Variations across time and context require careful research design. Integrating neuroimaging with behavioural data can offer insights into animal collectives, communication, and behavioural development across timescales, from proximal interactions to broader eco-evolutionary perspectives (Ioannou

& Laskowski, 2023). The success of such projects depends on fair and safe employment conditions for all personnel (Anagnostou *et al.*, 2022), including supporting indigenous communities in building their research capacity and fostering long-term collaborations (Claw *et al.*, 2018). By learning from their expertise without appropriation, researchers can enrich the scope of wildlife neuroimaging studies.

Discussions

In this study, I aimed to enhance the accuracy and utility of wildlife behavioural analysis by curating images of quadrupeds from three camera trap datasets. I labelled quadruped body parts in the camera trap images that could fine-tune the DeepLabcut Model Zoo Super Animal Quadruped for improved pose estimation performance on camera trap images in the near future. I also established a framework for designing rigorous and ethical neuroimaging studies on minimally captive free-behaving wildlife. This framework was divided into ten primary branches, each encompassing ten specific consideration points referencing selected articles related to the topic.

Emerging research has begun identifying behaviours in camera trap images (Norouzzadeh *et al.*, 2018; Schindler & Steinhage, 2021; Brookes *et al.*, 2023; Liu, Hou, *et al.*, 2023). Recent advancements in large vision-language models, such as CLIP, trained on millions of image-caption pairs, recognise specific behaviours from images (Gabeff *et al.*, 2024). WildCLIP has fine-tuned CLIP for behaviour recognition in camera trap images, which is beneficial as camera traps are rarely annotated beyond species-level identification. Dussert *et al.*, 2024 applied WildCLIP and similar models to predict behaviours like eating, moving, standing, and interacting. My strategy, however, uses pose estimation rather than language-based models for behaviour recognition.

Pose estimation offers some advantages over vision-language models for behaviour identification. It provides clearer interpretability by directly linking specific body poses with behaviours, allowing us to more easily troubleshoot why certain sex diversity-related behaviours were not recognised. Vision-language models often lack interpretability, making it challenging to refine the strategy to recognise sex diversity-

related behaviours (Li *et al.*, 2022; Salin *et al.*, 2023; Chen, Li, Dong, *et al.*, 2024). These models can also be limited by biases in training data that would require extensive data on sex diversity to expand beyond this bias. In contrast, pose estimation can be more feasible for incorporating community-informed approaches to generalise pose relationships related to sex diversity across species. Additionally, pose estimation captures spatiotemporal relationships that are important for behavioural dynamics.

After refining the DeepLabCut model for camera trap images, a detailed portfolio of poses associated with sex diversity could be generated. These pose relationships will help filter images using pose-based behaviour recognition tools. Neuroscientists can then manually inspect these images to identify behaviours of interest before conducting further analyses. My framework will guide the selection of species and behaviours suitable for neuroimaging studies, providing guidelines to ensure rigorous and ethical research practices.

Despite the potential of this approach, I recognise certain limitations. The number of images, species, and pose labels might not generalise across various camera trap datasets. Determining the performance after fine-tuning the Quadruped Model will help determine the extent of this limitation. An additional limitation was that my framework is based on existing written scholarly literature that could be accessed on scholarly databases without engaging with the community.

Expanding the range of species studied in neuroscience allows for more nuanced evolutionary interpretations of the nervous system, including those related to sex diversity (Cisek & Hayden, 2022). Emphasising sex diversity in neuroscience further underscores the importance of biodiversity, including diverse species and their behaviours (Mathuru *et al.*, 2020). Moreover, inquiries into sex diversity could contribute to conservation efforts by improving the fertility of animal species and focusing on their overall well-being (Whiten, 2021; Kappeler *et al.*, 2023; Gross *et al.*, 2024). Sex has also been observed as an important factor when determining what conservation strategies to adopt (Gissi *et al.*, 2024). Observable sex-related measures in sentinel species would also serve as early warning indicators of environmental changes (Hazen *et al.*, 2024).

Summary and Conclusions

In summary, my study provided a labelled dataset of camera trap images that could be used to fine-tune the DeepLabCut Model Zoo Super Animal Quadruped. This enables a pose-estimation-based strategy for behaviour recognition to screen for sex diversity in camera trap datasets. My framework supports the design of neuroimaging studies beyond laboratory environments, ensuring research rigour and ethics for behaviours and species relevant to sex diversity that could advance the conceptualisation of sex. These advancements could contribute to the evolutionary construction of how the nervous system interacts with sex diversity, enhancing biodiversity research and supporting global conservation efforts.

Recommendations

The immediate next step is to fine-tune the DeepLabCut Model Zoo Super Animal Quadruped Model, a feature that was just made available in version 3.0 of DeepLabCut, released in June 2024. Due to this release coinciding with the deadline for this dissertation, this was not included as a scope of this dissertation. This feature incorporates functions like memory replay to prevent catastrophic forgetting, where models forget previously learned data (Ye *et al.*, 2024). After successful pose estimation, a comprehensive pose relationship dataset will be generated from camera trap images containing sex diversity-related behaviours in wildlife. Collaboration with communities is crucial to synthesise a thorough list of behaviour patterns relevant to sex diversity beyond the limited understanding from scientists (Monk *et al.*, 2019b). The respective pose relationships could then be applied more robustly to various camera trap datasets to screen for sex diversity.

Recognising that behaviours are often transitional and vary over time, future strategies must consider the temporal adjacency of poses to capture these nuances in consecutive images (Calhoun & Hady, 2023). By integrating large vision-language models like WildCLIP with pose estimation, a more comprehensive behavioural recognition approach can be developed. Images obtained from each approach could then be used as additional training data for the other. These AI approaches also need to be

made more accessible to users with limited technical backgrounds like what has been done for Pytorch-Wildlife recently (Hernandez *et al.*, 2024).

Beyond pose estimation, designing rigorous and ethical neuroimaging studies requires interdisciplinary collaboration with ecologists, Indigenous Peoples, and other relevant communities (Chaudhury & Colla, 2021; Green *et al.*, 2023; John & John, 2023). This integrated strategy will enable researchers to construct a more robust, multidisciplinary approach to explore the relationship between the nervous system and sex diversity. While the automation of animal behavioural analysis has been celebrated, the societal implications of this change also need to be thoroughly considered (Trappes, 2023).

Figures & Legends

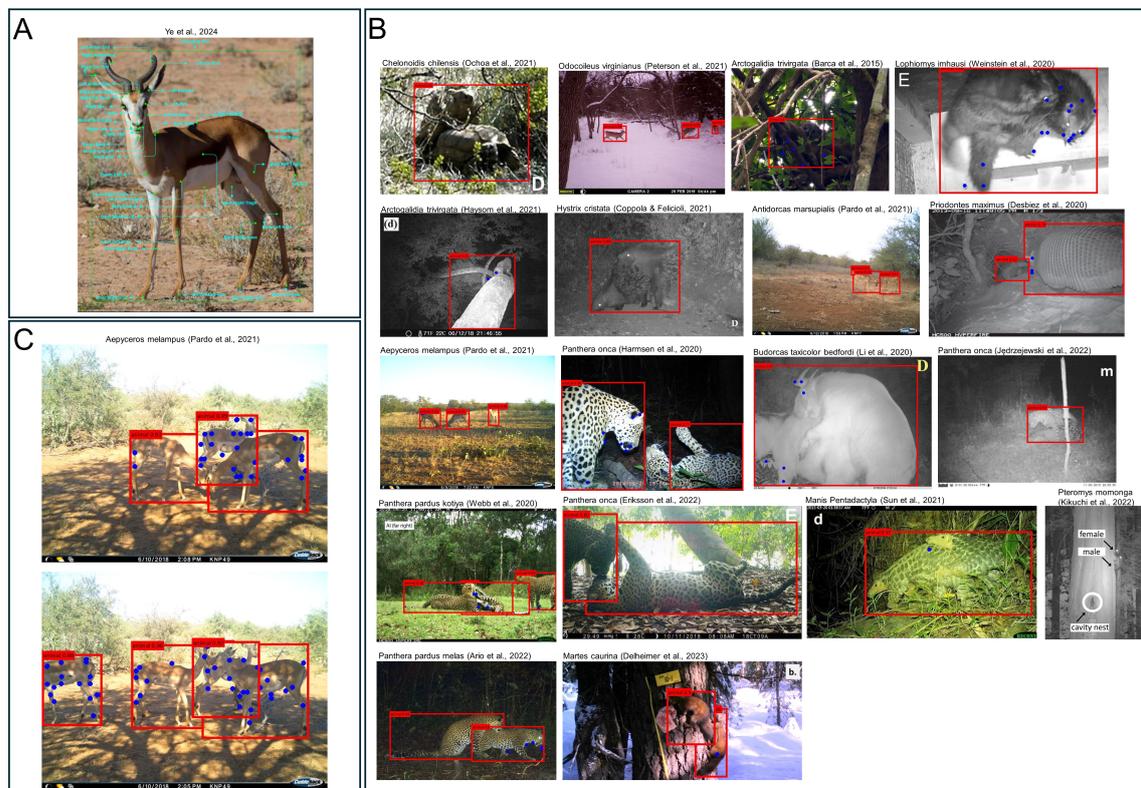


Figure 9 DeepLabCut Model Zoo Super Animal Quadruped could perform pose estimation on camera trap images of quadrupeds relating to sex diversity.

A) Example of a labelled image from DeepLabCut Model Zoo Super Animal Quadruped, showing the different body parts included (Ye *et al.*, 2024). B) Camera trap images that contained sexual behaviour between quadrupeds (Barca *et al.*, 2015; Desbiez *et al.*, 2020; Harmsen *et al.*, 2020; Li *et al.*, 2020; Webb *et al.*, 2020; Weinstein *et al.*, 2020; Coppola & Felicioli, 2021; Haysom *et al.*, 2021, 2021; Ochoa *et al.*, 2021; Peterson *et al.*, 2021; Sun *et al.*, 2021; Ario *et al.*, 2022; Eriksson *et al.*, 2022; Jędrzejewski *et al.*, 2022; Kikuchi *et al.*, 2022; Delheimer *et al.*, 2023). Pose estimation was performed on all images. MegaDetector animal detections are shown in bounding boxes, including the confidence of each detection. Pose estimation within each bounding box was carried out using the DeepLabCut Model Zoo Super Animal Quadruped. Poses detected with a likelihood threshold greater than 0.1 are indicated by blue dots. C) Example of pose estimation in Snapshot Kruger camera trap images containing social behaviour between

impalas. MegaDetector animal detections are shown in bounding boxes, including the confidence of each detection. Pose estimation within each bounding box was performed with DeepLabCut Model Zoo Super Animal Quadruped, with poses detected above the 0.1 likelihood threshold shown as blue dots.

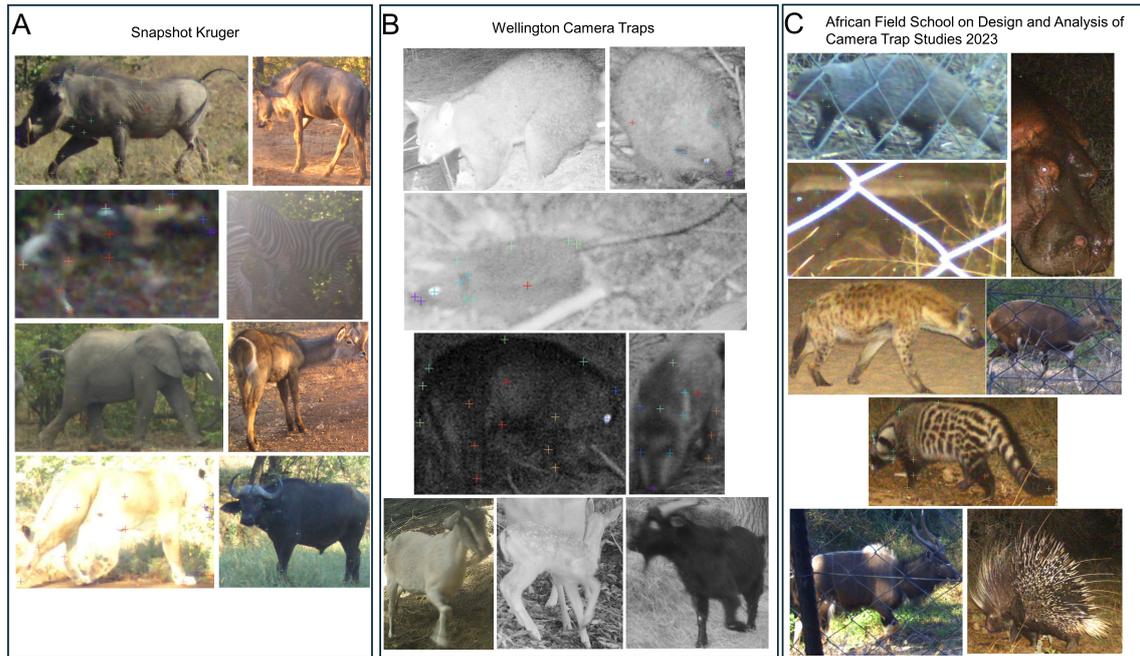


Figure 10 Additional quadruped camera trap images labelled for future memory replay fine-tuning of DeepLabCut Model Zoo Super Animal Quadruped specific for camera trap purposes.

A) Examples of selected camera trap MegaDetector animal detection crops from Snapshot Kruger (Pardo *et al.*, 2021) labelled with body parts for the DeepLabCut Model Zoo Super Animal Quadruped. B) Examples of selected camera trap MegaDetector animal detection crops from Wellington Camera Traps (Anton *et al.*, 2018) labelled with body parts for the DeepLabCut Model Zoo Super Animal Quadruped. C) Examples of selected camera trap MegaDetector animal detection crops from the African Field School on Design and Analysis of Camera Trap Studies 2023, labelled with body parts for the DeepLabCut Model Zoo Super Animal Quadruped.

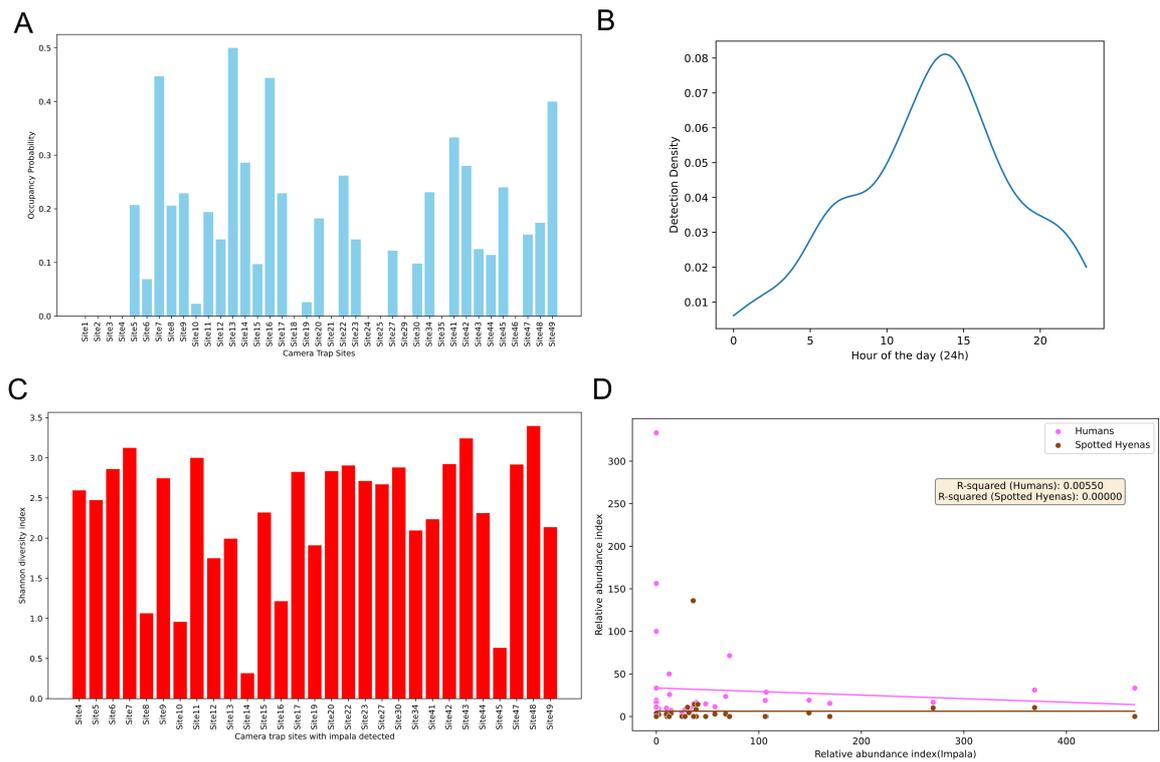


Figure 11 Camera trap analyses provide valuable information on species and ecosystems of interest relevant to neuroscience studies on wildlife animals.

A) Occupancy probability of impalas across sites in Snapshot Kruger. B) Detection density of impalas across hours of the day. C) Shannon diversity index of camera sites with impalas detected in Snapshot Kruger. D) Correlation of the relative abundance index of humans and spotted hyenas with the relative abundance index of impalas.

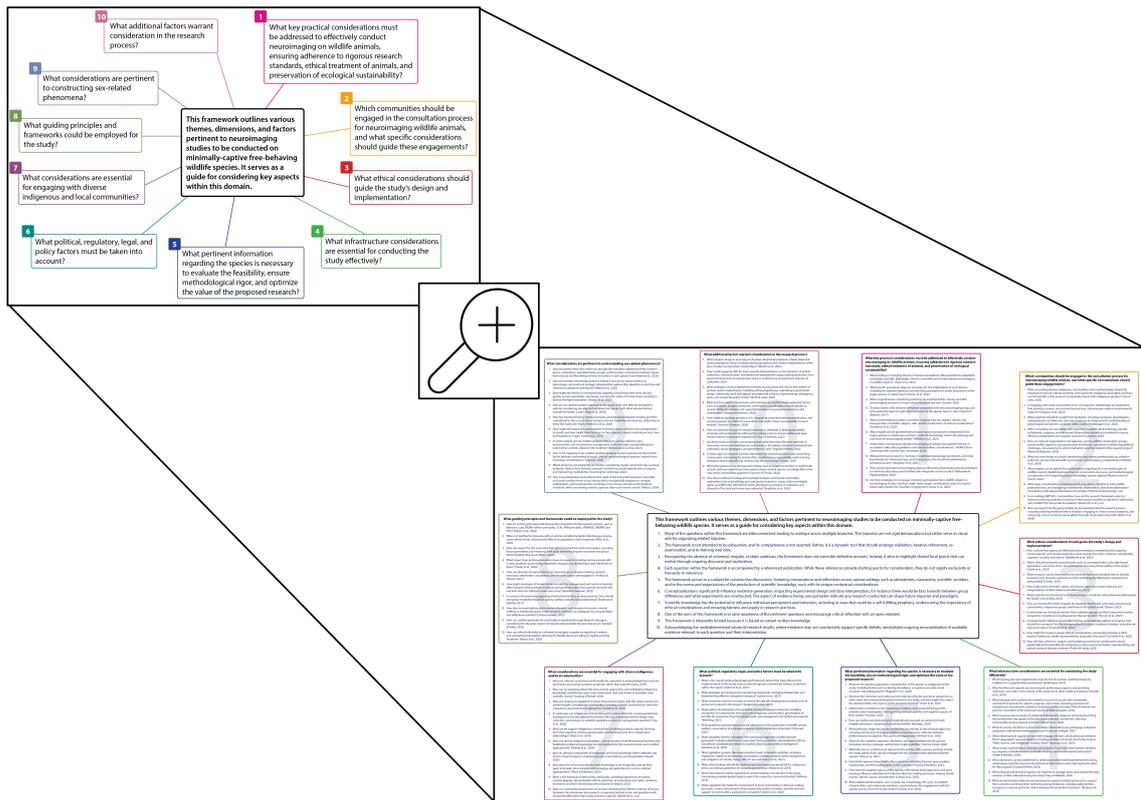


Figure 12 Framework for assessing the feasibility and ethics of conducting sex diversity neuroimaging studies on minimally captive free-behaving wildlife animals.

Framework for assessing the feasibility and ethics of conducting sex diversity neuroimaging studies on minimally captive free-behaving wildlife animals with 10 major branches. The full version is available at:

https://github.com/vchiang001/sexual_diversity_dissertation/blob/main/specifcaim3/wildlife_neuroscience_framework.png Both images were generated by Stephen Renjie Hu.

What key practical considerations must be addressed to effectively conduct neuroimaging on wildlife animals, ensuring adherence to rigorous research standards, ethical treatment of animals, and preservation of ecological sustainability? (Mancini, 2017; Soulsbury *et al.*, 2020; Cramer *et al.*, 2021; Hampton *et al.*, 2021; Shen *et al.*, 2021; Williams *et al.*, 2021; Gruber, 2022; Sueur *et al.*, 2023; Watanabe & Papastamatiou, 2023; ASAB Ethical Committee/ABS Animal Care Committee, 2024) Which communities should be engaged in the consultation process for neuroimaging wildlife animals, and what specific considerations should guide these engagements? (Adler *et al.*, 2020; Masset *et al.*, 2020; Chagnon *et al.*, 2022; Goldbogen *et al.*, 2023; John & John, 2023; Lambert, 2023;

Willette *et al.*, 2023; Cairns, 2024; Alexander *et al.*, n.d.; Palmer & Greenhough, n.d.) What ethical considerations should guide the study's design and implementation? (Carvalho *et al.*, 2023; Colditz, 2023; Dubova & Goldstone, 2023; Edelblutte *et al.*, 2023; Ferraro *et al.*, 2023; Gewin, 2023; Pichler & Hartig, 2023; Urai & Kelly, 2023; Donovan *et al.*, 2024; Smith *et al.*, 2024) What infrastructure considerations are essential for conducting the study effectively? (Fiorello *et al.*, 2016; Sikes & Animal Care and Use Committee of the American Society of Mammalogists, 2016; Patter & Blattner, 2020; Coleman & Wight, 2021; Couzin & Heins, 2022; Berizzi *et al.*, 2023; Jennings *et al.*, 2023; Kays & Wikelski, 2023; ASAB Ethical Committee/ABS Animal Care Committee, 2024; Reaser *et al.*, 2024) What pertinent information regarding the species is necessary to evaluate the feasibility, ensure methodological rigour, and optimize the value of the proposed research? (Ghai *et al.*, 2021; Shaw *et al.*, 2021; Colditz, 2023; Ehlman *et al.*, 2023; Galesic *et al.*, 2023; Kappeler *et al.*, 2023; Lasky & Bombaci, 2023; Beaulieu, 2024; Grimm, 2024; Smiley *et al.*, 2024) What political, regulatory, legal, and policy factors must be taken into account? (Sikes & Animal Care and Use Committee of the American Society of Mammalogists, 2016; Sellheim, 2018; Webb *et al.*, 2019; Grorud-Colvert *et al.*, 2021; Lawson *et al.*, 2021; Osborne *et al.*, 2021; Anagnostou *et al.*, 2022; Green *et al.*, 2023; Nandutu *et al.*, 2023; Wynberg, 2023) What considerations are essential for engaging with diverse indigenous and local communities? (Carey, 2015; Claw *et al.*, 2018; Ludwig & Poliseli, 2018; Yua *et al.*, 2022; Lamb *et al.*, 2023; Martin, 2023; Black & Tylianakis, 2024; Molnár *et al.*, 2024; Nepal, 2024; Werdel *et al.*, n.d.) What guiding principles and frameworks could be employed for the study? (Knutsson & Munthe, 2017; Franks *et al.*, 2020; Yerbury & Yerbury, 2021; Boyce *et al.*, 2022; Nustad & Swanson, 2022; Petkov *et al.*, 2022; Turnbull & Patter, 2022; Calhoun & Hady, 2023; Keij & van Meurs, 2023; Bliss *et al.*, 2024) What considerations are pertinent to constructing sex-related phenomena? (Benvenuto & Lorenzi, 2023; Gouvêa, 2023; Massa *et al.*, 2023; van Anders *et al.*, 2023; Aghi, Anderson, *et al.*, 2024; Aghi, Goetz, *et al.*, 2024; Cunningham & Benítez, 2024; Pape *et al.*, 2024; Smiley *et al.*, 2024; Velocci, 2024) What additional factors warrant consideration in the research process? (Mancini, 2017; Vannini & Vannini, 2020; Green, 2021; Gore & Bennett, 2022; Sommer & Ferraro, 2022; Brink & Lewis, 2023; Ioannou & Laskowski, 2023; Singleton *et al.*, 2023; Westlin *et al.*, 2023; Aguilar & Webb, 2024).

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CHAPTER 5

STEM CELL ENGINEERING OF HUMAN SEBOCYTES FOR STUDYING CELLULAR ASPECTS OF HUMAN SEX VARIABILITY

Introduction

Cellular, molecular and genetics related to human sex

Several reviews cover the cellular, molecular, and genetic research related to mammalian sex (Mullen & Behringer, 2014; Zambrano *et al.*, 2014; Arnold, 2017). Recent studies continue to explore sex at this level, including the development of the Wolffian and Müllerian ducts, and cell types such as Sertoli and Leydig cells (Alves *et al.*, 2023; Jia & Zhao, 2023; Kimura *et al.*, 2023; Souali-Crespo *et al.*, 2023; Kato *et al.*, 2024; Stocker *et al.*, 2024; Stévant *et al.*, 2024). However, much of this research has been conducted on rodents and immortalised cell lines, which may limit its relevance to humans.

Several conditions medicine has pathologized for human gonadal development have provided insights into some of the cellular, genetic, and molecular aspects of human sex. In congenital adrenal hyperplasia, XX individuals possess a type of steroidogenesis-related genes that may present ambiguous genitalia (Zhou *et al.*, 2022). DNA sequencing of peripheral blood leukocytes from these individuals has identified relevant genes. In Klinefelter syndrome, an extra X chromosome results in the XXY genotype and may reduce fertility (Mahyari *et al.*, 2021). Single-cell RNA sequencing (scRNA-seq) of testis biopsies from individuals with Klinefelter syndrome established 72 up-regulated

molecular pathways. In complete androgen insensitivity syndrome, XY individuals possess types of the androgen receptor gene that may manifest a vaginal opening (Zhou *et al.*, 2022). Whole exome sequencing of blood from these individuals established relevant mutations. In Turner syndrome, individuals have only one X chromosome or a structurally altered second X and may exhibit changes to puberty (Suntharalingham *et al.*, 2023). Whole exome sequencing of leukocytes from individuals with Turner syndrome established autosomal TIMP3 variants.

Despite progress in constructing the cellular, genetic, and molecular aspects of human sex through the study of disorders, this approach has limitations. First, the sample sizes may be small since medicine has not observed a high prevalence of these conditions (Imai *et al.*, 2013; Emmerich *et al.*, 2021; Mezinska *et al.*, 2021). Additionally, ethical considerations in accessing biological samples and conducting rigorous experiments on these individuals make causative explanations challenging. To overcome these limitations, *in vitro* models relevant to human physiology are essential for studying human sex variability (Kim, Koo, *et al.*, 2020; Marton & Paşca, 2020; Haniffa *et al.*, 2021; Ingber, 2022). These models can simulate developmental processes, provide accessible and scalable screening for molecular targets, enable longitudinal, real-time, high-resolution monitoring, and allow for controlled and reproducible experimental manipulation.

Skin is an important part of sex

The skin is often affected by gonadal development disorders, including webbed neck in Turner syndrome (Mohd Said *et al.*, 2023), acne in congenital adrenal hyperplasia (Hu, Wei, *et al.*, 2021), sparse pubic and axillary hair in androgen insensitivity syndrome (Lee, Kim, *et al.*, 2023), as well as mutated keratinocytes in patients with 46XX disorder of gonadal development (Dellambra *et al.*, 2022). Additionally, the skin exhibits sexual heteromorphism, such as in melanocyte stem cell behaviour and dermis in rodents (Prigent *et al.*, 2023; An *et al.*, 2024). Similar sexual heteromorphism has been observed in human samples (Lopes-Ramos *et al.*, 2020; Oliva *et al.*, 2020). While these differences cannot be solely attributed to gonadal development, emerging studies suggest that early-life hormonal programming can alter skin phenotypes. For instance, androgens influence

the skin before birth, and the skin on the genitalia undergoes significant changes during puberty (Rey, 2021; Lee, Kim, *et al.*, 2023).

In addition to studies linking gonadal development to the skin, the skin is also crucial in sexual behaviour. Human attractiveness is often judged based on skin appearance (Sakano *et al.*, 2021). The skin also contributes to sexual behaviour through the production of body odour (Ferdenzi *et al.*, 2020). Furthermore, touch, mediated through the skin, plays a significant role in human sexual behaviour (Elias *et al.*, 2023). Studies can incorporate these aspects of skin to better address sex variability, as well as examine how sexual characteristics, function, and behaviour develop in relation to the visual, olfactory, and tactile features of the skin (Frost *et al.*, 2022).

Major cell types in human skin include fibroblasts, basal cells, hair follicle cells, melanocytes, spinous cells, granular cells, endothelial cells, immune cells, sebocytes, keratinocytes, and pericytes (Kim, Chung, *et al.*, 2020; Zou *et al.*, 2021; Hailer *et al.*, 2023; Ma *et al.*, 2023; Zhou, Liang, *et al.*, 2023). Sebocytes are particularly relevant to human sex due to their role in gonadal steroid hormone accumulation and metabolism (Zouboulis *et al.*, 2022). Sebocytes, part of the sebaceous glands, produce sebum, which consists of lipids, antimicrobial compounds, and antioxidants (Güell & Schneider, 2023; Schmidt, Hansmann, *et al.*, 2024). Studies have established sexual heteromorphism in humans related to sebocytes, including differences in sebum production, hormone levels, and sebaceous gland size (Giacomoni *et al.*, 2009; Dahl *et al.*, 2018; Pineider, J; Eckert, K; McDonald, J; Harris-Tryon, T, 2021; Ceruti, JM; Leiros, GJ; Balana, ME, n.d.).

Sebocytes cross-communicate with the nervous system

The nervous system is well-established to undergo development related to sex (McCarthy, 2023; Kundakovic & Tickerhoof, 2024; Lafta *et al.*, 2024). However, it is important to note that the complex gene-environment interactions resulting in sexual heteromorphism in different brain regions do not support sex and gender essentialism (Donovan *et al.*, 2024). Many scholars have argued that human sex should not be reduced to a simple binary and that sociocultural factors play a critical role in shaping observed sex variability, rather than biology alone (Anderson & Falk, 2023; Danos *et al.*, 2023; Massa *et al.*, 2023; Sharpe *et al.*, 2023; Laine *et al.*, 2024, 2024; Smiley *et al.*, 2024;

Alexander *et al.*, n.d.). Additionally, historical discrimination and biases should be acknowledged for their impact on how human sex variability research shifted over time.

The skin, being the largest organ in the human body, interacts with the nervous system by responding to neurohormones, neuropeptides, and neurotransmitters through intra-, juxta-, para-, and autocrine pathways (Ramot *et al.*, 2021; Slominski *et al.*, 2022). The skin is also a crucial social organ, receiving information from social partners, including during sexual behaviour (Morrison *et al.*, 2010). The nervous system processes this information, such as during face perception and receiving olfactory signals emitted from the skin (Ferdenzi *et al.*, 2020; Hofer *et al.*, 2020; Damon *et al.*, 2021; Kondo & Hayashi, 2021; González-Álvarez & Sos-Peña, 2022; Hadders-Algra, 2022). Additionally, the skin plays a key role in social and affiliative touch, involving bidirectional neural communication during the act of touch and receiving touch from a social partner (Crucianelli & Ehrsson, 2023; Suvilehto *et al.*, 2023; Stevens *et al.*, 2024). Skin is also an important part of the mammalian genitalia, which is covered by sensory neurons that form the somatosensory end organ, Krause corpuscles (Qi *et al.*, 2024). Vibrotactile signals that emanate from the Krause corpuscles are projected to the dorsal grey commissure part of the spinal cord.

Sebocytes are rarely considered for their communication with the nervous system, yet evidence points to their vital role in neuroendocrinology (Clayton *et al.*, 2020). For example, sebum production is heavily regulated by growth hormones and androgens, which can originate from the nervous system. These growth hormones affect insulin growth factor 1 production in hepatocytes, which stimulates sebocytes (Okoro *et al.*, 2023). Additionally, neurohormones such as adrenocorticotrophic hormone and α -melanocyte-stimulating hormone influence sebocyte homeostasis (Clayton *et al.*, 2020). Sebocytes not only respond to neuroendocrine signals but also synthesise and release neurohormones. The nervous system interacts with sebocytes through cutaneous nerve fibres that release neuropeptides and neurotransmitters (Yang *et al.*, 2023). Receptors for neuropeptides Y2 and Y5 are highly expressed in sebaceous glands.

Roles of transposable elements in sex and sebocytes

Transposable elements (TEs) are increasingly recognised for their role in sex-related processes (Chiang, DeRosa, *et al.*, 2022). TEs, originating from other organisms or viruses, constitute nearly 50% of the mammalian genome and can mobilise to new genomic locations (Lawlor & Ellison, 2023). They may interact antagonistically or cooperatively with host genomes. TEs move through either a "cut and paste" mechanism (DNA transposons) or a "copy and paste" mechanism (retrotransposons) (Le Breton *et al.*, 2024). Retrotransposons are further divided into long terminal repeats (e.g. human mouse mammary tumour virus like-2) and non-long terminal repeats (e.g. long- and short-interspersed nuclear elements, LINE and SINE, respectively). LINES are autonomous, containing all necessary machinery for retrotransposition, whereas SINEs (e.g. Alu and SINE-VNTR-Alu) are not.

Some TEs have been exapted into the host genome, meaning they have adapted to perform functions different from their original roles (Gebrie, 2023). TEs can regulate gene expression at both transcriptional and post-transcriptional levels through several mechanisms. They account for 20% of cis-regulatory elements in the human genome, crucial for controlling nearby genes. These elements include promoters that start transcription, silencers that inhibit transcription, enhancers that increase transcription, and transcription factor binding sites. Additionally, some non-coding RNAs, such as microRNAs and long non-coding RNAs, may derive from TEs. MicroRNAs degrade or inhibit the translation of mRNA, while long non-coding RNAs act as scaffolds for protein complexes and guide chromatin-modifying complexes. Recently, TEs have also been established to shape the three-dimensional genome architecture (Lawson *et al.*, 2023).

Under certain conditions, TEs can be deleterious to the host, disrupting normal functions. Consequently, the host has developed pathways to regulate TEs (Lawlor & Ellison, 2023). These pathways include silencing TEs through piwi-interacting RNAs and DNA methylation and suppressing TE activities via mechanisms involving apolipoprotein B mRNA editing enzyme, catalytic subunit 3G, and Kruppel-associated box zinc fingers. Other pathways involve the formation of heterochromatin and histone modifications that silence TEs (Gebrie, 2023). Recently, scaffold attachment factor B proteins have been shown to play a dual role in preventing the retrotransposition of LINE1 and the exonisation of previously integrated TEs (Ilik *et al.*, 2024).

Advancements in gene-editing have facilitated the study of TEs (Feschotte, 2023; Han *et al.*, 2023). Recent reviews highlight their pivotal roles in cancer, ageing, and early human development (DiRusso & Clark, 2023; Yushkova & Moskalev, 2023; Liang *et al.*, 2024). Substantial evidence also underscored TEs' involvement in sex (Chiang, DeRosa, *et al.*, 2022). TEs were hypothesised to have given rise to sexual reproduction as a means of propagation (Daskalova, 2023). They tend to transpose and express in germ cells, ensuring their transmission to offspring. Additionally, piwi-interacting RNAs, which silence TEs, are speculated to originate in the gonads, balancing the expression of exapted TEs integral to gametogenesis and gamete function. TEs are abundant in X and Y chromosomes as well as gonadal-determining regions, which have been suggested to play a role in the differentiation of these chromosomes (Srikulnath *et al.*, 2022; Makova *et al.*, 2024).

Recent evidence continues to support TEs' role in sex. For instance, TEs have been studied in the W chromosome of the willow beauty moth, which belongs to the ZW gonadal determination system observed in various taxa (Hejníčková *et al.*, 2023). The study showed that the W chromosome is rich in TEs, supporting the idea that TEs drive ZW chromosome differentiation. In Medaka fish, TEs were expressed differently in the testes compared to the ovaries and clustered with sex-biased genes (Dechaud *et al.*, 2021). In mosquito fish, the W chromosomes were enriched with TEs, which actively differentiated the W chromosome (Müller *et al.*, 2023). In the Eurasian crow, the W chromosome was replete with active TEs, which were proposed to drive sex variability in the fitness of the crows (Warmuth *et al.*, 2022). In mammals, developing gametes in mice testes expressed genes derived from Ty3/Gypsy, a type of long terminal repeat TE (Laureau *et al.*, 2021). Similarly, in mice testes, gonadal determination was mediated by the expression of TEs in a time- and sex-related manner (Stévant *et al.*, 2024).

Recent studies have established possible relationships between TEs and sebocytes. One study established PIWI proteins to be strongly expressed in sebaceous glands (Pammer *et al.*, 2020). Specifically, mature sebocytes in human skin exhibited strong and consistent expression of piwi-like protein 2, unlike undifferentiated cells in the sebaceous glands, which showed weak or no expression. Similarly, in mice, sebaceous glands expressed piwi-like protein 2, which was thought to be involved in sebocyte differentiation. Another study established that in patients with squamous cell carcinoma

or basal cell carcinoma, LINE1 was expressed in sebocytes, possibly related to age (McKerrow *et al.*, 2023). Human endogenous retrovirus (HERV), a type of non-autonomous retrotransposon TE, was implicated in hepatocellular carcinomas (Chang *et al.*, 2023). In Taiwanese patients with hepatocellular carcinomas, different HERV levels were used to classify survival rates. Sebocyte quantities were higher in the group with low survival-related HERV activation compared to the high survival-related group. Given the role of sebocytes in sex, it is of interest how TEs may be relevant.

Sebocyte cell models

Several *in vitro* models have been created to study sebocytes, including the immortalised cell lines: SEB-1, Seb-E6E7, SEBO662, and SZ95 (Zouboulis, Schagen, *et al.*, 2008; Schneider & Zouboulis, 2018; Zouboulis *et al.*, 2020; Geueke & Niemann, 2021). The most widely used sebocyte cell line is SZ95, which has been applied in many recent studies (Footner *et al.*, 2023; Gao *et al.*, 2023; Kovács *et al.*, 2023; Liu, Luo, *et al.*, 2023; Su, Zouboulis, *et al.*, 2023; Zouboulis, Hossini, *et al.*, 2023; Kleemann *et al.*, 2024; Wei *et al.*, 2024; Zhang, Zouboulis, *et al.*, 2024). While most studies use two-dimensional cultures, three-dimensional models have also been developed. These include culturing SZ95 as spheroids, developing mouse sebaceous gland organoids from Blimp1+ epithelial cells, and creating 3D-SeboSkin, a co-culture of SZ95 with skin explants (Zouboulis *et al.*, 2020; Geueke & Niemann, 2021; Abdel-Naser *et al.*, 2023; Tang *et al.*, 2023; Zouboulis, Hou, *et al.*, 2023). Researchers have also turned to primary cultures of sebocytes, directly from freshly isolated tissues or organs (Forraz *et al.*, 2023; Kitajima *et al.*, 2023; Veniaminova *et al.*, 2023). However, cell lines and primary cultures present limitations, including finite expansion capacity, genomic instability, phenotypic heterogeneity, limited source material, and the inaccessibility of some tissues (Geraghty *et al.*, 2014; Antoni *et al.*, 2015; Horvath *et al.*, 2016; Kim, Koo, *et al.*, 2020; Richter *et al.*, 2021). These models often fail to replicate physiologically relevant conditions, potentially due to culturing effects or expansion from a subset of cells that do not represent the organ of interest. Additionally, sebocytes are notoriously difficult, tedious, and expensive to isolate from skin biopsies, with poor yield and non-standardised

dissociation protocols affecting the quality of generated sebocytes (Yang *et al.*, 2014; Onteniente & MARUOTTI, 2018).

Researchers are increasingly turning to human-induced pluripotent stem cells (hiPSCs) to overcome the limitations of traditional cell lines and primary cultures. HiPSCs, which are reprogrammed from non-invasively obtained human cells such as those from skin, urine, hair, and mouth, can self-renew indefinitely and differentiate into various cell types (Chehelgerdi *et al.*, 2023; Park *et al.*, 2023; Stöberl *et al.*, 2023; Chen, Li, & Wu, 2024; Inomata *et al.*, 2024). Cell models derived from hiPSCs tend to be more genetically stable and homogeneous, as they are created from carefully screened reprogrammed cells using specific differentiation protocols and can be replenished as needed (Jusop *et al.*, 2023; Palasantzas *et al.*, 2023; Xu *et al.*, 2023). In contrast, traditional cell lines and primary cultures rely on limited batches with finite subculturing capacity. The easily accessible source material for hiPSCs means there is a potentially unlimited supply of ethically obtained cell types that better reflect the biology of the donor compared to other *in vitro* models. These advantages have led to the use of hiPSCs in studies of human sex variability, development, reproduction, pathogenesis, and treatment outcomes (Waldhorn *et al.*, 2022). Sebocytes have been generated from hiPSCs. The most successful method involves creating multipotent skin precursor cells from hiPSCs using retinoic acid, bone morphogenetic protein 4, and epidermal growth factor, followed by sebocyte differentiation with a SMAD inhibitor, PPAR-delta agonist, and Hedgehog pathway inhibitor (Onteniente & MARUOTTI, 2018). These hiPSC-derived sebocytes have since been utilised in several studies (Cros *et al.*, 2023; Zerbinati *et al.*, 2023; Kleemann *et al.*, 2024; Knödlseeder *et al.*, 2024).

In vitro models like sebocytes present new opportunities for sex-related research. Genetic interactions involving X and Y chromosomes can be examined. Additionally, sebocytes have abundant gonadal hormone receptors that enable studies on the effects of gonadal hormones. Sebocyte models also allow comprehensive investigations into immune responses, metabolism, drug effects, environmental stressors, and epigenetics (Pitrez *et al.*, 2024). Studying TEs and their role in cis-regulatory elements, non-coding RNAs, and pathways that silence TEs, such as piwi-interacting RNAs and DNA methylation, is of particular interest in relation to human sex variability.

Specific Aim 4

A notable method for generating hiPSCs involves directed differentiation through transcription factor (TF) overexpression (Ng *et al.*, 2021; Joung *et al.*, 2023). This method offers benefits such as a short differentiation time of one-week, high yield, high efficiency, and the ability to differentiate multiple cell types simultaneously. However, only a few cell types directly related to human sex have been developed using this method, including Sertoli cells, ovarian granulosa cells, and gonad-like cells (Sepponen *et al.*, 2022; Yang *et al.*, 2022; Pierson Smela *et al.*, 2023). In this study, we developed a stem cell engineering strategy for sebocyte differentiation by directly activating TFs. By mining transcriptomic data and the literature, we identified TFs relevant to sebocytes and performed two rounds of hiPSC nucleofection with subsets of TF libraries. Sorted differentiated cells underwent transcriptomic analysis, mapping them to sebocytes in a single-cell atlas of the skin.

Methodology

Selecting transcriptional factors for cell fate engineering of sebocytes

For the literature search on TFs related to sebocyte differentiation, the terms “sebocytes” and “sebaceous” were queried with “transcription factors” in PubMed in March 2022. TFs were considered relevant to sebocyte development if they were experimentally demonstrated or reviewed in other studies. Dr Lily Li utilised the standard STAMPSCREEN pipeline to identify differentially expressed genes (DEGs) including TFs, from a single-cell transcriptome atlas of healthy human skin comprising over 400,000 cells (Tang, 2023). The DESeq2 package was employed to calculate log₂fc values for each aligned gene across the samples. Gene regulatory networks involving these DEGs and TFs were inferred using the GRNBoost2 algorithm within the Arboreto computational framework. Additionally, PageRank scores were computed for each TF in the resultant network using the NetworkX package. Based on this compiled list of TFs (Table 2), Round 1 sebocyte engineering was performed with 25 TFs available in glycerol stocks in the PBAN all-in-one Tet-On PiggyBac vector in the TFome. Round 2 sebocyte engineering used a subset of 30 TFs from the TFome, selected by consulting additional collaborators. Not all TFs were included either because they were not available in the

TFome or to limit the initial list to 30 TFs. A list of 30 TFs would give total possible combinations of cells being nucleofected 0 – 30 TFs by the equation, where k can range from 0 to 30, and C is the combination:

$$Total\ combinations = \sum_0^{30} C(30, k) \approx 1\ billion$$

A larger number of TFs would require a more laborious workflow, so a refined starting list likely to drive sebocyte differentiation was curated for subsequent iterations.

Transcription factor plasmid construction.

For Round 1 hiPSC differentiation, all TFs in the screening library were obtained from glycerol stocks from the TFome, already present in the PiggyBac vector, PBAN, and transformed in 5-alpha competent E. coli cells (NEB C2987H) (Ng *et al.*, 2021). PBAN is a doxycycline-inducible vector with puromycin selection, developed from PB-TRE-dCas9-VPR (Addgene no. 63800). Glycerol stocks were kept on dry ice, and sterile loops were used to scrape a small amount of stock, which was then spread on agar plates. The plates were cultured overnight at 37°C. Single colonies were picked and cultured with other TFs in liquid culture overnight (37°C, shaking). Plasmids were extracted using the endotoxin-free Midi Prep Plus Kit (Qiagen, 12943), quantified using NanoDrop, and stored at -20°C until use. Dr Songlei Liu constructed TFs for Round 2 sebocyte engineering. All TFs in the screening library were sourced from the TFome collection in pDONR format (Ng *et al.*, 2021). To generate a barcoded PBAN expression vector (pBAN-BC), the original PBAN was cleaved with AgeI and KpnI, and a gBlock (IDT DNA) carrying the same excised segment along with an additional 20-bp random barcode was ligated. Following bacterial transformation, individual colonies were cultured, and their plasmid DNA was isolated. Gateway cloning was used to transfer each TF from pDONR to the pBAN-BC vector. The barcode sequence for each TF was confirmed through Sanger sequencing.

Cell culture.

For hiPSC culture, we used the PGP1 line, derived from the dermal fibroblasts of a 55-year-old person who self-identified as a cisgender man (Church, 2005). The PGP1

line was quality tested, including karyotyping and Pluritest (Ng *et al.*, 2021). Cells were cultured feeder-free at 37°C, 5% CO₂, and 5% O₂ in mTESR Plus medium (Stemcell Technologies, 100-0276) on tissue-culture-treated plates coated with Cultrex (Bio-Techne Corporation, 604 3434-005-02) as per the manufacturer's instructions. Antibiotics were not used. Media were changed daily during standard expansion and passaging. Passaging was performed using TrypLE Express (Life Technologies, 12604013) for 5 minutes at 37°C every 3-4 days. After washing, cells were seeded into fresh plates at a 1:10 to 1:20 ratio and treated with 10 µM Y-27632 ROCK inhibitor (Millipore, 688001) for one day. Cell counts were conducted using an automated cell counter (Thermo Fisher Scientific, Countess II, AMQAX1000). Cells were cryopreserved using mFreSR (STEMCELL Technologies, 5854) at 2 million/mL with overnight cooling in a CoolCell LX (BioCision, BCS-405) at -80°C, followed by storage in vapor-phase liquid nitrogen for long-term preservation.

Generating inducible transcription factor human-induced pluripotent stem cell lines.

I performed Two rounds of cell differentiation, each with a distinct screening library: Round 1 with 25 TFs and Round 2 with 30 TFs (Table 2). Experimental duplicates were conducted for each round. These TFs were expressed in plasmids under the control of a doxycycline-inducible promoter. For each sample, 4 µg of TF plasmid pool was combined with 1 µg of piggyBac transposase expression plasmid (Super piggyBac Transposase - SystemBio PB210PA-1) at 0.5 ng/µL. For Round 1, as a proof-of-principle experiment, TF plasmids were pooled from several single colonies from glycerol stocks. This method has limitations as some colonies may have faster growth rates, resulting in a non-equimolar ratio of each TF plasmid. For Round 2, TF plasmids were prepared individually and mixed in equimolar ratios. Integration was performed using the P3 Primary Cell 4D-Nucleofector X Kit L (Lonza, V4XP-3024) with 600,000 hiPSCs, following the manufacturer's instructions, through electroporation in the 4D-Nucleofector X Unit (Lonza, AAF-1002X) with pulse setting CB-150. Subsequently, cells were seeded in Cultrex-coated 6-well plates in mTeSR Plus supplemented with 10 µM Y-27632 ROCK inhibitor. Cells were undisturbed for 48 hours post-electroporation. Selection for TF-integrated cells was performed by replacing the media with 1 µg/ml puromycin

(Gibco, A1113803). Media was changed daily with fresh puromycin for 3-5 days until 80% confluence was reached. At confluence, cells were frozen according to the procedures outlined in the “*Cell Culture*” section.

Inducing transcription factor expression in human-induced pluripotent stem cell lines.

Each experimental replicate of nucleofected hiPSC cell lines was seeded at 25,000 to 100,000 cells into one well of a 6-well plate in mTeSR Plus medium. Cells were treated with 10 μ M ROCK inhibitor, 1 μ g/ml puromycin, and 0.5 μ g/ml doxycycline (Sigma-Aldrich, D3072). Media were refreshed daily with the same formulation until the hiPSCs reached confluence at 3-4 days post-induction. On this day, cells were dissociated and stained for flow cytometry or fluorescent-activated cell sorting (FACS) without further passage to prevent the faster proliferation rate of hiPSCs from out-competing differentiated cells.

Flow cytometry and fluorescent-activated cell sorting

For flow cytometry analysis and FACS, cells were dissociated using TrypLE Express for 5 minutes at 37°C, suspended in a two-fold volume of Cell Staining Buffer (Biolegend, 420201), and centrifuged (200g, 3 minutes) to remove the digesting enzyme. Cells were then treated with 25 μ g/mL Human Fc Block (BD Biosciences, 564219) diluted in Cell Staining Buffer for 15 minutes on ice. Without removing the Human Fc Block, surface antigens were labelled in live cells with fluorophore-conjugated antibodies or isotype controls at concentrations recommended by the manufacturer (Table 5), and diluted in Cell Staining Buffer for 30 minutes on ice in the dark. Lipid staining was done similarly, except without isotype controls, and incubation was for 15 minutes at room temperature. After staining, cells were pelleted (200g, 5 minutes), washed twice with Cell Staining Buffer, and strained through a 35 μ m nylon mesh into a 5 mL round bottom polystyrene tube (Falcon, 352235). Flow cytometry data were acquired on a BD LSRFortessa Cell Analyzer and analyzed using FlowJo software (version 10.8.1). FACS sorting was conducted using a BD FACSAria instrument into 10 mL of fresh mTeSR Plus medium. I sorted cells exhibiting the lowest ~40% of TRA-1-60 expression, designated as TRA-1-60low, representing the differentiation gate.

Bulk RNA-sequencing library preparation.

FACS-sorted TRA1-60low cells were centrifuged (200g, 3 minutes), pelleted, and stored at -80°C until RNA extraction. RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, 74134), and RNA concentration was measured using the Qubit RNA HS Kit (Molecular Probes, Q32852). The samples were then sent to Novogene (Sacramento, California) for quality control using the Agilent 5400 and for polyA enrichment library preparation. Bulk RNA-Sequencing (RNA-seq) was performed on a NovaSeq platform with paired-end, 150-cycle reads.

Analysis of bulk RNA-sequencing data.

Dr. Lily Li and Zhengkuan Tang conducted the bulk RNA-seq data analysis. Raw reads were aligned to the GRCh38 human genome using STAR v2.7.11 to determine read mapping locations and quantified. Quality control was performed with FastQC v0.12.0 to identify sequencing errors, adapter contamination, or biases. Alignment files were indexed with SAMtools v1.18 for accessibility, and mapped reads were quantified with featureCounts in the SubRead package v2.0.6 to construct count matrices aligned to known human genes. Differential expression analysis was conducted using the DESeq2 package in R, including read normalization. Significantly dysregulated genes were defined as those with at least a 2-fold change in intensity values and a Benjamini-Hochberg false discovery rate correction (< 0.05). Data dimensionality was reduced using principal component analysis (PCA) in R with regularized-logarithm transformed raw counts. Visualization was achieved with a heatmap generated using the ComplexHeatmap package in R.

We also employed the enrichment theory of Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways to encode the relationship between a gene and a specific GO term or KEGG pathway into a numeric value. The GO enrichment score of a gene (denoted as g) and a GO term (denoted as GO_j) was calculated using the hypergeometric test P-value on two gene sets: $G(g)$ representing the genes annotated with g , and G_{GO} representing the genes associated with GO_j . This score is computed as follows:

$$\text{GO Enrichment Score} = 1 - \frac{\sum_{i=0}^{m-1} \binom{m}{i} \binom{N-n}{M-i}}{\binom{N}{M}}$$

Where N is the total number of human genes, M is the number of genes in G_{GO} , n is the number of genes in $G(g)$, and m is the number of genes common to both $G(g)$ and G_{GO} . A lower score indicates a stronger functional association between the gene g and the GO term GO_j . Similarly, the KEGG enrichment score of a gene g and a KEGG pathway K_j was computed using the hypergeometric test P-value on $G(g)$ and a gene set G_{KEGG} representing the genes annotated with K_j . The KEGG enrichment scores are given by:

$$\text{KEGG Enrichment Score} = 1 - \frac{\sum_{i=0}^{m-1} \binom{m}{i} \binom{N-n}{M-i}}{\binom{N}{M}}$$

Where N and n are the same as defined previously, and M represents the number of genes in G_{KEGG} . A lower KEGG enrichment score indicates a strong functional association between the gene g and the KEGG pathway K_j .

Single-cell RNA sequencing library preparation.

FACS-sorted TRA1-60low cells were centrifuged (200g, 3 minutes) and resuspended in mTeSR Plus for accurate cell counting. After counting, undifferentiated hiPSCs were added to the sorted cells at a 10% ratio. The samples were then processed with the Chromium Next GEM Single Cell 3' GEM, Library & Gel Bead Kit v3.1 (10× Genomics, PN-1000128). Targeted recovery was set at 6000 single cells. Libraries were prepared using the Chromium Next GEM Chip G Single Cell Kit (10× Genomics, PN-1000127) according to the manufacturer's instructions. During the Sample Index PCR step, 5 μ L of the PCR mix was combined with 0.5 μ L of 1000X SYBR Gold (Invitrogen, S11494) for a qPCR reaction. The ideal amplification cycle was identified as the cycle just before reaching half of the maximum total signal. Quality control was performed at the Biopolymers Facility at Harvard Medical School using TapeStation, and sequencing was conducted on a NextSeq 500, targeting a minimum of 30,000 reads per cell.

TF barcode amplicon library preparation.

After cDNA amplification in the 10x scRNA-seq protocol, the amplicons contained cell barcodes, unique molecular identifiers, and TF barcodes. These cDNAs served as templates for further amplification of TF-cell barcodes. Two consecutive PCR reactions were performed, each with a SYBR Gold spike-in qPCR to determine the

optimal cycle number as described in the "*Single-cell RNA sequencing Library Preparation*" section. For PCR1, primers NGS10x-F-i7-BC-PCR1F and i5000 were used. The 50 μ L PCR1 reaction mix included 25 μ L Q5 Hot Start High-Fidelity 2X Master Mix, 5 μ L amplified cDNA, 2.5 μ L of each primer (10 μ M), and 15 μ L nuclease-free water. The PCR1 program was 98°C for 30 seconds, followed by 11-13 cycles (determined by qPCR) of 98°C for 10 seconds, 67°C for 30 seconds, 72°C for 30 seconds, and a final extension at 72°C for 2 minutes. PCR1 products were purified using 1.2X SPRIselect beads and eluted in 20 μ L water. For PCR2, primers i7000, P5, and P7 were used. The 50 μ L PCR2 reaction mix included 25 μ L Q5 Hot Start High-Fidelity 2X Master Mix, 10 μ L PCR1 product, 2.5 μ L of each primer (10 μ M), and 7.5 μ L nuclease-free water. The PCR2 program was 98°C for 30 seconds, followed by 4-5 cycles (determined by qPCR) of 98°C for 10 seconds, 67°C for 30 seconds, 72°C for 30 seconds, and a final extension at 72°C for 2 minutes. PCR2 products were purified using the same protocol. Final libraries were submitted to the Biopolymers Facility at Harvard Medical School for MiSeq v3 sequencing with paired-end reads of 80 cycles from either direction after quality control.

Analysis of single-cell RNA sequencing data.

Our engineered cells were annotated using SKIN-TOSICA, a transformer-based tool inspired by One-Stop Interpretably Cell-type Annotation (TOSICA). SKIN-TOSICA employs a multi-head self-attention deep learning model, allowing interpretable annotation of cell types using biologically relevant features (Tang, 2023). The first layer of SKIN-TOSICA transforms genes into "tokens" using a method called Gene2vec, which is analogous to how words are processed in natural language processing. Each gene is represented as a token, a numeric vector capturing its characteristics. Then, a masking process is applied using a matrix based on expert knowledge of biological pathways. This means that connections between genes and pathways are filtered to retain only the most biologically meaningful interactions, reducing complexity and focusing on relevant data.

The resulting matrix, containing these filtered connections, is augmented by adding a "class token." This class token is a special trainable parameter that helps the model aggregate information as it processes the data. This augmented matrix is then fed

into the multi-head self-attention layer, which is a key component of transformer models. In the multi-head self-attention layer, the model processes the data by projecting the tokens into three different matrices: query, key, and value. The query matrix represents the current token, the key matrix represents the context of all tokens, and the value matrix holds the information to be passed along. Attention scores are calculated by multiplying the query with the transpose of the key, scaling by the inverse square root of the key's dimension (to stabilise training), and applying a softmax function to normalise the scores. These scores indicate the importance of each token in relation to others, highlighting significant interactions.

These attention scores are then used to weight the value matrix, effectively refining the information based on the most relevant interactions. This process is repeated multiple times (multi-head attention) to capture different aspects of the data. The final output, refined by these attention scores, is processed through a fully connected network to determine the probabilities of different cell types. Creating custom masks is essential for integrating skin-cell-specific knowledge into the model. These masks are constructed by extracting cell-type-specific DEGs from curated literature, ensuring the model focuses on genes and pathways specific to skin cell types. This tailored approach improves the model's ability to accurately identify and annotate sebocytes and other skin cell types in the data. SKIN-TOSICA consolidated datasets to create an atlas of over 400,000 cells, which could be annotated to major skin-related cell types: B Cells, Endothelial Cells, Fibroblasts, Hair Follicle Cells, Keratinocytes, Langerhans Cells, Lymphatic Cells, Mast Cells, Melanocytes, Myeloid Cells, Plasma Cells, Schwann Cells, Sebocytes, T Cells, and Vascular Smooth Muscle Cells.

Dr Li Li and Zhengkuan Tang processed the scRNA-seq data, starting with demultiplexing raw FASTQ files and extracting unique molecule identifiers using UMI-Tools. They aligned the data to the GRCh38 genome and quantified it with STAR, followed by doublet filtering, normalization, and log transformation using Scanpy 1.9.3. Quality control involved filtering barcodes based on the number of captured genes (400-6000) and total reads (1000 per barcode) and removing cells with high mitochondrial mRNA content (>20%). Batch correction was performed using BBKNN v1.5.1, resulting in a single-cell raw expression matrix. Highly variable genes were identified, and PCA was used for dimensionality reduction. Cell clustering was done using the Leiden

algorithm, and a UMAP plot was generated to visualize cell clusters mapped to the SKIN-TOSICA atlas. For cells mapping to sebocytes, their gene expression was further examined to identify highly expressed genes.

Generating single-guide RNAs for sebocyte reporter human-induced pluripotent stem cell lines

CRISPR/Cas9 single-guide RNAs (sgRNAs) targeting the final exons of melanocortin-5 receptor (MC5R) and keratin-7 (KRT7) were designed using Benchling, ensuring target sites were near the stop codon. Designs with over 20 off-targets, more than six 5' mismatches, or more than two editing distances to the target sequence were excluded. The final selection criteria included moderate GC content, minimal self-complementarity, and a higher E-score for better editing efficiency. Primer pairs for the selected sgRNAs (Table 6) were phosphorylated at the 5' end using T4 PNK (New England Biolabs, M0201S) following the manufacturer's recommendations. Phosphorylation increased ligation efficiency prevented self-ligation, and enhanced stability against exonucleases after annealing to the sgRNA oligo.

The sgRNA oligos were cloned into the pX459 Cas9/sgRNA expression plasmid (RRID: Addgene_62988). The pX459 plasmid was digested with the BbsI restriction enzyme and purified using the Monarch DNA Gel Extraction Kit (New England Biolabs, T1020S). Each sgRNA oligo was ligated into the digested plasmid using T4 DNA Ligase (New England Biolabs, M0202) according to the manufacturer's instructions. The ligated plasmids were transformed into 5-alpha competent *E. coli* (New England Biolabs, C2992H). Single colonies were verified through PCR amplification using U6 sequencing primer and sgRNA reverse primer. Sanger sequencing was performed with the U6 sequencing primer, and sequences were aligned against the intended vector using Benchling. Verified colonies were cultured overnight in Luria Broth with carbenicillin (37°C, 250 rpm), and plasmids were extracted using the Plasmid Plus Midi Kit (Qiagen, 12943). A portion of the culture was preserved in cryovials with 50% glycerol at -80°C for future use.

Generating left homology arms for sebocyte reporter human-induced pluripotent stem cell lines

Homology arms flanking the cut sites targeted by my sgRNAs were designed using Benchling, each spanning 1000 base pairs. Primers were used to amplify the left homology arm (5' homology arm) from PGP1 genomic DNA (Table 6) using Q5® High-Fidelity DNA Polymerase (New England Biolabs, M0494S). The left homology arm was then cloned in-frame with the C-terminal T2A-fluorescent reporter tandem Tomato (tdTomato) from pHDR-FOXL2-T2AtdTomato-PuroTK (RRID: Addgene_192892), replacing FOXL2, as used in a previous study (Smela *et al.*, 2022). This plasmid contained a Rox-PGK-PuroTK-Rox selection cassette and an MC1-DTA marker to discourage random integration.

The pHDR-FOXL2-T2AtdTomato-PuroTK plasmid was digested with AgeI to release the left homology arm of FOXL2 and the tdTomato cassette. The tdTomato was amplified from the undigested plasmid using primers (Table 6) with Q5® High-Fidelity DNA Polymerase (New England Biolabs, M0494S). All products, including the digested plasmid, tdTomato cassette, and left homology arm, were gel-purified using the Monarch DNA Gel Extraction Kit (New England Biolabs T1020S). Gibson assembly was performed with NEBuilder® HiFi DNA Assembly Cloning Kit (New England Biolabs, E5520S) to ligate the components in the correct order, following the manufacturer's guidelines. Subsequent steps, including transformation into 5-alpha competent *E. coli*, verification via PCR amplification, Sanger sequencing, and plasmid extraction, followed the same protocol outlined in the "*Generating single-guide RNAs for sebocyte reporter human-induced pluripotent stem cell lines*" section, with specific modifications using primers for the homology arm.

Results

Transcription factors were mined for cell fate engineering of induced pluripotent stem cells into sebocytes.

The experimental workflow is shown in Figure 13A. In brief, hiPSCs with or without reporter systems of sebocyte markers are nucleofected with TFs using a piggyBac transposon system. The TFs are activated through doxycycline induction, followed by FACS for differentiated cells (TRA1-60low) for transcriptomic analysis. To identify TFs

capable of differentiating hiPSCs into sebocytes, I conducted a comprehensive literature search (Horsley *et al.*, 2006; Smith *et al.*, 2008; Choi *et al.*, 2019). A transcriptome atlas of healthy human skin, incorporating both scRNA-seq and bulk RNA-seq datasets was also analysed (Li, Hajam, *et al.*, 2023; Tang, 2023). By determining DEGs in sebocytes compared to other cell types, additional TFs relevant to sebocyte differentiation were identified, either due to their own differential expression or their regulatory roles (Figure 13B). This resulted in a compiled list of potential TFs for sebocyte differentiation (Table 2). These TFs were introduced into hiPSCs using a PBAN all-in-one Tet-On PiggyBac vector, integrating the TFs into the genome with PiggyBac transposase and inducing TF expression with doxycycline (Figure 13C).

Proof-of-principle bulk RNA sequencing confirmed the feasibility of the cell engineering strategy.

To differentiate hiPSCs into sebocytes, an engineering design process was employed, iteratively testing and redesigning prototypes to approach an optimal end product (Brand, 2020). Given the challenge of predicting effective TF combinations for sebocyte differentiation, this trial-and-error approach was necessary. To demonstrate the feasibility of the workflow, the entire process was implemented, using bulk RNA-seq as the readout. Prototyping with bulk RNA-seq is cost-effective and less technically and bioinformatically challenging than scRNA-seq (Hegenbarth *et al.*, 2022). Although it lacks single-cell resolution, bulk RNA-seq provides valuable insights into the overall success of the differentiation protocol and sample processing methods.

Many of the TFs of interest were present in the human TFome collection, the first comprehensive human TF library (Ng *et al.*, 2021). Several TFs in the library were already available in glycerol stocks of PBAN all-in-one Tet-On PiggyBac vectors. A list of 25 TFs were selected from the TF list curated for sebocyte differentiation, that were available in glycerol stocks for Round 1 of sebocyte engineering. Large volumes of these TF plasmids were prepared using Maxi-Prep. The hiPSC line used was PGP1, generated from fibroblasts (Ng *et al.*, 2021). PGP1 cells were cultured in coated plates between passages 28–33 in preparation for TF nucleofection. In two independent experiments, I nucleofected 600,000 PGP1 cells with PiggyBac TF vectors and transposase DNA at a mass ratio of 4:1. Cells with successful genomic integration were selected using

puromycin, and TF expression was induced by applying doxycycline for three consecutive days. Compared to samples without doxycycline, those with doxycycline exhibited noticeable morphological changes (Figure 14A). Using flow cytometry, we gated approximately 40% of cells with low levels of the pluripotency marker TRA1-60 (Figure 14B). These cells were FACS-sorted, RNA-extracted, and sent to Novogene for bulk RNA-sequencing.

PCA was performed to obtain dimensionally reduced representations of the hiPSCs and human sebocytes from existing bulk RNA-seq datasets, reducing them to two principal components (Figure 14C). The engineered cells differed from hiPSCs in both principal components and were similar to human sebocytes in one principal component, indicating progress toward achieving my cell fate engineering objective. Analysis of DEGs (Figure 14D) established several sebocyte-related markers, including FoxO1 for sebaceous lipogenesis, LRG6 for sebocyte differentiation, and SOX9 for promoting sebocyte proliferation (Table 3). Additionally, the engineered cells exhibited DEGs associated with gonadal determination, such as AMH (involved in estrogen signalling), SRY (critical for gonadal determination), and DMRT2 (also crucial for gonadal determination). We also identified differential expression of TE-related genes, such as APOBEC3G (involved in TE silencing), CSMD2 (harbours TEs), and PIWIL2 (associates with piwi-interacting RNAs that regulate TEs) (Table 3). However, engineered cells display significant heterogeneity, as each cell randomly integrated a unique set of TFs, averaging about seven TFs per cell (Liu *et al.*, 2022). This extensive heterogeneity made it challenging to specifically interrogate individual cell types with bulk RNA-seq. Despite this, the feasibility of the pipeline was demonstrated, providing a high-resolution readout and direction for further refining the sebocyte engineering design.

Single-cell RNA-sequencing mapped engineered cells to sebocytes.

After demonstrating the feasibility of the workflow, two components were added to refine the TF combination for sebocyte differentiation (Appleton *et al.*, 2022). The first component involved incorporating barcodes into the TF vectors to identify and quantify exogenous TFs in engineered cells. The second component included scRNA-seq to capture the heterogeneity of the engineered cells and determine if any of them resembled

sebocytes transcriptomically. Another subset of TFs was selected from the TF list curated for sebocyte differentiation for Round 2 of cell fate engineering (Table 2). This round included 30 TFs from the TFome. A 20bp barcode specific to each TF was cloned between the stop codon and polyA sequence in the PBAN all-in-one Tet-On PiggyBac vector. Following nucleofection, puromycin selection, and doxycycline induction, ~40% of the cells expressing low levels of TRA1-60 were FACS-sorted for scRNA-seq (Figure 15A), with 10% non-induced hiPSCs included as undifferentiated controls.

Approximately 6000 cells were partitioned into nanolitre-scale gel beads in emulsion to produce barcoded full-length cDNA. The cDNA was then used for scRNA-seq library preparation, and the barcode part of the cDNA was amplified along with the cell barcodes for single-cell DNA sequencing. Samples were processed with quality control at Harvard Medical School Biopolymers Facility prior to sequencing.

We used SKIN-TOSICA, a tool for identifying different skin cell types in scRNA-seq datasets through a Transformer-based architecture (Tang, 2023). SKIN-TOSICA employs self-attention, enabling the model to weigh the importance of each gene by considering their interactions. Genes are transformed into tokens and masked using a matrix based on expert knowledge of biological pathways. This ensures only meaningful connections among genes and pathways are considered, enhancing the model's interpretability and accuracy. The self-attention process involves projecting tokens as query, key, and value matrices, which are then multiplied and scaled to highlight significant interactions. Additionally, SKIN-TOSICA uses custom masks relevant to skin cells, created by extracting DEGs from curated literature. This custom mask improved the model's ability to accurately identify sebocytes and other skin cell types in transcriptome data. SKIN-TOSICA consolidated a single-cell transcriptome atlas of over 400,000 human skin cells and annotated 15 major skin cell types (Figure 15B). Using this tool, a Uniform Manifold Approximation and Projection (UMAP) of the engineered cells was constructed and mapped to the skin atlas, finding six cells aligned with sebocytes (Figure 15C). Due to proprietary constraints, the specific exogenous TFs driving differentiation to these sebocytes were not disclosed in this dissertation. However, this information will inform future iterations of cell fate engineering to further optimize and refine the sebocyte differentiation protocol.

The transcriptome patterns of these engineered sebocytes (Figure 15D) can generate research hypotheses about how sex-related genetics contribute to the observed gene expression in sebocytes. Additionally, these cells could be studied to explore how sebocytes express sex-related genes through functional pathways and gene regulatory networks (Table 4). For instance, we observed high expression of EEF1A1 in engineered sebocyte-like cells, which is related to temperature-dependent sex dynamics (Wang *et al.*, 2022). Similarly, SERBP1 is involved in progesterone metabolism (Shilenok *et al.*, 2023). Several TE-related genes were also highly expressed in these cells, including NCL, which regulates totipotent gene expression via LINE1 (Wang *et al.*, 2024), and GRID2, which contains a polymorphism that increases transcription of a fusion product of LINE1 and GRID2 (Akagi *et al.*, 2008).

Gene-editing strategies were designed to have cells fluoresce once they start expressing sebocyte markers.

Given that the scRNA-seq workflow was optimised to construct sequencing libraries for ~6000 cells, it is crucial that the sorted cells represent my population of interest. While I sorted the cells expressing low levels of TRA1-60, it is possible that many of these cells are not sebocyte-like. To isolate more sebocyte-like cells, I examined the properties of sebocytes that could aid in their identification. Since sebocytes produce sebum, an oily and waxy material (Geueke & Niemann, 2021), I stained cells from Round 2 for the presence of sebum using Nile Red and BODIPY (Seo *et al.*, 2020; Na *et al.*, 2022). In cells with low TRA1-60 levels after doxycycline induction (differentiated cells) and in cells with high TRA1-60 levels without doxycycline induction (undifferentiated hiPSCs), I observed lower fluorescence of both Nile Red and BODIPY in cells with low TRA1-60 (Figure 16A). Based on these lipid stains, it remained inconclusive which cells were sebocyte-like, likely because the dyes stained lipids generally, not specifically sebum (Wolins *et al.*, 2018; Boumelhem *et al.*, 2022). Additionally, even if some engineered cells were sebocyte-like, they may not yet be mature enough to produce sebum (Shamloul & Khachemoune, 2021).

To explore other strategies to isolate sebocyte-like cells, I searched in the literature for sebocyte-specific markers suitable for live cell sorting, as intracellular markers require cell permeabilisation, which would kill the cells. I identified mucin-1 and

MC5R as potential sebocyte markers (Denisenko *et al.*, 2020) (Zhang *et al.*, 2006; de Bengy *et al.*, 2019). Using flow cytometry, I measured the expression of mucin-1 and MC5R in duplicate nucleofection samples from Round 2. Cells with low TRA1-60 expression after doxycycline induction showed lower levels of mucin-1 and MC5R compared to cells with high TRA1-60 expression without doxycycline induction (Figure 16B). However, these results again could not help me determine which cells were sebocyte-like. This could relate at least in part to issues with antibody staining (Ayoubi *et al.*, 2023; Brooks *et al.*, 2024).

Given the inconclusive results with lipid stains and sebocyte markers, I pivoted to using gene-editing to make cells fluoresce when they express specific markers (Smela *et al.*, 2022). I focused on MC5R, a well-recognised sebocyte marker (de Bengy *et al.*, 2019). Using a CRISPR-Cas9 strategy, I designed sgRNAs targeting the last exon of MC5R near the stop codon. The sgRNAs were cloned into the pX459 plasmid containing Cas9 nuclease. Sanger sequencing confirmed the alignment of the sgRNA sequence with the target (Figure 16C). To insert fluorescent tags, the Cas9 nuclease cuts the gene and facilitates homology-directed repair with a donor plasmid containing the fluorescent tag cassette (Zhong *et al.*, 2020; Labun *et al.*, 2021). I cloned the left homology arm of MC5R into a donor plasmid with a tdTomato fluorescent tag used in a previous study (Kramme *et al.*, 2022), and Sanger sequencing verified the sequence alignment (Figure 16C). This set the stage for cloning the right homology arm and creating an MC5R hiPSC reporter line. Once constructed, the plasmid could be co-electroporated with the Cas9/sgRNA plasmid into PGP1, followed by the direct activation of the TF workflow as previously described (Kramme *et al.*, 2022). Since MC5R is a marker of mature sebocytes and would not identify less mature sebocyte-like cells, I also chose KRT7 as a sebocyte progenitor marker (Veniaminova *et al.*, 2023; Thalheim & Schneider, 2024). The same gene-editing strategy was implemented for KRT7, with Sanger sequencing verifying the sequences of my sgRNA and left homology arm (Figure 16D).

Discussions

In this study, I developed a strategy to engineer stem cells for sebocyte differentiation via direct activation of TFs. Initially, TFs were identified as relevant to

sebocytes through extensive literature review and transcriptome analysis. Subsets of TFs from the TFome were then selected and incorporated into a PiggyBac transposase system, allowing genome integration and controlled expression in the presence of doxycycline. I performed two rounds of nucleofection using TF libraries: the first with bulk RNA-seq to validate the protocol and the second with scRNA-seq to refine the TF library for optimising sebocyte differentiation. To further enhance this approach, a strategy was developed to create a reporter hiPSC line using CRISPR/Cas9 technology. This would enable the sorting of differentiated cells expressing sebocyte markers for the downstream workflow that could be more targeted towards sebocyte-like cells.

Other studies have used hiPSCs to generate human sebocytes (Cros *et al.*, 2023; Zerbinati *et al.*, 2023; Kleemann *et al.*, 2024; Knödlseeder *et al.*, 2024). Their protocols involved stimulating hiPSCs with retinoic acid, BMP4, and epidermal growth factor to create multipotent skin precursor cells over 5–15 days. This was followed by differentiation into sebocytes using additional factors over another 5–15 days: Suppressor of Mothers against Decapentaplegic protein, a peroxisome proliferator-activated receptor-delta agonist, and a Hedgehog pathway inhibitor. Verification included immunoexpression of KRT7 and MUC1, lipid content assessment, lipogenesis induction, and expression of sebocyte-related genes. My strategy reduces the differentiation timeline to three days, and once established, could achieve higher yields and efficiency. This method not only differentiates sebocytes rapidly but also supports parallel programming for the simultaneous differentiation of multiple cell types, facilitating the creation of organoids, assembloids, and tissue/organ-on-a-chip systems (Palasantzas *et al.*, 2023). While I did not conduct functional assays to confirm the lipogenic activities of the engineered cells in this study, future work will optimise TF combinations for sebocyte engineering and include functional assays to ensure robust lipogenesis in the engineered cells.

The significance of this study lies in generating human sebocytes to investigate the cellular, molecular, and genetic factors related to human sex, particularly how the nervous system communicates with the skin. Using TEs such as Alu SINE RNA in these cells under conditions that better represent human physiology than conventional models

could help determine if they regulate gonadal steroid hormone receptors, which are abundant in human sebocytes (Clayton *et al.*, 2020). For instance, in the C6 rat glioma immortalised cell line, researchers have observed the regulatory role of rodent B2 SINE in controlling glucocorticoid receptor transcription (Bartlett *et al.*, 2023). Human sebocyte models could clarify which factors regulate these TEs and their relevance to neuroendocrinological communication with the skin, such as exposure to different gonadal steroid hormones. Epigenetic mechanisms like DNA methylation that regulate TEs, including those influencing promoters and imprint control regions, could be sex-related (Kramme *et al.*, 2022). While transcribed TEs could be detected with scRNA-seq and several packages have been developed to analyse this type of data (McKerrow & Fenyő, 2019; He *et al.*, 2021; Benedetto *et al.*, 2022; Rodríguez-Quiroz & Valdebenito-Maturana, 2022; Lee, Barbour, *et al.*, 2023), this was outside the scope of this dissertation. Future studies could examine TEs in our scRNA-seq datasets, considering TEs delineate cell types that may not be identified by standard mRNA expression (Reyes-Gopar *et al.*, 2023).

This study has several limitations. I did not assess variability in the differentiation propensity between hiPSC lines, which may affect the generalizability for scaling up the process to multiple individuals. Additionally, I did not explore the effects of different media compositions or the potential enhancement of differentiation through exogenous factors. My approach only allowed for one-time induction of TF expression, unlike *in vivo* development where TFs are sequentially induced (Haniffa *et al.*, 2021). Furthermore, I could not control the timing and level of TF induction, and my readout did not provide data to optimize these parameters. Other limitations included the random integration of TFs, which could pose unknown risks, and the assumption that TF combinations have an additive effect, which may not always be the case. It was also unclear whether the TF-induced sebocyte-like cells resulted from the canonical function of these TFs or forced overexpression.

Developing physiologically relevant human *in vitro* models, such as sebocytes, benefits transgender, non-binary, and gender-diverse individuals, some of whom may require gender-affirming hormonal therapy. hiPSCs can be reprogrammed from non-

invasive samples to obtain various cell types more physiologically relevant to these individuals and assess their responses to gonadal steroid hormones (Aghi, Goetz, *et al.*, 2024). Studying these models could help construct the complex interactions between the cellular, molecular, and genetic aspects with other factors related to sex/gender, such as sociocultural influences (Aghi, Anderson, *et al.*, 2024).

Summary and Conclusions

I have developed a stem cell engineering strategy for sebocyte differentiation through the direct activation of TFs. Transcriptomic analysis identified engineered cells resembling sebocytes, guiding us toward optimised TF combinations for effective differentiation. The reporter line system further refines this strategy by preferentially isolating cells expressing sebocyte markers for targeted transcriptomic analysis. The successful generation of human sebocyte models offers a unique opportunity to explore the cellular, molecular, and genetic factors related to human sex and their impact on neuroendocrine communication with the skin. Additionally, these models could provide a physiologically relevant platform for improving gender-affirming care for sex/gender-diverse individuals by studying individual-specific genetics in response to environmental factors.

Recommendations

Future directions include iteratively updating the TF list using the hiPSC reporter lines and creating polycistronic cassettes for precise TF combinations to optimise the cell fate engineering process (Appleton *et al.*, 2023). Further steps should involve testing different hiPSC lines, adjusting medium compositions with exogenous factors, developing functional testing pipelines for sebocytes, and evaluating the maintenance of engineered sebocytes in culture. Controlled genomic integration should be achieved by targeting safe-harbour loci or using non-integrating episomal vectors (Cho *et al.*, 2023; Csatári *et al.*, 2024). In the longer term, sequential, temporal, and quantitative control of TF induction can be accomplished by developing tunable gene circuits and titratable promoters (Azizoglu *et al.*, 2021; Velazquez Sanchez *et al.*, 2023; Xie *et al.*, 2023; Mao *et al.*, 2024). Ultimately, this approach will enable parallel programming of sebocyte-

related cell types that facilitate the generation of assembloids to investigate cellular, molecular, and genetic factors related to human sex (Ng *et al.*, 2021; Bellon, 2024; Zhao & Haddad, 2024). Furthermore, defining a cell type requires interrogation with advancements in gene expression analysis to ensure it is data-driven (Marx, 2024). Beyond gene expression for defining cell types, it is crucial to consider lineage history and spatiotemporal observations of the cell's physical structure and organisation (Domcke & Shendure, 2023; Rafelski & Theriot, 2024).

Developing human sebocytes as a cell model using hiPSCs could elucidate important cellular, molecular, and genetic aspects of human sex, especially given that sebocytes are highly responsive to gonadal steroid hormones (Güell & Schneider, 2023). Validating findings on sebocyte development from animal studies using human sebocytes would benefit this research (Zouboulis *et al.*, 2022; Güell & Schneider, 2023). Sebocytes communicate with the nervous system through neuroendocrinological pathways, as the sebaceous gland is richly vascularised and replete with microcirculation. Whether sebocytes are directly innervated remains unclear. Establishing how these communication pathways regulate sebum production and sebocyte behaviour is crucial (Clayton *et al.*, 2020). Given the important roles of TEs in sex, I am particularly interested in their roles in relation to sebocytes. Future studies should investigate how sebocyte interactions with the nervous system contribute to neuronal plasticity and diversity through TE effects on transcriptional networks and three-dimensional genome dynamics (Lawson *et al.*, 2023; Le Breton *et al.*, 2024; Ravens *et al.*, 2024).

Alternative methods for generating sebocytes could offer new avenues for investigating sex variability. Sebocytes could be transdifferentiated from donor tissue without reprogramming to hiPSCs (Pitrez *et al.*, 2024). This approach is advantageous when preserving non-genomic features such as epigenetic signatures, which could be erased during hiPSC reprogramming. Transdifferentiation also retains cellular heterogeneity from the donor tissue, resulting in polyclonal sebocytes that reflect the diverse cell population of the original tissue. In contrast, sebocytes derived from hiPSCs are monoclonal, originating from a homogeneous hiPSC population. Despite this monoclonality, hiPSC-derived sebocytes could still provide a platform for investigating

non-genomic biological relationships related to sex variability. Researchers can introduce environmental stressors or elicit molecular effects through gene editing, enabling the exploration of various factors influencing sex variability within these cells. This could be useful in constructing how sex variability affects gender-diverse individuals at the cellular level and in testing gender-affirming hormonal therapies relevant to specific individuals.

Existing frameworks for studying human sex primarily focus on medical therapeutics for conditions related to fertility, reproduction, and gonadal development that have been pathologized by medicine. While these topics may have value, research must also explore broader aspects of human sex variability such as non-binary gender identities, asexuality, and diverse sexual experiences (Kennis *et al.*, 2024). While neuroscience studies have begun to explore these topics in humans (Aghi *et al.*, 2022; Goberdhan, V; Darolti, I; Mank, JE; Corral-Lopez, A, 2023; Aghi, Goetz, *et al.*, 2024), there remains a significant gap in constructing the molecular, cellular, and genetic foundations related to human sex. This research gap affects scientific discourse, societal perceptions, policies, and funding priorities, around the LGBTQ2S+ communities, which have been historically excluded from scientific inquiry (Danos *et al.*, 2023; Smiley *et al.*, 2024; Alexander *et al.*, n.d.). Therefore, prioritising research that addresses the molecular, cellular, and genetic aspects of human sex is crucial for advancing gender-affirming care and avoiding the wrongful pathologisation of sex variability. Community-informed approaches and the involvement of researchers with transgender, non-binary, and gender-diverse identities are essential to addressing these gaps. This also begins with recognising that sex exists across multiple levels and how they dynamically interact in a non-essentialist way. Constructing the interplay between the nervous system and the skin could be part of this endeavour to engage with the multidimensionality of sex/gender. More broadly, these approaches can help improve the social, political, and medical conditions of sex/gender-diverse individuals (Sharpe *et al.*, 2023; Aghi, Goetz, *et al.*, 2024; Smiley *et al.*, 2024).

Figures & Legends

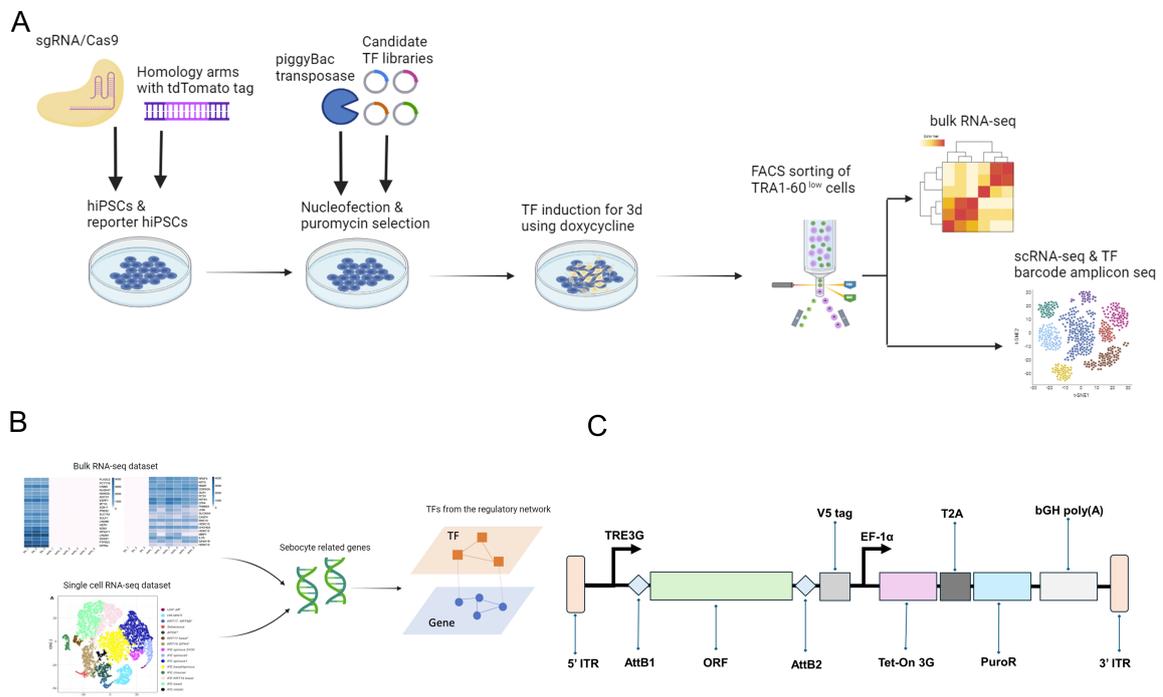


Figure 13 Strategies for human induced pluripotent stem cells engineering of sebocytes

A) The process starts with introducing piggyBac transposase and a library of candidate transcription factors (TFs) into hiPSCs via nucleofection. Successful integration of the TFs is confirmed by selecting for puromycin resistance. TF expression is induced by applying doxycycline for three days, prompting differentiation. Cells with low TRA-1-60 expression, indicative of reduced pluripotency, are isolated using fluorescence-activated cell sorting (FACS). These cells are then prepared for bulk and single-cell RNA sequencing. Single-cell sequencing includes amplifying TF barcodes to identify which exogenous TFs drive differentiation. This image was created with the assistance of Dr Lily Li. B) Sebocyte-related genes are identified by analysing differentially expressed genes from bulk and single-cell RNA sequencing datasets. TFs are included if they are differentially expressed or regulate genes within differentially expressed gene regulatory networks. This image was created by Dr Lily Li. C) The PBAN all-in-one Tet-On PiggyBac vector includes inverted terminal repeats (ITRs) for integration into the genome via piggyBac transposase. The Tet-On system uses doxycycline to activate the Tet-On 3G transcriptional activator, which binds to the tetracycline-responsive element 3G (TRE3G),

driving the expression of the target TF. AttB1 and AttB2 sites facilitate Gateway cloning, while the V5 tag aids in detection and purification. The vector contains the human elongation factor 1 alpha (EF-1 α) promoter for high expression levels and the T2A self-cleaving peptide for co-expression of multiple proteins from a single transcript. The puromycin resistance gene (PuroR) ensures the selection of cells that have integrated the vector, and the bovine growth hormone polyadenylation signal (bGH PolyA) facilitates transcription termination.

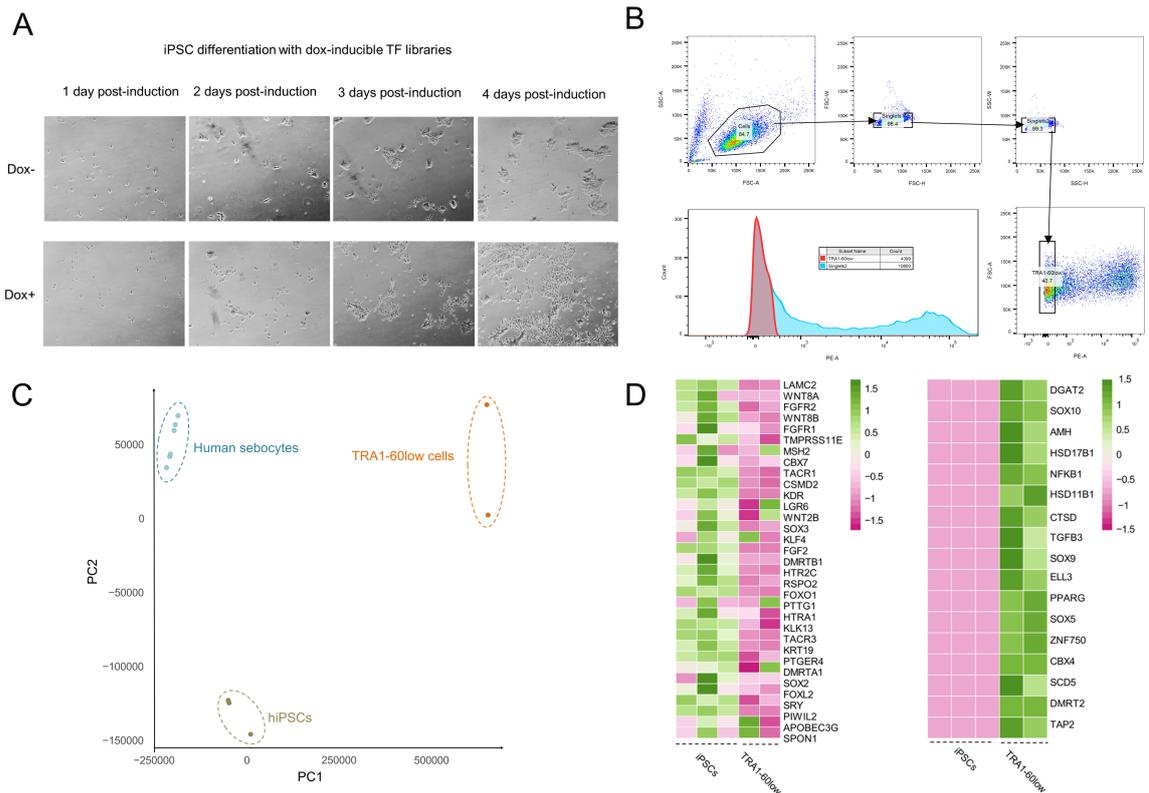


Figure 14 Bulk RNA-sequencing established the feasibility of proof-of-principle cell fate engineering strategy for obtaining sebocytes.

A) Representative images display changes in cell morphology following induction with and without doxycycline in hiPSCs nucleofected with TF libraries. B) Flow cytometry analysis measures TRA1-60 expression, an indicator of pluripotency. The gating strategy sequentially filters cells using forward (FSC-A) and side (SSC-A) scatter areas, followed by singlet selection based on forward (FSC-W, FSC-H) and side (SSC-W, SSC-H) scatter widths and heights. TRA1-60 low cells are identified through PE fluorescence signals via antibody staining. The density plot outlines these gating steps, while a histogram overlays the distribution of TRA1-60 expression levels, highlighting TRA1-60 low cells for further analysis. C) Principal component analysis (PCA) compares transcriptome profiles of undifferentiated iPSCs, human sebocytes, and TRA1-60 low cells sorted for bulk RNA sequencing. This comparison helps assess the differentiation status and transcriptional similarity of engineered cells to sebocytes. This image was created with assistance from Dr Lily Li. D) A heatmap shows differentially expressed genes relevant to sebocyte biology, transposable elements, and sex-related variables. The colour gradient represents

the level of gene regulation, with shades of pink indicating downregulation and intensities of green indicating upregulation. This image was created by Dr Lily Li.

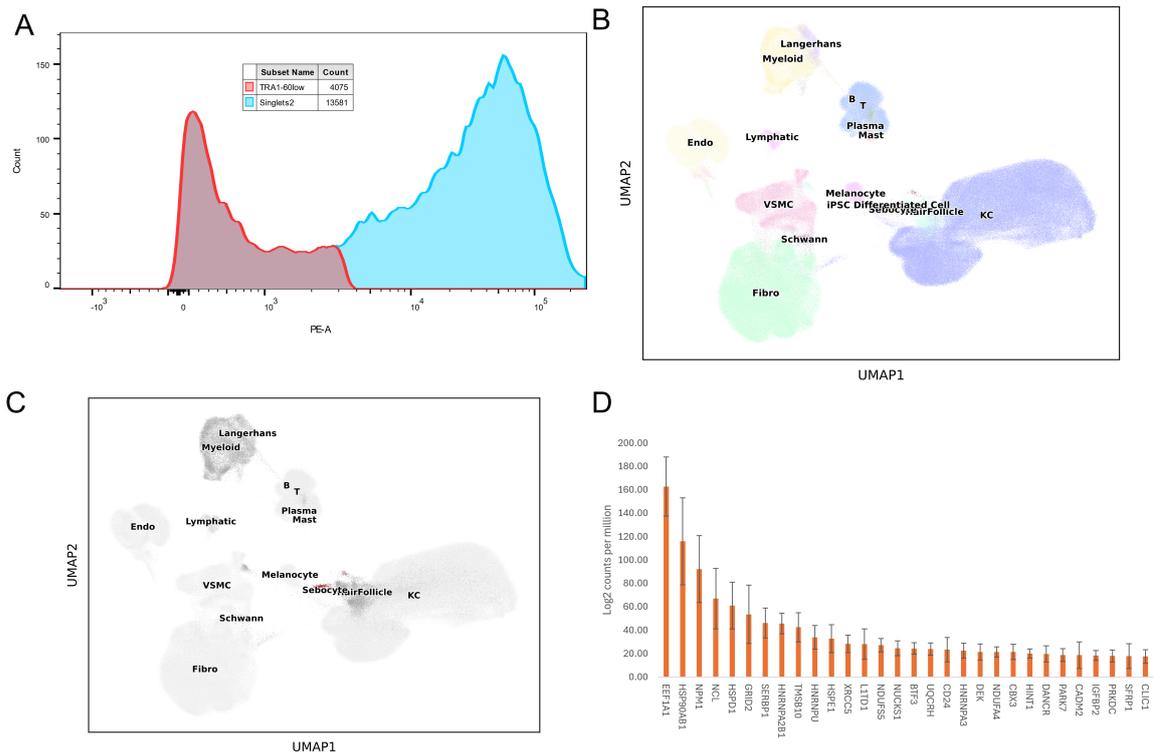


Figure 15 Single-cell RNA-sequencing mapped engineered cells to sebocytes

A) A histogram displays the fluorescence intensity of phycoerythrin (PE) signals from TRA1-60 antibody staining in sorted TRA1-60 low cells intended for single-cell RNA sequencing. The PE signals from all gated singlet cells are included to illustrate the selection of TRA-1-60 low cells. B) The Uniform Manifold Approximation and Projection (UMAP) plot provides an annotated visualization of major skin cell types, including B cells, endothelial cells (Endo), fibroblasts (Fibro), hair follicle cells, keratinocytes (KC), Langerhans cells, lymphatic cells, mast cells, melanocytes, myeloid cells, plasma cells, Schwann cells, sebocytes, T cells (T), and vascular smooth muscle cells (VSMC). This image was created by Zhengkuan Tang. C) SKIN-TOSICA mapping enables the classification of engineered cells to skin cell types. Engineered cells that resemble human sebocytes transcriptomically are mapped as red colour in the UMAP. This image was created by Zhengkuan Tang. D) Selected genes highly expressed in the engineered cells mapped to sebocytes are depicted in log₂ counts per million. These genes, chosen for their relevance to sex-related characteristics or transposable elements, provide insights into the transcriptional landscape of engineered sebocyte-like cells.

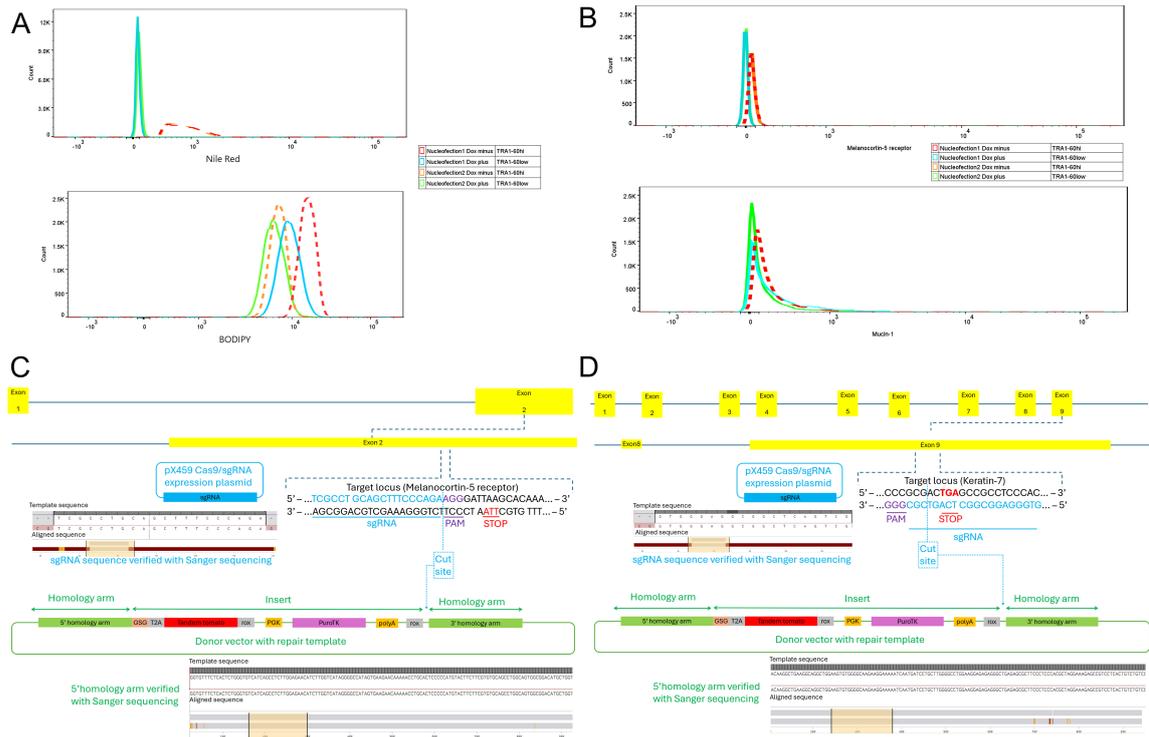


Figure 16 Gene-editing strategies were designed to have cells fluoresce once they start expressing sebocyte markers

A) Flow cytometry was used to count cells stained with Nile Red and BODIPY. Cells were gated based on TRA1-60 expression and analysed in transcription factor nucleofection duplicates treated with (solid line) or without (dashed line) doxycycline to induce transcription factor expression. B) A flow cytometry histogram shows the expression levels of melanocortin-5 receptor and mucin-1 in cells gated on TRA1-60 expression. Duplicates of transcription factor nucleofection were treated with (solid line) or without (dashed line) doxycycline to induce transcription factor expression. C) The CRISPR/Cas9 strategy involves inserting a tandem tomato fluorescent tag into the C-terminal exon of the melanocortin-5 receptor near the stop codon (STOP). The single-guide RNA (sgRNA) is shown with the cut site and protospacer adjacent motif (PAM). This sgRNA was cloned into the pX459 Cas9/sgRNA expression plasmid and verified through Sanger sequencing. Homology arms were designed to flank the cut site and insert a cassette containing glycine-serine-glycine linker (GSG), a self-cleaving T2A peptide from *Thoesa asigna* virus, the tandem tomato tag, and a selection marker consisting of rox, phosphoglycerate kinase (PGK), puromycin resistance, thymidine kinase (PuroTK),

and a polyadenylation signal (polyA). The left homology arm was cloned into the donor vector and validated through Sanger sequencing. D) The CRISPR/Cas9 strategy for tagging the C-terminal exon of keratin-7 with a tandem tomato fluorescent marker near the stop codon (STOP) is depicted. The sgRNA is shown with its cut site and protospacer adjacent motif (PAM). This sgRNA was cloned into the pX459 Cas9/sgRNA expression plasmid and verified through Sanger sequencing. Homology arms were created on either side of the cut site to insert a cassette similar to that used for the melanocortin-5 receptor, containing the GSG linker, T2A peptide, tandem tomato tag, rox, PGK, PuroTK, and polyA. The left homology arm was then cloned into the donor vector and validated through Sanger sequencing.

Tables

Table 2 Transcription factors selected for sebocyte engineering

Transcription Factor	Engineering Round	Barcodes (applicable to Round2)	Selection Evidence
ATF2	1		From Gene regulatory network
ATF3	1		Sebocyte transcriptome differential expression
CEBPB	1		(Zouboulis, Baron, <i>et al.</i> , 2008)
CEPBD	Not yet included		Gene regulatory network to sebocyte transcriptome
CTCF	1		Gene regulatory network to sebocyte transcriptome
CTNNB1	Not yet included		Gene regulatory network to sebocyte transcriptome
DBP	2	TTCGTA C TACGCCACGCA T	Gene regulatory network to sebocyte transcriptome
DLX6	2	TGTCAGTGGATATCAACGT T	Gene regulatory network to sebocyte transcriptome
E2F1	1		Gene regulatory network to sebocyte transcriptome
EGR1	1		Gene regulatory network to sebocyte transcriptome
ESR1	1		Gene regulatory network to sebocyte transcriptome
ESRRG	1		Sebocyte transcriptome differential expression
ESX1	2	TGGCGAATATGTGAGGGTT A	Gene regulatory network to sebocyte transcriptome
EVX1	2	TTAGAACGATGTGGAGGAG T	Gene regulatory network to sebocyte transcriptome
EZH1	1		Gene regulatory network to sebocyte transcriptome
FEV	2	TTATCCGCAACGCTTGCTTT	Gene regulatory network to sebocyte transcriptome
FOXA1	Not yet included		Gene regulatory network to sebocyte transcriptome
FOXF2	1		Gene regulatory network to sebocyte transcriptome
GRHL1	2	GGCTAACATAGGGACCTCA T	Gene regulatory network to sebocyte transcriptome
HDAC1	Not yet included		Gene regulatory network to sebocyte transcriptome
HLF	2	TCATGTTGGACGCCTCGCG T	Gene regulatory network to sebocyte transcriptome
HOXA1	Not yet included		Gene regulatory network to sebocyte transcriptome
HOXA9	2	GGTGCTTGCCTGAGGCTAC A	Gene regulatory network to sebocyte transcriptome
HOXB7	1		Gene regulatory network to sebocyte transcriptome
HOXC12	2	TATTGGCTCGACTGAACCT T	Gene regulatory network to sebocyte transcriptome
HOXD12	2	TTCTACATCGACGTTGGTC C	Gene regulatory network to sebocyte transcriptome
HSFY2	2	ACTGGCGGTCAGTTATGAT G	Gene regulatory network to sebocyte transcriptome
JUN	Not yet included		Gene regulatory network to sebocyte transcriptome
JUND1	Not yet included		Gene regulatory network to sebocyte transcriptome
KLF4	Not yet included		Gene regulatory network to sebocyte transcriptome
KLF5	Not yet included		Gene regulatory network to sebocyte transcriptome

LBX1	2	GAGGATGTGGTGTGTCTCA G	Gene regulatory network to sebocyte transcriptome
LMX1B	2	TTGCTATGGGCTGCCTAGA T	Gene regulatory network to sebocyte transcriptome
MEF2B	2	GTGTGTGAGACATCATGGT G	Gene regulatory network to sebocyte transcriptome
MEF2D	2	GTGAGGATGACTGAGGCGT G	Gene regulatory network to sebocyte transcriptome
MEOX1	2	CAGTGTCTCTGTGTGCCAT C	Gene regulatory network to sebocyte transcriptome
NFIC	Not yet included		Gene regulatory network to sebocyte transcriptome
NFKB1	Not yet included		Gene regulatory network to sebocyte transcriptome
NOBOX	2	TGGCCTCGTATTGGAGTTG C	Gene regulatory network to sebocyte transcriptome
OLIG1	2	CTGTCCCATTGGAAGATCG T	Gene regulatory network to sebocyte transcriptome
OLIG2	2	CGTTACAGGATGGTGTGTG G	Gene regulatory network to sebocyte transcriptome
POU2F1	1		Gene regulatory network to sebocyte transcriptome
POU4F2	1		Gene regulatory network to sebocyte transcriptome
PPARA	1		(Zouboulis, Baron, <i>et al.</i> , 2008)
PPARD	1		(Zouboulis, Baron, <i>et al.</i> , 2008)
PPARG	1, 2	ATGCCAGAGGAAATGAAG GT	(McNairn <i>et al.</i> , 2013)
PRDM1	1,2	TTGGTCCGATGCACCACAT T	(Horsley <i>et al.</i> , 2006)
RELA	1		Gene regulatory network to sebocyte transcriptome
RNF14	Not yet included		Gene regulatory network to sebocyte transcriptome
RUNX1	1		Sebocyte transcriptome differential expression
RUNX3	Not yet included		Gene regulatory network to sebocyte transcriptome
SMAD3	1		Gene regulatory network to sebocyte transcriptome
SMAD4	Not yet included		Gene regulatory network to sebocyte transcriptome
SOX9	1, 2	CTTCATAAGGAGGCGTGA G	(Shi <i>et al.</i> , 2017)
SP1	1		Gene regulatory network to sebocyte transcriptome
STAT3	Not yet included		Gene regulatory network to sebocyte transcriptome
STAT6	Not yet included		Gene regulatory network to sebocyte transcriptome
TBX1	2	TTGTGTTTCGAGCGGTGGG A	Gene regulatory network to sebocyte transcriptome
TBX20	2	GAGTCAGTTGGCAAGGTG G	Gene regulatory network to sebocyte transcriptome
TCF7L1	2	GCTGGACACCATTTGTTGT G	Gene regulatory network to sebocyte transcriptome
TEF	2	ACTGATTCGGCACTTGAAT T	Gene regulatory network to sebocyte transcriptome
TLF4	Not yet included		Gene regulatory network to sebocyte transcriptome
TP63	2	AAATTCTCAAGTGCACGTC A	Gene regulatory network to sebocyte transcriptome
TSC22D3	Not yet included		Gene regulatory network to sebocyte transcriptome
USF1	Not yet included		Gene regulatory network to sebocyte transcriptome
USF2	Not yet included		Gene regulatory network to sebocyte transcriptome
VAX1	2	AACATATGGTCTGCTACGG G	Gene regulatory network to sebocyte transcriptome

VAX2	2	GGAGGATAAGGGTGAAAG CA	Gene regulatory network to sebocyte transcriptome
VDR	1		(Zouboulis, Baron, <i>et al.</i> , 2008)
XBP1	Not yet included		Sebocyte transcriptome differential expression
ZBTB10	1		Sebocyte transcriptome differential expression
ZNF24	1		Gene regulatory network to sebocyte transcriptome
ZNF333	2	TAAGCTGTTAGTACTCGGC A	Gene regulatory network to sebocyte transcriptome

Table 3 Differentially expressed genes in bulk RNA sequencing comparing induced-pluripotent stem cells with TRAI-60low cells

Gene	log ₂ Fold Change	Adjusted p-value	Relevance	Reference
SOX10	10.39	0.000000	SOX10 has been implicated in disorders of gonadal development.	(Sreenivasan <i>et al.</i> , 2022)
SOX9	6.05	0.000000	SOX9 facilitates proliferation, differentiation, and lipogenesis in sebocytes.	(Li, Zeng, <i>et al.</i> , 2023)
DMRT2	6.03	0.000170	DMRT2 is involved in gonadal development, including the development of sex organs.	(Kikkawa & Osumi, 2021)
PPARG	5.78	0.000000	Sebaceous gland reprogramming could be facilitated through PPARG.	(Liu, Ji, <i>et al.</i> , 2023)
HSD11B1	5.42	0.000340	Ovoid immature Leydig cells express HSD11B1	(Zhan <i>et al.</i> , 2020)
SOX5	4.71	0.000000	Gene variants of SOX5 are involved in the pathogenesis of gonadal development	(Qian <i>et al.</i> , 2021)
HSD17B1	4.40	0.000000	HSD17B1 is expressed in the foetal testis converts A-dione to T.	(Sipilä <i>et al.</i> , 2020)
TGFB3	3.70	0.000000	TGFB3 may be required in the early step of sebaceous gland development	(Lin <i>et al.</i> , 2011)
ELL3	3.62	0.000000	Long Interspersed Nuclear Element-1 5' untranslated regions are marked by ELL3, which function as enhancers to regulate naïve pluripotency in embryonic stem cells	(Meng <i>et al.</i> , 2023)
TAP2	3.58	0.000000	TAP2 had low expression in subjects with two copies of Alu-1023, and it was highly expressed in subjects without any copies of Alu-1023.	(Koks <i>et al.</i> , 2021)
AMH	3.56	0.000460	Oestrogen inhibits AMH during the critical time of gonadal determination.	(Ruan <i>et al.</i> , 2024)
ZNF750	3.43	0.000040	ZNF750, which controls epithelial homeostasis by regulating epidermal differentiation, is expressed in ocular and extraocular sebaceous carcinoma	(Cheng <i>et al.</i> , 2022)
SCD5	3.39	0.000000	In receptor tyrosine-protein kinase erbB-3-depleted sebaceous cells, there was an upregulation of SCD5.	(Dahlhoff <i>et al.</i> , 2015)
CTSD	3.23	0.000000	CTSD stains intensely in sebaceous glands.	(Koenig <i>et al.</i> , 2020)

NFKB1	3.15	0.000000	NFKB1 was altered in human SZ95 sebocytes after 24 h pre-incubation with linoleic acid.	(Zouboulis, Hou, <i>et al.</i> , 2023)
CBX4	2.98	0.000000	5-aza-dC affects gonadal development, which correlates with inhibited CBX4.	(Ribas <i>et al.</i> , 2017)
DGAT2	2.18	0.000000	perilipin 2 affects sebaceous lipogenesis, which correlates with the decrease in DGAT2	(Camera <i>et al.</i> , 2014)
PTTG1	-2.02	0.000000	Gene expression signatures of transposable elements meta-cells included PTTG1	(Brocks <i>et al.</i> , 2019)
MSH2	-2.07	0.000000	MSH2 is inserted with the transposable element TC1 in <i>C. elegans</i>	(Degtyareva <i>et al.</i> , 2002)
APOBEC3G	-2.07	0.000000	APOBEC3G silence TEs	(Lawlor & Ellison, 2023)
SPON1	-2.08	0.000000	Intronic endogenous retroviruses polymorphism exhibited truncated Spon1	(Li <i>et al.</i> , 2012)
KLF4	-2.09	0.000000	KLF4 facilitates lipid production in immortalized human sebocytes via regulating the expression of sterol regulatory element-binding protein 1	(Li, Zeng, <i>et al.</i> , 2023)
SOX2	-2.17	0.000000	SOX2 was shown to repress Long Interspersed Nuclear Element-1-H transcription in neural stem cells.	(Hermant & Torres-Padilla, 2021)
LGR6	-2.20	0.003280	Lgr6+ cells contribute exclusively differentiated progeny to the sebaceous gland	(Teder-Laving <i>et al.</i> , 2023)
DMRTA1	-2.24	0.000000	DMRT genes encode a large family of transcription factors involved in gonadal development. In vertebrates, Dmrt- family genes are primarily involved in the development of sex organs.	(Kikkawa & Osumi, 2021)
FGFR1	-2.34	0.000000	FGFR1 plays a role in neurons that control the secretions of downstream gonadal hormones by producing Gonadotropin hormone-releasing hormone, which affects gonadal development before birth or during puberty	(Ying <i>et al.</i> , 2020)
CBX7	-2.45	0.000000	CBX7 is a component of the histone-modifying complex protein regulator of cytokinesis 1 and is linked to gonadal determination.	(Renn & Hurd, 2021)
WNT2B	-2.47	0.005048	Wnt2b is expressed in foetal gonads and may be relevant for germ cell survival and maintenance.	(Overeem <i>et al.</i> , 2021)
HTRA1	-2.76	0.000000	In the postnatal ovary, which in many ways is still a developmental stage, bone morphogenetic protein antagonists are expressed in oocytes and granulosa cells, including Htra1.	(Patton <i>et al.</i> , 2021)

FGFR2	-2.77	0.000000	Loss of function Fgfr2 mutations have been linked to X, Y-linked disorders. A large heterozygous genomic deletion including FGFR2, was detected in a 46, XY individual with ambiguous genitalia.	(Bird <i>et al.</i> , 2020)
FOXO1	-3.50	0.000000	FoxO1 and sebaceous lipogenesis	(Melnik, 2010)
FOXL2	-3.64	0.000941	FOXL2 encodes a transcription factor that regulates genes in gonadal development, and ovarian function.	(Tucker, 2021)
TACR3	-4.21	0.000000	Kispeptin1 neurons in the arcuate nucleus of the hypothalamus express TacR3.	(Rønnekleiv <i>et al.</i> , 2022)
CSMD2	-4.23	0.000000	Exapted elements accumulate in CSMD2.	(Kamm <i>et al.</i> , 2013)
HTR2C	-4.30	0.000000	HTR2C is X-linked	(Pinsonneault & Sadée, 2015)
PIWIL2	-4.30	0.000000	Piwi-interacting RNAs originate from transposable elements and silence them, which are associated with PIWIL2.	(Loubalova <i>et al.</i> , 2023)
KDR	-4.32	0.000000	KDR is activated in undifferentiated sebocytes, and it should participate in sebocytes in the proliferative state, such that the synthesis and metabolism of lipids promote sebaceous gland secretion.	(Wu <i>et al.</i> , 2019)
PTGER4	-4.32	0.000000	PTGER4 is important for the luteal cycle	(Braun & Jewgenow, 2022)
TMPRSS11E	-4.34	0.000000	TMPRSS11E expression is epithelial-specific and includes the testes.	(Rittié <i>et al.</i> , 2016)
KLK13	-4.53	0.000000	KLK13 is expressed in sebaceous glands	(Komatsu <i>et al.</i> , 2003)
SRY	-4.59	0.000060	SRY initiates testes formation	(Sreenivasan <i>et al.</i> , 2022)
KRT19	-4.67	0.000000	Sebocytes expresses Krt19	(Liu, Hong, <i>et al.</i> , 2023)
SOX3	-5.00	0.000000	Sox3 has been implicated in X, Y-linked disorders	(Sreenivasan <i>et al.</i> , 2022)
LAMC2	-5.03	0.000000	Piwi-interacting RNA in the intron of lamc2 could silence it.	(Chattopadhyay <i>et al.</i> , 2022)

FGF2	-5.10	0.000000	Fgf2 controls Leydig cell development and steroidogenesis	(Liang <i>et al.</i> , 2021)
TACR1	-5.64	0.000027	Kisseptin1 neurons in the arcuate nucleus of the hypothalamus express TacR1.	(Rønnekleiv <i>et al.</i> , 2022)
RSPO2	-5.80	0.000010	RSPO2 is an oocyte-specific gene	(Qin <i>et al.</i> , 2022)
WNT8B	-6.53	0.000000	Mice testes express WNT8B	(Kerr <i>et al.</i> , 2014)
WNT8A	-6.79	0.002260	Temperature alters levels of WNT8A in gonads.	(Rhen <i>et al.</i> , 2021)
DMRTB1	-7.35	0.000090	DMRTB1 is involved in gonad development	(Kikkawa & Osumi, 2021)

Table 4 Highly expressed genes in single-cell RNA sequencing mapping of engineered sebocytes using SKIN-TOSICA

Gene	Average log ₂ reads per million	Standard Error	Relevance	Reference
EEF1A1	163	25	EEF1A1 regulates temperature-dependent gonad determination	(Wang <i>et al.</i> , 2022)
HSP90AB1	116	37	Effects on HSP90AB1 have been found to differ between pups that were identified as different sex	(Barua <i>et al.</i> , 2016)
NPM1	92	29	NPM1 mutations exhibit sex variability	(Juliusson <i>et al.</i> , 2020)
NCL	67	26	NCL regulate totipotent gene expression with LINE1	(Wang <i>et al.</i> , 2024)
HSPD1	61	20	There are sex-variable expressions of HSPD1	(Schuldt <i>et al.</i> , 2021)
GRID2	54	25	Polymorphisms of GRID2 harbour differences in LINE1 that alter their expression in the testes	(Akagi <i>et al.</i> , 2008)
SERBP1	46	13	SERBP1 interact with PGRMC1 to regulate progesterone	(Shilenok <i>et al.</i> , 2023)
HNRNPA2B1	46	9	HNRNPA2B1 is an rCGG repeat binding protein where rCGG repeats activate the TE, Gypsy.	(Liu & Shi, 2021)
TMSB10	43	13	TMSB10 has sex variable expression.	(Zelco & Joshi, 2023)
HNRNPU	34	10	HNRNPU dissociates LINE1 RDE-containing RNA from euchromatin and acts on LINE1 RNP.	(Attig & Ule, 2019)
HSPE1	33	12	HSPE1 were differentially expressed in germ cells from BPA exposure	(Karmakar <i>et al.</i> , 2017)
XRCC5	28	7	XRCC5 regulate neuronal genome rearrangements with the TE-derived PGBD5.	(Zapater <i>et al.</i> , 2023)
L1TD1	28	13	L1TD1 associates with LINE-1 transcripts and interacts with LINE-1 ORF1p protein, thereby facilitating LINE-1 retrotransposition.	(Kavaklıoğlu <i>et al.</i> , 2024)
NDUFS5	27	6	NDUFS5 showed a dense appearance in the medial preoptic nucleus. Localization of Ndufs5 cells overlapped with gonadal steroid receptor and aromatase enzyme expressing neuronal clusters	(Lékó <i>et al.</i> , 2021)
NUCKS1	25	6	NUCKS1 was sex-variably expressed in Parkinson's disease	(Nordengen <i>et al.</i> , 2023)
BTF3	24	5	BTF3 is linked to gonadal phenotype in chickens	(Liu <i>et al.</i> , 2020)

UQCRH	24	5	UQCRH over-expressing mice exhibited sex variability delay to the onset of hyperglycaemia.	(Jacobs <i>et al.</i> , 2023)
CD24	23	10	In a mouse carcinogenesis model, CD24 exhibits sex-variability bladder tumour formation and metastasis	(Overdevest <i>et al.</i> , 2012)
HNRNPA3	23	6	HNRNPA3 highly expressed in the synaptic oogonia interacts with X chromosome inactivation escape and synaptonemal complex genes in single X chromosome oogonia.	(McGlacken-Byrne <i>et al.</i> , 2023)
DEK	21	7	There is a sex variable DEK expression in the anterior cingulate cortex and its association with dementia severity in schizophrenia	(O'Donovan <i>et al.</i> , 2018)
NDUFA4	21	4	NDUFA4 is a candidate gene in the sex-variability of diabetes in a rodent model	(Barkalifa <i>et al.</i> , 2010)
CBX3	21	7	CBX3 participate in TE silencing	(Hadziselimovic <i>et al.</i> , 2011)
HINT1	20	4	HINT1 exhibits sex variability in schizophrenia	(Jackson <i>et al.</i> , 2012)
DANCR	20	7	DANCR expression in the lung is a sex variable, relevant to SARS Cov2	(Meydan <i>et al.</i> , 2020)
PARK7	19	5	DNA methylation of PARK7 is a sex variable in Parkinson's disease	(Kochmanski <i>et al.</i> , 2022)
CADM2	19	11	CADM2 is associated with age at first sexual intercourse and may play a role in risk-taking behaviours and may be a gene involved in the nexus of cognitive and reward-related processes that underlie them.	(Strawbridge <i>et al.</i> , 2018)
IGFBP2	18	4	There are sex-variable levels of IGFBP2 in the serum and the hypothalamus in response to low-fat diet	(Guerra-Cantera <i>et al.</i> , 2020)
PRKDC	18	5	PRKDC expression levels may relate to testosterone	(Armoskus <i>et al.</i> , 2014)
SFRP1	18	10	SFRP1 regulate gonadal development in mice embryo	(Warr <i>et al.</i> , 2009)
CLIC1	18	6	Effects of CLIC1 knockout was sex variable	(Ulmasov <i>et al.</i> , 2017)

Table 5 Information on the antibodies and dyes

Antibody/Dye	Vendor	Catalogue Number	IgG	Clonality	RRID	Dilution per million cells
PE Mouse anti-Human TRA-1-60	BD Pharmingen	560193	Mouse (BALB/c) IgM, kappa	TRA-1-60	AB_1645539	20uL
Alexa Fluor 488 Mouse IgG2A	R&D Systems	IC003G	Isotype control	20102	AB_10718683	5uL
PE Mouse IgM, kappa	BD Pharmingen	555584	Isotype control	G155-228	AB_395960	20uL
Alexa Fluor 488 Mouse anti-MC5R	R&D Systems	FAB8205G	Mouse IgG2A	821012	AB_3095670	5uL
APC Human anti-MUC1	BioLegend	355608	Mouse IgG1, lambda	16A	AB_2565312	5uL
APC Mouse IgG1, kappa	BioLegend	400122	Isotype control	MOPC-21	AB_2665396	5uL
BODIPY™ 493/503 (4,4-Difluoro-1,3,5,7,8-Pentamethyl-4-Bora-3a,4a-Diaza-s-Indacene)	Invitrogen	D3922	N/A	N/A	N/A	1uL(10ug)
Nile Red	Invitrogen	N1142	N/A	N/A	N/A	1uL(30pmol)

Table 6 Information on the primers and oligos

Oligo ID#	Sequence	Purpose
NGS10x-F-i7-BC-PCR1F	GGAGTTCAGACGTGTGCTCTTCCGATCTCTTTCCAAGCACCTGCTACA TAG	Transcription factor amplicon-sequencing
i5000	AATGATACGGCGACCACCGAGATCTACACaactcgctACACTCTTTCCTAC ACGACGCTCTTC 671 CGATCT (lower case region represents a sample-specific barcode)	Transcription factor amplicon-sequencing
i7000	CAAGCAGAAGACGGCATAACGAGATtcgecttaGTGACTGGAGTTCAGACGT GTGCTCTCCGA674 TCT (lower case region represents a sample-specific barcode)	Transcription factor amplicon-sequencing
P5	AATGATACGGCGACCACCGA	Transcription factor amplicon-sequencing
P7	CAAGCAGAAGACGGCATAACGA	Transcription factor amplicon-sequencing
VSCC011_KRT7_sg RNA 1 Fwd	CACCGGTGGGAGGCGGCTCAGTCG	Reporter hiPSC lines
VSCC012_KRT7_sg RNA 1 Rev	AAACCGACTGAGCCGCTCCCACC	Reporter hiPSC lines
VSCC015_MC5R_s gRNA 1 Fwd	CACCGCGCCTGCAGCTTTCAGAA	Reporter hiPSC lines
VSCC016_MC5R_s gRNA 1 Rev	AAACTTCTGGAAAGCTGCAGGCGC	Reporter hiPSC lines
VSCC029_KRT7sg RNA1AmpR FWD	agtccttcggtcctccgatcgttgcagaagtaagtggccg	Reporter hiPSC lines
VSCC030_KRT7sg RNA1AmpR_REV	CTAAAGGTGTCTGTGGCCTAcggggaaatgtgcgcggaac	Reporter hiPSC lines
VSCC031_KRT7sg RNA1HAL FWD	gttcgcgcacattccccgTAGGCCACAGACACCTTTAGGATTTATGATACG	Reporter hiPSC lines
VSCC032_KRT7sg RNA1HAL_REV	CCTCTGCCCTCGCCAGAGCCCGGGCACTCCTGCGACTG	Reporter hiPSC lines
VSCC033_KRT7sg RNA1Cassette_FWD	CAGTCGCAGGAGTGCCCGGGGCTCTGGCGAGGGCAGAGG	Reporter hiPSC lines
VSCC034_KRT7sg RNA1Cassette_REV	AGCCGGTTGGCGCCTACCGGTGGATGTGGAATGTGTGCGAGG	Reporter hiPSC lines
VSCC035_MC5Rs gRNA1AmpR_FWD	agtccttcggtcctccgatcgttgcagaagtaagtggccg	Reporter hiPSC lines
VSCC036_MC5Rs gRNA1AmpR_REV	CACCTCTGTCTTGGCAGCAcggggaaatgtgcgcggaac	Reporter hiPSC lines
VSCC037_MC5Rs gRNA1HAL FWD	gttcgcgcacattccccgTGCTGCCAAGACAAGAGGTG	Reporter hiPSC lines
VSCC038_MC5Rs gRNA1HAL_REV	CCTCTGCCCTCGCCAGAGCCTGGAAAGCTGCAGGCGATC	Reporter hiPSC lines
VSCC039_MC5Rs gRNA1Cassette_FWD	GATCGCTGCAGCTTTCAGGCTCTGGCGAGGGCAGAGG	Reporter hiPSC lines
VSCC040_MC5Rs gRNA1Cassette_REV	AGCCGGTTGGCGCCTACCGGTGGATGTGGAATGTGTGCGAGG	Reporter hiPSC lines
U6	ACTATCATATGCTTACCGTAAC	Reporter hiPSC lines

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CHAPTER 6

GENERAL CONCLUSION

In this dissertation, I aimed to adopt sex/gender frameworks that produce rigorous, precise, and transparent science to advance conceptual constructions of sex/gender (Massa *et al.*, 2023; McLaughlin *et al.*, 2023; Aghi, Anderson, *et al.*, 2024; Ridgeway & Saperstein, 2024; Smiley *et al.*, 2024). Furthermore, I pivoted the implications of my research toward how it is applicable to sex/gender justice in the more-than-human world.

In Specific Aim 1, I developed automated behavioural analysis methods to assess sexual behaviour in B6D2F1 hybrid mice that display steroid-independent sexual behaviour (McGill & Tucker, 1964; McInnis *et al.*, 2016; David, Wyrosdic, Wan, *et al.*, 2022). I created classifiers for mount/intromission and ejaculation using DeepLabCut pose estimation from multi-cage-stacked video recordings in night vision under red light. This method significantly reduces the time researchers would spend manually annotating video recordings of sexual behaviours. In the future, it could capture the variability and individuality in rodent sexual behaviour beyond binary classifications of male and female behaviour (McLaughlin *et al.*, 2023; Volsche *et al.*, 2023).

To study sexual behaviour in a collective context, I developed automated behavioural analysis methods for the East African cichlid, *Astatotilapia burtoni* in Specific Aim 2. These cichlids exhibit lekking and have been used in neuroscience studies on social hierarchy and courtship (Fernald & Hirata, 1977; Culbert *et al.*, 2023;

Santos *et al.*, 2023). I created a DeepLabCut pose estimation model capable of tracking up to 24 cichlids in a tank and an analytical pipeline for kinematic analysis in a two-separated cichlids setup. This work advances the automation of tracking African cichlids, a process not yet seen in publications, and lays the foundation for achieving individual identification of each cichlid in a large community. I discussed the limitations of conducting neuroscience studies on sexual behaviour only using dyads that are singly isolated until experimentation (Sliwa, 2021; Vogt *et al.*, 2024). Such studies lack generalizability to diverse social and mating systems across species, such as lekking (Clutton-Brock, 2021; Kao, Hund, *et al.*, 2023; Rathore, Isvaran, *et al.*, 2023). Future efforts to construct a comprehensive framework around animal sexual behaviour could enhance animal welfare by integrating sexual behaviour considerations into ethical standards (Coria-Avila *et al.*, 2022; Cavallino *et al.*, 2023).

Specific Aim 3 tackled the challenge of using animals in the lab and captivity for sexual behaviour research (Mathuru *et al.*, 2020; Lambert, 2023). Studying wildlife offers insights into the neuroscience of sexual behaviour in natural environments that resemble more closely to where their brains have evolved (Hawkes *et al.*, 2021a; Cisek & Hayden, 2022). As camera traps are widely used to study sex diversity in the wild (Caravaggi *et al.*, 2020; Nazir & Kaleem, 2024), I aimed to develop a pipeline for examining sex diversity in camera trap datasets. I curated a quadruped camera trap dataset that could be used for future fine-tuning of the pose estimation performance of the DeepLabCut Model Zoo Super Animal Quadruped. The results of this provide a detailed portfolio of pose relationships associated with sex diversity, which could be used to identify sex diversity in camera trap datasets. I also created a framework to help neuroscientists evaluate the feasibility and ethics of conducting neuroimaging studies in minimally captive free-behaving wildlife. Observing sexual behaviour in the wild from a neuroscience perspective could contribute to conservation efforts for species and ecosystems (Boyce *et al.*, 2022; Clark *et al.*, 2023; Aguilar & Webb, 2024).

Specific Aim 4 focused on stem cell engineering with human sebocytes, a cell type relevant to human sex and neuroscience. I developed a pipeline that directly activates TFs in hiPSCs, creating cells that resemble human sebocytes transcriptomically. Once

optimized, these human sebocytes could be used to study sex-related mechanisms, such as TEs (Chiang, DeRosa, *et al.*, 2022). Human models capturing individual molecular, cellular, and genetic factors of sex could improve gender-affirming care for transgender, non-binary, and gender-diverse individuals, and thus help address gaps in the constructions of human sex variability (Aghi *et al.*, 2022; Aghi, Goetz, *et al.*, 2024)(Aghi, Goetz, *et al.*, 2024; Smiley *et al.*, 2024).

These aims touched upon various aspects of sex variability and diversity, emphasizing rigorous, precise, and transparent science to promote sex/gender justice and advance the constructions of sex/gender. Science constructs the world it measures including through the conceptual frameworks shaping the evidence obtained (Dubova & Goldstone, 2023; van Anders *et al.*, 2023). Therefore, I was mindful of how I conceptualised sex/gender, including the language I used. I acknowledged that sex/gender is complex, dynamic, and context-dependent, reflecting variability and diversity across taxa and genera (Massa *et al.*, 2023; McLaughlin *et al.*, 2023; Smiley *et al.*, 2024). I carefully considered what studies meant by sex/gender, whether they claimed it as a causal mechanism, and also how non-scientific inquiries into sex/gender could be beneficial (van Anders *et al.*, 2023; Pape *et al.*, 2024). It is important to transform science into a field that is inclusive of sex/gender diverse individuals and to use it to achieve sex/gender justice. Given science's significant impact on the lives of sex/gender diverse individuals and the extent to which current Western society is constructed around sex/gender conceptualisations, this transformation is crucial (Sharpe *et al.*, 2023; Aghi, Anderson, *et al.*, 2024; Ridgeway & Saperstein, 2024).

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BIOGRAPHICAL SKETCH

Vic Shao-Chih Chiang has been privileged to receive research training from various labs in food science and nutrition at the University of Auckland. Their experiences included working on grain infestation control under Prof Conrad Perera, and a postprandial multiomics clinical trial under Prof Siew-Young Quek and Prof David Cameron-Smith. This was followed by muscle nutrition microRNA studies in the same lab. They later pivoted their research focus towards neuroscience, guided by Prof Shin-ichi Ohnuma at UCL on the extracellular proteoglycans in the brain, and Prof Nancy Ip at HKUST on microglial multiomics. Before their PhD, they also learned from Prof Minmin Luo at Beijing Tsinghua, conducting research on reward circuit optogenetics.

Their volunteering experiences helped shape their commitment to social equity. Serving diverse communities in London, they worked to improve the nutrition of solitary living elderlies at Clarion Housing and health equity for refugees in Hong Kong with Health in Action. These helped motivate them to co-found the social impact organisation Savyn in Toronto, which focuses on developing just and equitable approaches to healing trauma in diverse communities fighting marginalisation. The same impetus to support underserved communities led them to this PhD. They will return to Savyn after their PhD, with initiatives such as leveraging neuroscience and machine learning to advance digital psychotherapies co-designed with the communities they serve including sex/gender-diverse individuals.

Vic has long been an advocate for epistemological diversity, hosting debates as part of Open Mind Discussion Forums since their time in London. Together with their husband, who shares these values, they run a video channel in Han that explores a range of topics, including sex/gender, through an epistemologically diverse lens. After their PhD, they will also finish their second book in Han together, which contemplates films as a medium of epistemological diversity. This book draws from films from a wide array of countries such as Lebanon, Lesotho, Romania, Chile, and Cambodia. This commitment to epistemological diversity is also reflected in Vic's PhD.