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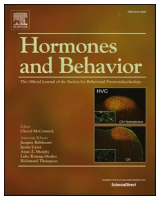

Uncovering the seasonal brain: Liquid chromatography-tandem mass spectrometry (LC-MS/MS) as a biochemical approach for studying seasonal social behaviors☆

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ABSTRACT

Many animals show pronounced changes in physiology and behavior across the annual cycle, and these adaptations enable individuals to prioritize investing in the neuroendocrine mechanisms underlying reproduction and/or survival based on the time of year. While prior research has offered valuable insight into how seasonal variation in neuroendocrine processes regulates social behavior, the majority of these studies have investigated how a single hormone influences a single behavioral phenotype. Given that hormones are synthesized and metabolized via complex biochemical pathways and often act in concert to control social behavior, these approaches provide a limited view of how hormones regulate seasonal changes in behavior. In this review, we discuss how seasonal influences on hormones, the brain, and social behavior can be studied using liquid chromatography-tandem mass spectrometry (LC-MS/MS), an analytical chemistry technique that enables researchers to simultaneously quantify the concentrations of multiple hormones and the activities of their synthetic enzymes. First, we examine studies that have investigated seasonal plasticity in brain-behavior interactions, specifically by focusing on how two groups of hormones, sex steroids and nonapeptides, regulate sexual and aggressive behavior. Then, we explain the operations of LC-MS/MS, highlight studies that have used LC-MS/MS to study the neuroendocrine mechanisms underlying social behavior, both within and outside of a seasonal context, and discuss potential applications for LC-MS/MS in the field of behavioral neuroendocrinology. We propose that this cutting-edge technology will provide a more comprehensive understanding of how the multitude of hormones that comprise complex neuroendocrine networks affect seasonal variation in the brain and behavior.

1. Introduction

In vertebrates, distinct physiological mechanisms underlie phenotypes associated with specific life-history stages, and these processes are influenced by naturally occurring shifts in environmental cues, such as photoperiod (i.e., day length), temperature, humidity, and food availability (Stevenson et al., 2017; reviewed in Wingfield, 2018). In particular, temperate- and arctic-reproducing organisms show dramatic variation in annual reproductive rhythms, including endocrine cycles, gonadal changes, and associated social behaviors, that enable individuals to maximize reproductive success when abiotic and biotic resources are abundant (Fig. 1; reviewed in Pradhan et al., 2015; Ramenofsky and Wingfield, 2017). Reproductive behavior and secondary sexual characteristics are regulated through interconnected endocrine networks of biomolecules, such as peptides, amines, binding proteins, steroid hormones, receptors, and enzymes (Fig. 1; reviewed in Payne and Hales, 2004; Pradhan et al., 2015; Taff and Vitousek, 2016). Once produced, hormones can activate intracellular biochemical pathways and/or bind to nuclear response elements to modulate the expression of genes, enabling cells and whole organisms to express these phenotypes during future responses (reviewed in Balthazart et al., 2003; Hoffmann et al., 2012; Maruska et al., 2013; Silverthorn, 2019). Recent

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advances in high-throughput technology provide the opportunity to sequence and quantify the transcriptome, enabling the exploration of molecular mechanisms underlying a phenotype at the transcript level and, thus, greatly advancing the field of behavioral neuroendocrinology (reviewed in Balthazart, 2020).

1.1. Prohormones, enzymes, and the physiological landscape

While new tools have greatly expanded the physiological landscape, most studies that have investigated hormone-function relationships focus on establishing a link between one hormone and one phenotype (reviewed in Soma, 2006). Because the physiological milieu is comprised of a cocktail of hormones that produces pleiotropic effects in different organs, there is a disconnect in the spatial specificity of the location of hormonal action and its consequent measurement, particularly given that researchers commonly focus on measuring blood-borne hormones (reviewed in Bass and Grober, 2009; Schmidt et al., 2008; Schmidt and Soma, 2008). Often, prohormones circulate at high concentrations in the blood and bind to receptors with lower affinity compared to their more active end-products (Demas et al., 2007; reviewed in Schmidt et al., 2008; Soma, 2006). For example, steroid hormones, one class of hormones that regulate seasonal reproductive phenotypes, are synthesized in peripheral organs and within discrete regions of the central nervous system either *de novo* from cholesterol or via *in situ* metabolism from circulating prohormones (Figs. 1–2; reviewed in Do Rego et al., 2012). Testosterone (T), a sex steroid that is produced primarily by Leydig cells in the testes, can be converted within other tissues to either 17β-estradiol (E_2) via the enzyme aromatase (ARO) or to 5α-dihydrotestosterone (DHT) via the enzyme 5α-reductase (Fig. 2). These metabolites bind to estrogen and androgen receptors, respectively, with higher affinity than prohormones. In addition to the gonads, steroidogenic enzymes have been identified in almost all tissues across vertebrate taxa, including the brain, indicating that these tissues are capable of producing steroids *de novo* (Aizawa et al., 2010; Callard et al., 1979; reviewed in Do Rego et al., 2012; Kibaly et al., 2005; Kusakabe, 2002; Payne and Hales, 2004; Schulz et al., 2008; Weiss and Ford, 1977). Therefore, determining the activity of steroid-synthesizing and steroid-metabolizing enzymes is critical for elucidating the steroidogenic potential of tissues and how the function of these enzymes may change seasonally (reviewed in Pradhan and Soma, 2012).

In the brain and other tissues, conversion of prohormones to their more active end-products can also occur over relatively short time-scales through the rapid regulation of active steroidogenic enzymes (reviewed in Cornil and Charlier, 2010; Norris and Carr, 2020; Peterson et al., 2005; Pradhan et al., 2014b; Remage-Healey et al., 2008). Steroidogenic enzymes are important endogenous modulators of steroid concentrations that regulate both natural and pathological states (reviewed in Brocca and Garcia-Segura, 2018; reviewed in Do Rego et al., 2012; Hirotsu et al., 2015; Luu-The et al., 2008); however, which enzymes are active and the direction of activity is determined by endogenous

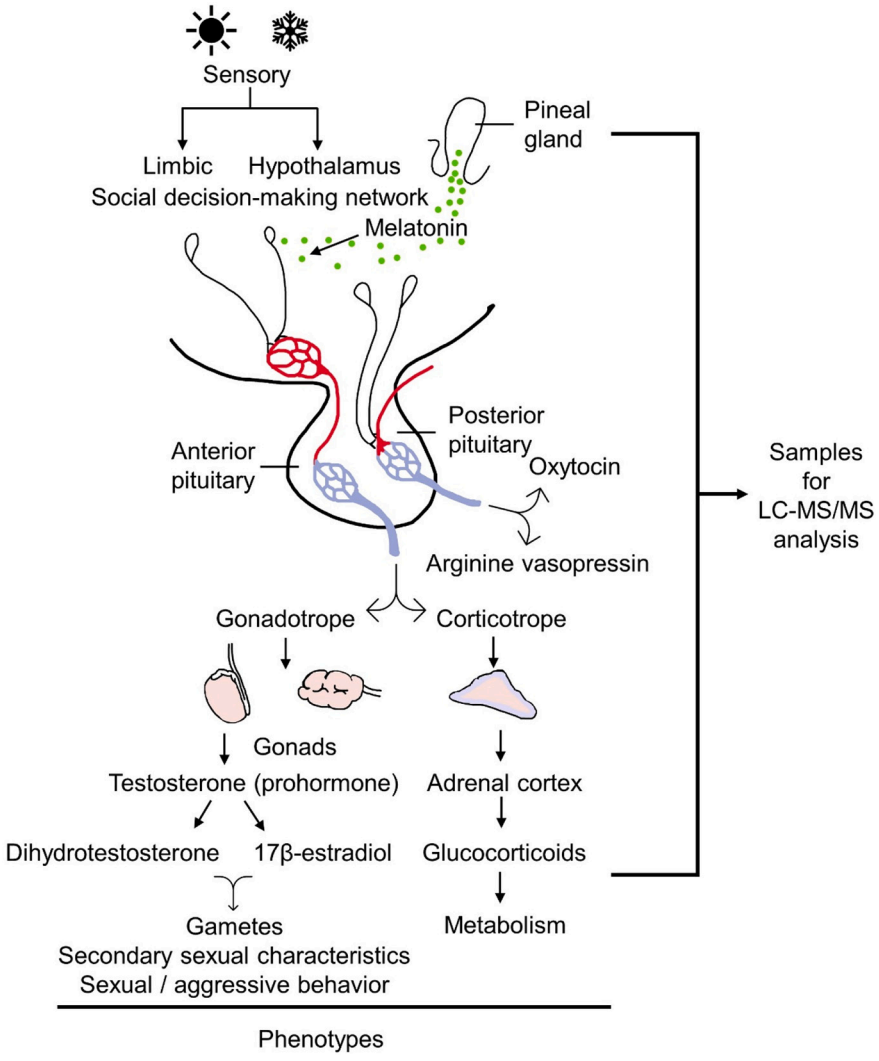

Fig. 1. Simplified conceptual framework for studying the neuroendocrine regulation of seasonal phenotypes. These mechanisms are influenced by environmental cues such as photoperiod (i.e., day length), which is biochemically encoded via the secretion of melatonin from the pineal gland, and sensory cues, which are detected and integrated by limbic and hypothalamic nuclei within the social decision-making network. Hormones that control reproduction, metabolism, and social behavior, including steroids, nonapeptides, and their precursors, can be extracted from a variety of tissues, such as endocrine glands (e.g., pituitary gland, gonads, adrenal cortex, and brain) and blood, and measured via liquid chromatography-tandem mass spectrometry (LC-MS/MS).

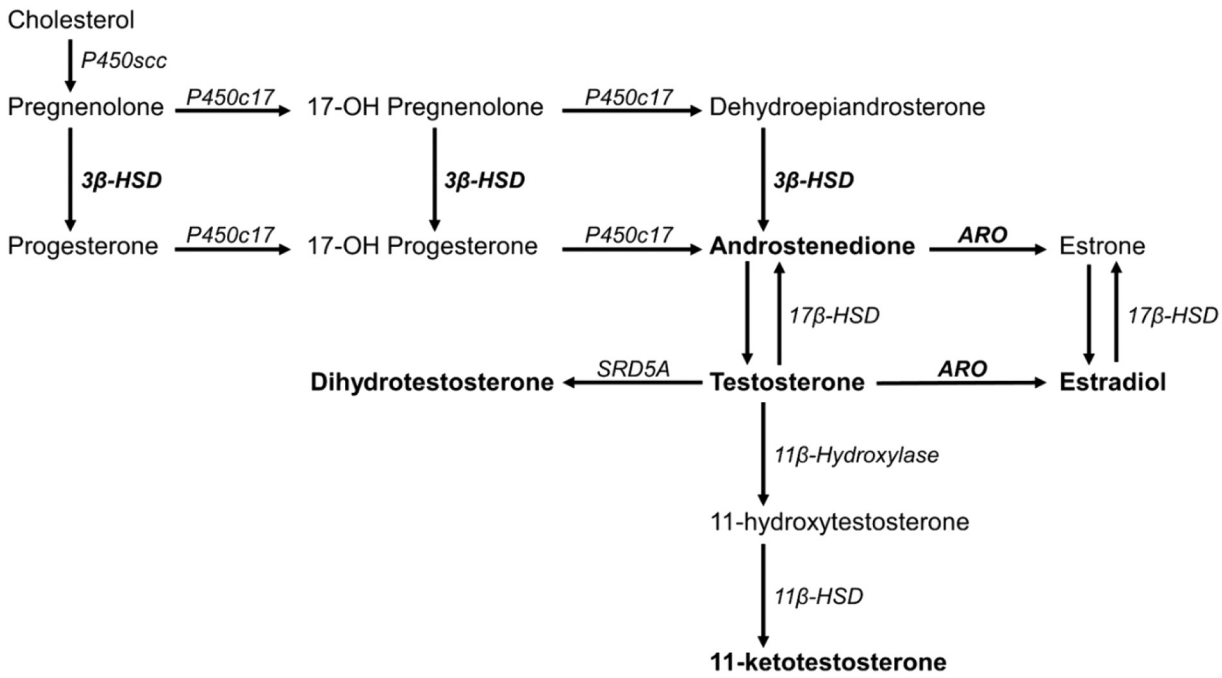

Fig. 2. Simplified diagram of sex steroid synthesis. Steroids are in normal font, and enzymes are in italics. Steroids and enzymes that have been a major focus of research investigating seasonal plasticity in the neuroendocrine regulation of sexual behavior and aggression (Section 2) are in bold. Abbreviations: ARO, aromatase; P450scc, cholesterol side-chain cleavage enzyme; P450c17, cytochrome P450c17; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 11β-HSD, 11β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; SRD5A, 5α-reductase.

posttranslational mechanisms and the microenvironment of the specific tissue, such as antioxidants, cofactor concentrations, phosphatases, and kinases (Balthazart et al., 2005; reviewed in Bull et al., 2002; Casagrande and Hau, 2018; Comito et al., 2016; Edmondson and Gadda, 2011; Sherbet et al., 2007). Sudden and dynamic changes in the external social environment, such as aggressive encounters, can rapidly affect changes in steroidogenic enzyme activity in the brain via these mechanisms (Charlier et al., 2011; reviewed in Cornil and Charlier, 2010; Pradhan et al., 2010b; Pradhan et al., 2014b; Remage-Healey and Bass, 2004; Remage-Healey et al., 2008). More predictable influences on the expression and activity of steroid-synthesizing enzymes in the brain and other peripheral tissues are associated with seasonal transitions or different phases within a particular season (Breton et al., 2015; Canoine et al., 2007; Freking et al., 2000; reviewed in Schlinger et al., 2006; Soma et al., 2003). For example, the breeding season can be comprised of morphological transformations and/or resource acquisition that influence the timing and expression of reproductive behavior via activation of the hypothalamus-pituitary-gonadal axis (Fig. 1; Wallen and Schneider, 1999; Wingfield et al., 2001). Thus, the specificity of temporal and spatial variation in steroidal mechanisms and function can be enhanced by examining changes at the biochemical level (reviewed in Balthazart et al., 2003; Pradhan et al., 2010a) and under appropriate environmental contexts (Fuxjager et al., 2009; Pradhan et al., 2014a).

While few neuroendocrinology laboratories investigate enzymology, the most popular approaches for measuring steroids are radioimmunoassays and enzyme immunoassays. These antibody-based techniques are commercially available, relatively easy to set up and use, and have low cross reactivities with closely related hormones; however, these methodologies often facilitate the measurement of a single hormone of interest and require a considerable volume of sample, a limitation that can be particularly problematic for scientists who study seasonal social behaviors. Thus, research that examines how a behavioral phenotype is regulated by multiple hormones rapidly increases the logistics of steroid quantification, such as time, materials, cost, and sample quantity. Conversely, nonapeptides, another group of hormones that have been studied extensively with respect to seasonal changes in social behavior, are mostly commonly investigated using *in situ* hybridization, immunohistochemistry, and receptor autoradiography to identify sites of anatomical localization. In vertebrates, this group of hormones includes oxytocin (OT), mesotocin (the non-mammalian tetrapod homolog of OT), isotocin (the teleost homolog of OT), arginine vasopressin (AVP), and arginine vasotocin (AVT; the non-mammalian homolog of AVP), all of which are synthesized in the magnocellular neurons of the supraoptic and paraventricular nuclei of the hypothalamus (Fig. 1; Castel and Morris, 1988; Maruska et al., 2007; Sawchenko et al., 1984). Traditionally, the functions of both nonapeptides and steroid hormones have been examined using *in vivo* pharmacological manipulations of either the synthesis or mechanisms of action of hormones (i.e., manipulation of enzymes or receptors, respectively).

1.2. New approaches to hormone measurements

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is an analytical chemistry technique that has emerged in the field of behavioral neuroendocrinology as a tool to simultaneously measure the concentrations of multiple hormones and the activity of synthetic enzymes (reviewed in Gravitte et al., 2021; Taves et al., 2011). In recent years, LC-MS/MS has been implemented in a growing number of field- and laboratory-based studies for several reasons: 1) it is more sensitive than traditional antibody-based techniques, 2) it uses automated methods, thereby reducing time spent for sample preparation and costs of using multiple assay kits (reviewed in America and Cordewener, 2008; Cross and Hornshaw, 2016), 3) it is highly specific for metabolites of interest and has little cross-reactivity with closely-related hormones, 4) it is versatile across sample types, 5) it allows for simultaneous measurement of multiple hormones and/or synthetic enzymes from a single sample without having to use different time-intensive separation methods for androgens and estrogens, such as those used in thin-layer chromatography (reviewed in Alomary et al., 2001; Soldin and Soldin, 2009; Pradhan et al., 2008; Pradhan et al., 2010a; Wudy et al., 2018; see Section 3), and 6) it enables researchers to measure the biochemical conversion of any precursor molecule, many of which can be purchased

at a relatively low cost. Traditional biochemical studies use tritium-labeled precursors, which can be both dangerous and expensive and often need to be repurified prior to use, a laborious process that requires significantly more time in the laboratory (Soma et al., 2003). Additionally, the structure of some steroids, such as 11-hydroxytestosterone, a precursor to 11-ketotestosterone (11-KT, a potent androgen in fishes; Fig. 2), does not allow for a tritium label (D.S. Pradhan, personal communication with Perkin Elmer). Due to this limitation, this conversion pathway has not been investigated in depth biochemically. Moreover, LC-MS/MS provides the opportunity to identify novel biomolecules in samples, an application that makes this technique ideal for studying the evolution of hormones and behavior using non-traditional animal models (reviewed in Wood et al., 2021; De Haes et al., 2015). Together, these advantages make LC-MS/MS a valuable tool that will enable researchers to study how endocrine networks, or collections of hormones, enzymes, and receptors that act together to regulate physiology processes and behavior, vary across the annual cycle.

In this review, we first discuss studies that examine seasonal changes in brain-behavior interactions. Specifically, we focus on the roles of sex steroids and nonapeptides in regulating seasonal changes in sexual and aggressive behavior across five major vertebrate taxa (fishes, amphibians, reptiles, birds, and mammals). Second, we explain the operation of LC-MS/MS, from sample preparation and collection to data analysis and interpretation. Finally, we discuss comparative studies that have used LC-MS/MS to investigate the neuroendocrine processes controlling social behavior, both within and outside of a seasonal context, and present current and future applications for LC-MS/MS in the field of behavioral neuroendocrinology. We propose that this cutting-edge methodology should be more widely used in this field and will enable researchers to gain a more wholistic understanding of how the myriad of hormones that comprise neuroendocrine networks regulate seasonal plasticity in social behavior.

2. Seasonal changes in the neuroendocrine processes regulating social behavior

The role of systemic hormones in regulating seasonal changes in physiology and behavior is well-established (reviewed in Ketterson and Nolan, 1992; Wingfield et al., 2019). However, these studies provide little insight into the cellular and subcellular mechanisms underlying local shifts in hormone secretion and signaling and how such changes influence behavior across seasons. These processes are especially fine-tuned within neuroendocrine tissues such as the brain, which consists of distinct regions that exhibit differing sensitivities to hormones and, thus, regulate disparate behaviors. Over the past few decades, considerable progress has been made in characterizing seasonal influences on the neuroendocrine regulation of social behavior, particularly in non-traditional animal models (fishes – reviewed in Forlano et al., 2015; Silva et al., 2020; amphibians and reptiles – reviewed in Krohmer and Lutterschmidt, 2011; Wilczynski et al., 2017; birds – reviewed in Schlinger and London, 2006; Wingfield et al., 2018; mammals – reviewed in Albers, 2012; Munley et al., 2018). Through these studies, we are beginning to understand the proximate mechanisms underlying social behavior, including seasonal shifts in the synthesis, metabolism, and signaling of hormones within the brain.

Two groups of hormones, sex steroids and nonapeptides, regulate behaviors that are important for enhancing reproductive success and survival based on the time of year. In particular, hormonal control of social behavior occurs within nodes of the social behavior network (SBN), a collection of reciprocally-connected nuclei in the basal forebrain, hypothalamus, and midbrain of teleost fishes, amphibians, reptiles, birds, and mammals (reviewed in Crews, 2003; reviewed in Goodson, 2005; Newman, 1999; O'Connell and Hofmann, 2011). In mammals, these nodes include the anterior hypothalamus (AH), bed nucleus of the stria terminalis (BNST), lateral septum (LS), medial amygdala (MeA), periaqueductal gray (PAG), preoptic area (POA), and ventromedial hypothalamus (VMH). Seasonal changes in neuroendocrine signaling also occur within the mesolimbic reward system, a neural circuit that enables animals to evaluate the salience of stimuli in their native environment, including social stimuli, and reinforces responses to salient stimuli (reviewed in Alcaro et al., 2007; Modi and Sahin, 2019). In mammals, this circuit is mainly comprised of telencephalic and dopaminergic projections from the ventral tegmental area. Because the SBN and the mesolimbic reward system share overlapping nodes (the BNST, LS, and MeA) and are sensitive to sex steroids and nonapeptides, these neural circuits have been proposed to form a larger social decision-making network, an evolutionarily conserved circuit that integrates information about external stimuli with internal physiological state to generate adaptive behaviors (reviewed in O'Connell and Hofmann, 2011, 2012). Collectively, this complex network of neural circuits is important in regulating behavioral responses to seasonal fluctuations in environmental resources, particularly those that are directly or indirectly associated with an organism's fitness. While many social behaviors vary seasonally, we will highlight two examples of seasonal plasticity in the brain and behavior: sexual behavior and aggression.

2.1. Sexual behavior

Depending on breeding strategy, sexual behavior is expressed with the goal of producing offspring via heterosexual mating. Although Frank Beach's descriptions of the different phases and reciprocal interactions between mammalian partners were described almost 50 years ago (Beach, 1976), understanding both the nuances and specific mechanisms by which sex steroids control reproductive behaviors, especially in seasonally breeding animals, is still a topic of active investigation (reviewed in Balthazart et al., 2004; Freeman and Rissman, 1996; Krohmer and Lutterschmidt, 2011). To study sexual behavior in seasonally breeding species, laboratory-based investigations typically manipulate photoperiod (i.e., day length), the primary environmental cue that many seasonal breeders use to anticipate changes in season and alter their physiology and behavior accordingly (reviewed in Dawson et al., 2001; Nelson et al., 2010; Stevenson et al., 2017). Specifically, animals are typically housed in experimental light cycles that mimic seasonal changes in their natural environment; long-day photoperiods (i.e., long days, LDs) simulate the summer breeding season, whereas short-day photoperiods (i.e., short days, SDs) reflect the winter non-breeding season. In contrast, field studies rely on ambient changes in season, allowing for the exploration of naturally occurring variation in the neuroendocrine regulation of reproductive behavior. Both laboratory- and field-based research has demonstrated that the steroids T and E_2 and the steroidogenic enzyme ARO are important in regulating seasonal plasticity in sexual behavior, primarily using systemic or neural implants and by manipulating the activity of ARO via pharmacological inhibitors.

To date, the neural actions of sex steroids on vertebrate sexual behavior have mainly been investigated in Japanese quail (*Coturnix japonica*; reviewed in Ball and Balthazart, 2010; Balthazart et al., 2004), musk shrews (*Suncus murinus*; reviewed in Freeman and Rissman, 1996), green anole lizards (*Anolis carolinensis*; reviewed in Wade, 2011), and red-sided garter snakes (*Thamnophis sirtalis parietalis*; reviewed in Krohmer and Lutterschmidt, 2011). Prior work has demonstrated that manipulations of systemic or neural T levels and ARO activity alter sexual behavior in both castrated and gonadally-intact animals. Peripheral T implants restore sexual behavior in castrated female musk shrews and facilitate copulatory behavior in gonadally-intact male green anoles (Neal and Wade, 2007; Rissman et al., 1990), and stereotaxic implants of T, T propionate, E_2, and E_2 benzoate in brain regions that regulate reproduction (i.e., POA, VMH, dorsomedial hypothalamus) increase sexual behavior in castrated male Japanese quail and female shrews (Riters et al., 1998; Sharma and Rissman, 1994; Veney and Rissman, 2000; Watson and Adkins-Regan, 1989a). Likewise, pharmacological inhibition of ARO via 1,4,6-androstatriene-3,17-dione (ATD),

racemic vorozole, and/or 4-hydroxyandrostenedione suppresses sexual behavior in male quail and female shrews and reduces courtship behavior in male red-sided garter snakes (Foidart et al., 1994; Krohmer, 2020; Rissman et al., 1990; Watson and Adkins-Regan, 1989b). This behavioral phenotype, however, is rescued in male snakes given both ATD and E_2 implants (Krohmer, 2020), suggesting an evolutionarily conserved role for ARO in regulating sexual behavior.

Moreover, previous studies suggest that seasonal variation in reproductive behavior is associated with neuroendocrine and anatomical changes in the brain, including variation in steroidogenic enzymes, soma size, and dendritic spine density, in behaviorally relevant regions. Whole brain ARO activity and ARO mRNA density in the POA are higher in male and female green anoles during the breeding season compared to the non-breeding season (Cohen and Wade, 2010a, 2010b; Neal and Wade, 2007), and T implants increase soma size in the POA and amygdala of breeding males and females (Neal and Wade, 2007; O'Bryant and Wade, 2002). In male red-sided garter snakes, T and E_2 implants increase dendritic spine density in the anterior hypothalamus preoptic area (AHPOA) of actively courting, castrated males relative to castrated males given control implants (Krohmer and Jurkovic, 2020), and this response is inhibited in male snakes implanted with ATD (Krohmer, 2020). Treatment with ATD reduces sexual behavior and ARO activity in the medial basal hypothalamus/POA in gonadally-intact female musk shrews (Rissman et al., 1990), and administration of the non-steroidal ARO inhibitor vorozole alters the distribution of ARO-immunoreactive cells in the POA, hypothalamus, MeA, and BNST (Rissman et al., 1996). Similarly, injections of racemic vorozole, 4-hydroxyandrostenedione, and ATD reduce the number of ARO-immunoreactive cells in the POM, BNST, and tuberal hypothalamus of male Japanese quail (Foidart et al., 1994; Watson and Adkins-Regan, 1989b). Collectively, these studies suggest that the neural aromatization of androgens is important in regulating vertebrate reproductive behavior. It is unclear, however, whether additional steroids, enzymes, and receptors within this biochemical pathway change seasonally and how these neuroendocrine processes may influence sexual behavior.

There is also evidence that AVT regulates sexual behavior in amphibians and birds (reviewed in Goodson and Bass, 2001; Wilczynski et al., 2017). Intracerebroventricular (ICV) injections of AVT stimulate amplectic clasping (i.e., courtship behavior) and enhance appetitive responses to female visual and olfactory stimuli in male rough-skinned newts (*Taricha granulosa*), whereas treatment with the AVP antagonist d(CH2)5Tyr(*Me*)AVP or an anti-AVT immune serum inhibits sexual behavior (Moore and Miller, 1983; Thompson and Moore, 2000). Breeding male newts also have a higher density of AVT-immunoreactive cells in several brain regions associated with reproduction, including the dorsal POA and ventral arcuate nucleus, compared to non-breeding males (Zoeller and Moore, 1988). Interestingly, intracranial DHT and E_2 implants cause AVT-like increases in clasping behavior in males (Moore and Miller, 1983), and treatment with both DHT and AVT implants or T and AVT implants produce male-typical behavioral responses towards female olfactory stimuli (e.g., amplectic clasping and proximity to female-scented model) in ovariectomized females (Moore et al., 1992; Thompson and Moore, 2003), indicating that both steroids and AVT control neural pathways that integrate sexual stimuli into male-typical courtship behaviors. In contrast, ICV injections of AVT reduce sexual behavior in castrated male Japanese quail treated with T, and these behaviors are restored after administering the V1 receptor antagonist dPTyr(*Me*)AVP (Castagna et al., 1998), suggesting that AVT inhibits sexual behavior in this species. Further research is necessary to investigate whether other nonapeptides (e.g., oxytocin), in addition to AVT, regulate reproductive behavior and to determine whether similar neuroendocrine mechanisms are utilized by other vertebrate taxa, such as fishes and mammals.

2.2. Aggression

Aggression is perhaps one of the most extensively studied social behaviors with respect to seasonal changes in the brain and behavior (fishes – reviewed in Quintana et al., 2021; Silva et al., 2020; amphibians and reptiles – reviewed in Wilczynski et al., 2017; birds – reviewed in Heimovics et al., 2018; Wingfield et al., 2018; mammals – reviewed in Munley et al., 2018; Soma et al., 2015). Aggressive behavior is universally exhibited across animal taxa and enables individuals to compete for access to limited resources in their environment (e.g., territories, mates, and food; Jalabert et al., 2018; Nelson, 2006). Therefore, aggression can be exhibited by male or female conspecifics or by members of different species. Aggressive behavior is further classified based on the social context in which it is elicited, and some of the most well-studied subtypes of aggression include inter-male aggression, predatory aggression, territorial defense, and maternal aggression (Brain, 1979; Moyer, 1971; reviewed in Scott, 1966). Regardless of social context, aggressive encounters are a costly investment with respect to energy, predation risk, physical injury, and time, and individuals must evaluate the costs and benefits of competing for these resources and make a decision that results in maximal fitness payoffs. Thus, many species are highly aggressive during the breeding season, when competition for access to these resources is extensive and the ability to acquire a mate and actively defend a territory is essential to increasing an individual's chances of reproductive success. There are some species, however, that display equivalent or higher levels of territorial aggression during the non-breeding season despite gonadal regression, suggesting that these animals face additional selective pressures that favored the evolution of alternative neuroendocrine mechanisms, which are independent of gonadal steroids, to regulate aggressive behavior throughout the year (reviewed in Munley et al., 2018; Soma et al., 2008). Such species have been particularly useful in elucidating seasonal and neuroendocrine influences on aggression, as these animals provide an opportunity to explore the role of extragonadal hormones, such as neurosteroids and nonapeptides, in regulating seasonal aggression in an ecologically relevant context.

While numerous studies have examined the role of gonadal steroids in regulating aggressive behavior (reviewed in Cunningham et al., 2012; Soma, 2006), less is known about the neural actions of sex steroids on seasonal aggression, and most of this research has characterized how a single steroid, enzyme, or receptor influences this behavior. Prior work in songbirds has demonstrated seasonal plasticity in steroid concentrations, steroidogenic enzymes, and steroid receptors in brain regions associated with aggressive behavior (reviewed in Heimovics et al., 2018; Pradhan and Soma, 2012). Male song sparrows (*Melospiza melodia*), which display high levels of territorial aggression year-round (except during molt; Wingfield and Hahn, 1994) have lower levels of T and E_2 in the POA, AH, and nucleus taeniae (the avian homolog of the MeA) during the non-breeding season compared the breeding season (Heimovics et al., 2016). Conversely, non-breeding male sparrows have higher activity and/or mRNA expression of ARO and 3β-hydroxysteroid dehydrogenase (3β-HSD), an enzyme that converts pregnenolone to progesterone and dehydroepiandrosterone (DHEA) to androstenedione (Fig. 2), in several brain regions that regulate aggressive behavior, including the ventromedial telencephalon (contains the nucleus taeniae), caudal telencephalon (contains the LS), POA, and BNST (Pradhan et al., 2010b; Soma et al., 2003; Wacker et al., 2010). Interestingly, breeding male sparrows have higher androgen receptor (AR) mRNA expression in the POA than non-breeding males, but there are no seasonal differences in ERα and ERβ mRNA expression in brain regions associated with aggressive and/or sexual behavior (Wacker et al., 2010), suggesting that region-specific changes in steroids and their synthetic enzymes may contribute to non-breeding aggression. It is important to note, however, that similar changes within the sex steroid synthesis pathway are not exhibited by all seasonally breeding songbirds. For example, male spotted antbirds (*Hylophylax n. naevioides*), which are

highly territorial year-round, have higher estrogen receptor α (ERα) mRNA expression in the POA and AR mRNA expression in the nucleus taeniae during the non-breeding season than the breeding season (Canoine et al., 2007), a distinct response from that exhibited by non-breeding male song sparrows. Collectively, these results indicate that seasonal variation in neurosteroids and their signaling mechanisms are important in controlling aggressive behavior in songbirds, but that some of the seasonal changes that occur within this biochemical pathway are species-specific.

Seasonal plasticity in neural steroidogenic enzymes and steroid receptors has also been documented in rodents (reviewed in Laredo et al., 2014b; Munley et al., 2018). To date, seasonal influences on the neural actions of sex steroids and aggression in rodents have primarily been studied in Siberian hamsters (*Phodopus sungorus*) and a few species of deer mice (*Peromyscus* sp.) that display elevated territorial aggression during the non-breeding season [i.e., beach mice (*P. polionotus*), deer mice (*P. maniculatus*), and California mice (*P. californicus*); Jasnow et al., 2000; Trainor et al., 2006; Scotti et al., 2007]. Treatment with the ARO inhibitor fadrozole reduces aggression in SD male beach mice, whereas fadrozole administration increases aggression in LD males. E_2 injections, however, prevent the effects of fadrozole on these behavioral phenotypes (Trainor et al., 2007a). In contrast, LD and SD female Siberian hamsters display no difference in the density of ARO-immunoreactive cells in the PAG and two brain regions that regulate reproduction (the paraventricular nucleus of the hypothalamus and ventral tegmental area; Rendon et al., 2020). Furthermore, Siberian hamsters and deer mice show seasonal changes in the expression of estrogen receptors in brain regions associated with aggressive behavior. SD male and female Siberian hamsters show higher ERα abundance in brain regions associated with aggressive behavior, including the PAG, LS, MeA, and/or BnST, relative to LD hamsters (Kramer et al., 2008; Rendon et al., 2017). In contrast, there are no seasonal difference in ERα abundance in brain regions associated with reproductive behavior (i.e., POA, arcuate nucleus, and the anteroventral periventricular nucleus of the hypothalamus) in female hamsters. SD male beach mice and deer mice also exhibit increases in ERα abundance and expression in the BnST, but show reductions in ERβ abundance and expression in the BnST and MeA relative to LD males (Trainor et al., 2007b). Selective activation of either ERα or ERβ is also associated with elevated aggression in SD male beach mice (Trainor et al., 2007a). Intriguingly, there are no differences in ERα and ERβ immunostaining in the LS, POA, BNST, or MeA between seasonal phenotypes of male California mice (Laredo et al., 2013; Trainor et al., 2008), a species that displays elevated aggression in response to SDs, yet does not exhibit gonadal regression (Nelson et al., 1995; Trainor et al., 2008). Thus, these results suggest that estrogen receptors may act to increase non-breeding aggression in rodents that are reproductively responsive to changes in photoperiod.

Although fewer studies have investigated the neural actions of sex steroids on seasonal aggression in fishes, there is some support that these neuroendocrine mechanisms may be evolutionarily conserved across vertebrates, particularly in weakly electric fish (reviewed in Quintana et al., 2021; Silva et al., 2020). In the solitary species *Gymnotus omarorum*, non-breeding males display high levels of aggression despite low plasma 11-KT levels, and this behavioral phenotype is independent of gonadal steroids. Specifically, castration does not affect aggressive behavior in non-breeding males, yet administration of the ARO inhibitor fadrozole reduces aggression in these animals (Jalabert et al., 2015). In a follow up study, transcriptomic analysis revealed that among non-breeding male *Gymnotus omarorum*, dominant males have higher expression of transcripts associated with estrogen synthesis (e.g., ARO and GREB1, an estrogen-responsive gene that is involved in regulating social behavior), whereas subordinate males show increased expression of transcripts involved in the conversion of active androgens into non-aromatizable androgens (e.g., DHEA sulfotransferase, an enzyme that converts DHEA to sulfated DHEA, and cytochrome P450 1B1, an enzyme that converts DHEA to 16a-hydroxyl-DHEA and estrone/E_2 to estriol/2-hydroxyestradiol; Eastman et al., 2020). While further investigation is needed to characterize how different components of sex steroid signaling (i.e., steroids, steroidogenic enzymes, and steroid receptors) regulate aggression in teleosts and to compare these pathways between species and across seasonal phenotypes, these data suggest that neurosteroids may contribute to non-breeding aggression in weakly electric fish.

Thus far, the role of nonapeptides in regulating aggression has been studied almost exclusively during the breeding season (reviewed in Albers, 2012; Kelly and Wilson, 2020; Wilczynski et al., 2017). Consequently, few studies have investigated how seasonal variation in nonapeptides and their receptors influence aggressive behavior. Increased aggression during the non-breeding season occurs independently of AVP and the vasopressin V_{1a} receptor (V_{1a}R) in Syrian hamsters (*Mesocricetus auratus*); AVP injections into the AH increase aggression in LD male hamsters, but does not affect aggression in SD males. Conversely, administration of Manning compound, a V_{1a}R antagonist, into the AH inhibits aggression in LD males, but does not alter aggression in SD males (Caldwell and Albers, 2004), indicating that AVP controls territorial aggression during the breeding season, but not the non-breeding season in male Syrian hamsters. Furthermore, administration of the ARO inhibitor letrozole downregulates oxytocin receptor mRNA expression in the BNST, but not the POA in LD male California mice, demonstrating that oxytocin receptor gene expression is estrogen-dependent in some brain regions. Treatment with melatonin, a pineal hormone that serves as the body's biochemical cue for changes in photoperiod, reduces oxytocin receptor mRNA expression in the POA, and this effect is suppressed in LD male mice given the melatonin receptor antagonist luzindole (Laredo et al., 2014a), suggesting that oxytocin receptor expression may be inversely related to territorial aggression in this species. In contrast to the mammalian species described above, the size of AVT-immunoreactive somata and the density of AVT fibers in brain regions associated with social behavior and sensory processing do not change seasonally in butterflyfish (*Chaetodon* sp.; Dewan et al., 2008). Additional studies are necessary to characterize how seasonal variation in nonapeptides and their receptors may contribute to aggressive behavior and to determine whether nonapeptides may act alone or in concert with neurosteroids to regulate seasonal aggression in vertebrates.

3. LC-MS/MS: a versatile and sensitive technique for hormone quantification

Although previous research has provided invaluable insight into how seasonal variation in neuroendocrine processes regulate social behaviors, such as sexual behavior and aggression, the majority of these studies have focused on measuring how a single hormone affects a single behavior. Given that hormones are synthesized and metabolized via complex biochemical pathways and often act in concert to control behavior, these approaches provide a limited view of how hormones modulate seasonal changes in social behavior. Thus, it is critical that the methodologies used to characterize the neuroendocrine mechanisms underlying seasonal plasticity in behavior capture the intricacy of these pathways, specifically by enabling researchers to measure the concentrations of multiple hormones and the activities of their synthetic enzymes within the brain. Below, we discuss the uses, guidelines, practical considerations, and applications for one technique that has recently emerged as a tool for studying seasonal influences on neuroendocrine networks: LC-MS/MS.

3.1. What is LC-MS/MS?

Although high-performance liquid chromatography (HPLC) has been used in endocrinology studies since the 1960s (reviewed in Shackleton, 2010), the coupling of liquid chromatography with mass spectrometry is relatively new. The liquid chromatography (LC) columns in these

instruments elute loaded chemicals into analyte bands of different sizes that correlate with different masses of chemical compounds. This elution process through the LC column is dependent both on the polarity of the solvents used and the amount of time the chemicals are given to separate (reviewed in Shackleton, 2010). Both HPLC and LC-MS/MS instruments utilize two types of phases (i.e., solvents) to move samples through the LC column. The stationary phase prepares the column between each use, whereas the mobile phase moves samples through the column. While HPLC was a revolutionary methodology for its time, one of its biggest limitations is that it requires a high volume of samples and standards for accuracy (reviewed in van de Merbel, 2019). Unlike HPLC, however, LC-MS/MS includes a LC column coupled to a mass spectrometer, a highly-sensitive instrument that measures the mass-to-charge ratio of chemical compounds in each sample, thereby enabling the quantification of metabolites with great precision and accuracy (Fig. 3; Cross and Hornshaw, 2016; reviewed in van de Merbel, 2019).

To date, several types of LC-MS/MS instruments have been developed that are suitable for measuring hormones. The type of biomolecule and the level of resolution needed for sample analysis, however, needs to be considered to determine the type of instrument that should be used. The most commonly used LC-MS/MS instrument is the tandem quadrupole, also known as a triple quadrupole (TQ) LC-MS/MS. This instrument is primarily suitable for measuring steroid hormones, and its high sensitivity is a good starting point for determining which solvents should be used during the elution process and what type of LC column to use (Agilent Technologies, 2012a). TQ LC-MS/MS uses two quadrupole mass analyzers in a series that are separated by a collision cell, a device in which fragmentation of a chemical compound occurs via an inert gas and produces a charge that the instrument registers. Precursor ions are separated in the first mass analyzer and enter the collision cell, where they are fragmented. Then, each product ion is separated and detected in the second mass analyzer. This process allows the instrument to measure the mass of the selected chemical by its charge (i.e., whether the compound has had a hydrogen added (+) or a hydrogen removed (−) during the fragmentation process; Polymer Solutions News Team, 2017). Because the identities of precursor and product ions must be defined and the settings for fragmentation must be optimized prior to TQ LC-MS/MS analysis, this instrument can only quantify known chemical compounds. Conversely, quadrupole time-of-flight (QTOF) LC-MS/MS is ideal for screening and identifying unknown compounds. This instrument generates high resolution-accurate mass (HRAM) spectral data, from which the chemical formula of an unknown compound can be predicted. This molecular formula can be investigated by searching databases that contain information on known chemical compounds, which yield a list of candidate chemical structures for the unknown sample compound. The instrument software can then be used to predict the fragmentation pattern of each candidate structure and compare it to the observed fragmentation pattern in the HRAM mass spectral data, allowing researchers to find the candidate structure that best matches the unknown sample compound (Agilent Technologies, 2015; Polymer Solutions News Team, 2017).

3.2. General guidelines for LC-MS/MS

3.2.1. Sample collection and preparation

Behavioral neuroendocrinologists measure hormones from a wide variety of proxies based on the type of analysis being conducted (i.e., direct or indirect measurements; reviewed in Pradhan et al., 2015). Blood, plasma, and serum are some of the most common samples used for direct hormone measurements, and these samples represent hormone concentrations in general systemic circulation. Hormones can also be directly quantified within specific tissues, where they are either synthesized or active (reviewed in Pradhan et al., 2015; Schmidt et al., 2008). In contrast, indirect hormone measurements are performed using samples that are collected non-invasively, such as urine, feces, hair, feathers, and ambient water (for aquatic and semi-aquatic organisms). Although specimens used for indirectly quantifying hormones are generally easier to collect, they are often heterogenous, more variable among individuals, and include chemicals such as pheromones, solid or dissolved metabolic waste, and toxins (Moosmang et al., 2019; Pradhan et al., 2014a). Generally, any sample type can be used for LC-MS/MS analysis, as long as it is adequately extracted, purified, and filtered to remove particles that could interfere with LC column integrity and instrument sensitivity. Furthermore, aspects of sample collection and preparation prior to LC-MS/MS analysis should be standardized, regardless of the sample type being used. Specifically, samples should be weighed as they are freshly collected, stored appropriately and consistently at low temperatures (−20 to −80 °C) to maintain integrity, and weighed just prior to use in assays in order to determine yield. In addition, pooled samples that represent variation in sample matrices must also be created during method development (Moosmang et al., 2019). For small sample quantities, it is particularly important to be attentive to techniques that allow for preconcentration of compounds and separation of metabolite fragments (reviewed in Zwiener and Frimmel, 2004).

Because LC-MS/MS separates chemical compounds that are dissolved in liquids, samples that are concentrated in organic solvents that match the mobile phase of the protocol must be injected into the instrument (see Section 3.2.2). Prior to LC-MS/MS analysis, tissue samples must be homogenized to break up the tissue and disrupt the cells (Fig. 3). Tissue homogenization is typically performed in aqueous buffer or organic solvent using a hand-held or bead mill homogenizer. Either whole homogenates of tissue samples or partially purified supernatants, which are obtained following centrifugation and are more likely to have higher concentrations of hormones and/or enzymes (Peterson et al., 2005; Pradhan et al., 2010a; Pradhan et al., 2008; Pradhan et al., 2014b), can be used for LC-MS/MS analysis. Following homogenization, extraction is performed to separate hormones from the other components of the sample (Fig. 3). Two techniques are primarily used for hormone extraction: 1) liquid-liquid extraction, in which organic solvents are used to isolate hormones, and 2) solid-phase extraction, in which hormones are separated from samples after they are loaded onto a primed and equilibrated sorbent (most commonly, silica-bonded C_{18} columns; reviewed in Taves et al., 2011). Previous studies that have used LC-MS/MS to quantify hormones in comparative animal models have used a variety of homogenization and hormone extraction techniques

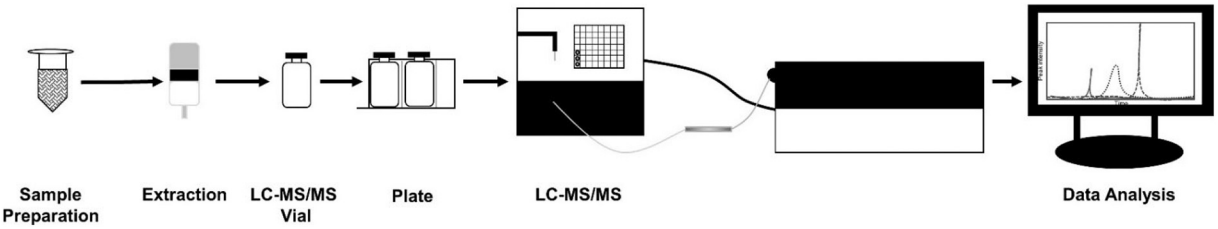

Fig. 3. Workflow for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Following sample collection, hormones are typically extracted using liquid-liquid or solid-phase extraction techniques, dried, resuspended in organic solvent, and transferred to glass vials prior to LC-MS/MS analysis.

Table 1
A summary of behavioral neuroendocrinology studies that have used liquid chromatography-tandem mass spectrometry to measure sex steroids or nonapeptides within a behavioral and/or seasonal context.

Reference	Study species	Sex	Behavior	Variable/ manipulation	Sample type	Hormones	Extraction	LC-MS/MS analysis
Ericsson et al., 2014	*Gallus gallus*	♀	Exploratory, foraging, grooming, anxiety-like	Acute restraint stress	Blood	PREG, PROG, DHEA, A4, T, E_1, E_2, E_3, B	LLE	TQ LC-MS/MS
Jalabert et al., 2021	*Melospiza melodia*	♂	N/A	Season	Brain, blood, plasma	PREG, PROG, DHEA, A4, T, DHT, E_1, E_2, 17α-E_2, B	LLE	QTRAP UHPLC-MS/MS
Kulczykowska et al., 2015	Family *Labridae*	♀	Mutualistic	Species	Brain	AVT, IT	SPE	TQ LC-MS/MS
Munley et al., 2021	*Phodopus sungorus*	♂	Aggression	Season	Brain, blood	PROG, DHEA, T, E_2, F	SPE	QTRAP LC-MS/MS
Odekunle et al., 2019	*Asterias rubens*	♂, ♀	Feeding	N/A	Radial nerve cord	AT[a,b]	LLE	QTOF UPLC-MS/MS
Pirger et al., 2010	*Helix pomatia*	♂, ♀	Hibernation	Temperature	Brain, hemolymph	Neuropeptides[a]	SPE	MALDI-TOF/TOF MS
Pradhan et al., 2014b	*Lythrypnus dalli*	♂	Parenting	11β-HSD inhibitor	Brain, testes	11-KT, B	SPE	TQ LC-MS/MS
Pratavieira et al., 2014	*Apis mellifera*	♀	Eusocial	Age	Brain	AmTRP-5, AST-1[a,c]	N/A	MALDI-TOF/TOF MS
Prior et al., 2016	*Taeniopygia guttata*	♂, ♀	Affiliative	Sex	Plasma	PREG, pregnan-3,17-diol-20-one, PROG, DHEA, A5, T, AN, ADIOL	LLE	TQ UPLC-MS/MS

Abbreviations: A4, androstenedione; A5, androstenediol; ADIOL, androstanediol; AN, androsterone; AT, asterotocin; AVT, arginine vasotocin; B, corticosterone; DHEA, dehydroepiandrosterone; DHT, 5α-dihydrotestosterone; 17α-E_2, 17α-estradiol; E_1, estrone; E_2, 17β-estradiol; E_3, estriol; F, cortisol; 11β-HSD, 11β-hydroxysteroid dehydrogenase; IT, isotocin; 11-KT, 11-ketotestosterone; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LLE, liquid-liquid extraction; MALDI-TOF, matrix-assisted laser desorption/ionization tandem time-of-flight; MS, mass spectrometry; PREG, pregnenolone; PROG, progesterone; QTOF, quadrupole time-of-flight; QTRAP, quadrupole ion trap; SPE, solid phase extraction; T, testosterone; TOF, time-of-flight; TQ, triple quadrupole; UHPLC, ultra-high-performance liquid chromatography; UPLC, ultra-performance liquid chromatography.

[a] Indicates studies in which QTOF or MALDI-TOF/TOF mass spectrometry was used to identify previously unknown neuropeptides.

[b] AT is a vasopressin/oxytocin-type neuropeptide that has only been identified and characterized in *Asterias rubens*.

[c] AmpTRP-5 is a tachykinin peptide, and AST-1 is an allatostatin peptide. These neuropeptides have only been identified and measured in *Apis mellifera*.

(Table 1); thus, it is critical that the efficacy of these methods is evaluated prior to use and chosen appropriately based on the application (i.e., biochemistry assays versus hormone measurements). After hormone extraction, samples are generally eluted into LC-MS sample vials (preferably amber vials with resealable caps; Fig. 3) using an organic solvent and concentrated via drying, a process that is typically carried out using a speed vacuum or radio inert gas, such as nitrogen. Samples must then be resuspended in organic solvent prior to injection into the LC-MS/MS instrument.

3.2.2. *Measuring hormones via LC-MS/MS*

Before conducting LC-MS/MS analysis, the LC column should be primed for use. To prepare or "condition" the column, inorganic solvent must flow through the column and then be ejected into a waste container. This process can be routinely performed by either temporarily editing the LC-MS/MS method or by creating a separate protocol for column conditioning that can be saved for future use. The best solvents to use for conditioning depend on the manufacturer, and this information is generally provided in the LC column manual (Agilent Technologies, 2012b; Restek, 2018; Waters Corporation, 2012). Moreover, the elution time that is selected for conditioning will depend on the type of LC column, which is identified by the size of carbon particles packed into the column. A tightly packed column is characterized by smaller packing particles (e.g., 1–3 μm), which typically require longer elution times, whereas columns with particles >4 μm usually require shorter elution times.

Like most high-throughput technologies, protocols can be programmed into the LC-MS/MS software, allowing for easy retrieval for future use. These programs allow the user to control the volume of sample being injected onto the LC column, to specify the speed at which inorganic and organic solvents are moved through the instrument, and to adjust the elution time (Agilent Technologies, 2012a). Although the elution time is dependent on the chemical structure of the metabolite of interest and the type of LC column being used, this step is typically altered to account for adding a hydrogen to the metabolite, which involves running the machine in positive mode, or for subtracting a hydrogen ion, which involves running the instrument in negative mode. This addition or subtraction of a hydrogen ion enables the instrument to register and measure the chemical compound of interest more effectively. After the LC-MS/MS protocol has been programmed or uploaded into the software, a worklist must be created. This "map" specifies the position of each LC-MS/MS vial on the loading plate (Fig. 3). Once this information has been added the to the worklist, the identity of each sample and the volume of sample that will be injected into the instrument can also be programmed (Agilent Technologies, 2015). After these data have been entered, the samples can be run through the instrument. During the analysis, the instrument software will automatically save any data that is collected from LC-MS/MS, which will allow the user to analyze the data in real time or at a later date.

When developing a LC-MS/MS protocol, it is necessary to perform fine-tuning, a process that ensures the peak signal intensities of the compounds of interest are sharp, with little inference from the organic solvent (reviewed in America and Cordewener, 2008). Before starting to fine-tune a protocol, relevant literature should be reviewed, and a published protocol should be referred to that analyzed chemical compounds that are similar in mass and charge to the metabolites of interest, if available. Importantly, the inorganic and organic solvents used for LC-MS/MS analysis must be classified as "LC-MS grade." This label indicates that the solvent was developed using high purification preparation methods and has high UV transmittance, making it suitable and reliable for use in LC-MS/MS applications. The first step in the fine-tuning process is to adjust the run time through the LC column to baseline, a setting that enables the instrument to run without any sample present on the column until it has been adequately conditioned. Once the ideal time frame for the LC column has been determined, the elution gradient should be adjusted. This technique alters the composition of the mobile phase (i.e., an aqueous/organic solvent mixture) during the course of a chromatographic run. Typically, it is best to start with 95% inorganic

solvent and 5% organic solvent and gradually increase the proportion of organic solvent over the course of the elution process, such that the final step of the gradient (i.e., when the metabolite is read by the instrument) is 0% inorganic solvent and 100% organic solvent (Agilent Technologies, 2012a, 2015). Finally, the flow rate of the solvents and the vacuum speed for the LC-MS/MS instrument should be adjusted (reviewed in America and Cordewener, 2008).

3.2.3. Data analysis and interpretation

A feature of LC-MS/MS that makes it such a versatile and attractive methodology is the ability to assess limits of quantification and detection for each metabolite of interest. Moreover, LC-MS/MS is highly sensitive, selective, accurate, and precise, enabling researchers to measure minute concentrations of multiple metabolites of interest with strong specificity. During LC-MS/MS analysis, each metabolite is separated based on its mass-to-charge ratio as it runs through the LC column. As the mobile phase passes through the column, each metabolite elutes off of the column at particular time, known as the 'retention time' (Podwojski et al., 2009). The retention time is influenced by the LC column, the organic and inorganic solvents used for the mobile phase, the temperature of the machine, and the temperature of the room in which the LC-MS/MS instrument is housed (Podwojski et al., 2009). The LC-MS/MS software records the retention time of each metabolite of interest during the elution process and generates a chromatograph, which shows the peak signal intensity of each metabolite based on its mass-to-charge ratio (Fig. 3). For each metabolite, the peak height (i.e., maximum amplitude of the curve) and peak area (i.e., area under the curve) can be extrapolated by the software and can be used to calculate metabolite concentrations in each sample.

Typically, one of two mathematical approaches are used to determine the concentrations of metabolites following LC-MS/MS analysis. Perhaps the most commonly used method in biological research is to generate calibration curves, in which reference standards for metabolites of interest are diluted and linear standard curves are created by plotting the peak signal intensities from LC-MS/MS analysis against known concentrations of reference standards. Concentrations of each metabolite can then be interpolated using these calibration curves. Alternatively, metabolite concentrations can be calculated using isotopic internal standard quantification (also known as absolute quantification or internal calibration), in which the amount of an isotopically-labeled analog of each metabolite of interest is multiplied by the ratio of the fragment ion signal for the endogenous metabolite to the fragment ion signal for the isotopically-labeled analog (Bennett et al., 2008; Nilsson and Eklund, 2007). Isotopic internal standard quantification, which has been shown to be highly accurate and precise and produces similar results to calibration curves, provides a simplified alternative to this more traditional approach without compromising analytical quality (Nilsson and Eklund, 2007).

Studies that investigate the neuroendocrine mechanisms underlying social behavior typically use multiple treatment groups, test several independent variables, and measure a large number of dependent variables. Similarly, LC-MS/MS allows for the quantification of a potentially unlimited number of hormones from a single sample, an approach that can also be used to compare the concentrations of multiple hormones and the activities of their synthetic enzymes across multiple tissues or in multiple regions within a given tissue (e.g., the brain). Thus, using multiple one-way analyses of variance (ANOVAs) or separate repeated-measures ANOVAs to assess the effects of various treatments on concentrations of each individual hormone obtained via LC-MS/MS analysis may be inappropriate, as such analyses cause a considerable increase in false discovery rate. Furthermore, most hormones that are quantified via LC-MS/MS are part of the same biochemical pathway, and neither these hormones nor the tissues or regions in which they are measured act in isolation to regulate behavior. Thus, these data violate one of the assumptions of univariate ANOVAs: that samples must be independent of one another. To provide an integrated understanding and interpretation of LC-MS/MS results, statistical approaches that reflect the coordination and complexity of these biochemical pathways must be used. Examples of such analyses include multivariate analyses (e.g., MANOVA, PERMANOVA, MANCOVA), model selection, factor analyses (e.g., principal component analysis, discriminant factor analysis), classification and regression trees (e.g., CART), and network analyses. Depending on the biological question being investigated, the best strategy for statistical testing may be to *a priori* determine the number of hormones to analyze to reduce the total number of statistical comparisons being made. For example, biomolecules that are measured during LC-MS/MS analysis that show little variation in concentration between treatment groups may not allow for meaningful statistical comparisons. This *a priori* approach, in addition to the use of the statistical tests described above, will reduce the rate of type I and type II errors when analyzing and interpreting data acquired via LC-MS/MS. Finally, studies measuring multiple metabolites through LC-MS/MS could use computational and bioinformatics approaches, similar to those for transcriptomics and genomic data.

3.3. Practical considerations

While LC-MS/MS can be an extremely powerful tool for behavioral neuroendocrinologists who study non-traditional animal models in the field or laboratory, especially those who are limited by sample quantity (e.g., seasonal biologists), the accessibility of LC-MS/MS technologies in academic research institutions, particularly those that pursue fundamental questions in science, is far behind industrial pursuits. This major gap in resource availability has significantly reduced the pace of advancement in basic scientific discovery. While companies such as Agilent Technologies, the major producer of these analytical instruments, provide educational opportunities via instrument training and workshops for different aspects of the LC-MS/MS workflow, the required learning curve is rather steep for biologists compared to analytical chemists. Set-up and operational costs are important factors that play into the accessibility of LC-MS/MS: 1) the instrument and yearly service contracts are costly, 2) the instrument needs to be housed in a dedicated facility where other laboratory assays are not performed, especially those that generate volatile gases/fumes, 3) the instrument requires frequent maintenance and changing parts, which is generally provided by full-time supporting technicians who are experts in analytical chemistry, 4) the processing of biological samples typically requires additional preparation steps, whereas drug discovery studies use purified proteins and synthetic chemicals for analyses, 5) depending on their initial skill level and prior preparation, researchers may need to consult with a more experienced user for initial training and troubleshooting as questions arise, and 6) there is often a communication gap between biologists and chemists due to the nature of experimental questions and traditional separations in these scientific fields.

In order to circumvent some of these issues, LC-MS/MS instruments at academic research institutions are often housed in shared or core facilities for multiple users. Another approach has been for non-expert scientists to send samples to other facilities to process samples for a fee. While the initial set-up for LC-MS/MS can be expensive, the maintenance costs of this technology are minimal and the methods are time-efficient due to automation once they are optimized. For example, while some parts need to be replaced only by specialized personnel from instrumentation companies, it is possible to receive institutional discounts if multiple year package services are purchased. Utilization of LC-MS/MS applications can also be seen as an opportunity for biologists and chemists to break down traditional barriers, engage in multidisciplinary discovery projects from inception, and write collaborative proposals as co-investigators trying to mutually answer research questions, rather than merely offering an exchange of services. Such collaborations would also provide a platform for the next generation of scientists to learn instrumentation skills that would better prepare them for the workforce. We propose that this synergistic approach might increase the

opportunity for both disciplines to apply for grants that foster cross-disciplinary collaborations and would increase the quality and impact of the research findings and applications.

4. LC-MS/MS as a novel biochemical approach to studying the seasonality of brain-behavior mechanisms

4.1. Neuroendocrine studies using LC-MS/MS approaches

To date, few studies have used LC-MS/MS to characterize brain-behavior interactions, and even less have performed this analysis using a seasonal framework. Thus, research that has measured sex steroids or nonapeptides via LC-MS/MS within a behavioral and/or seasonal context is diverse with respect to study system, sex, behavior, sample type, hormones, and type of LC-MS/MS analysis (Table 1). In general, behavioral neuroendocrinology studies have utilized triple quadrupole (TQ) or quadrupole ion trap (QTRAP) LC-MS/MS (an instrument that is similar to a TQ LC-MS/MS, except that the third quadrupole can also be operated as an ion trap with compound screening and identification applications) to quantify sex steroid concentrations in circulation and tissues. Similar to previous work in this field, these studies support a role for sex steroids in regulating social behavior, both within and across the seasons. SD male Siberian hamsters display increased aggression, but have lower levels of DHEA, T, and E_2 in the LS, AH, MeA, and/or PAG than LD males. Interestingly, timed melatonin administration produces similar changes in aggression and neurosteroid levels in LD males, and SD and melatonin-treated LD males both show negative relationships between neural T, E_2, and cortisol concentrations and aggressive behavior (Munley et al., 2021), suggesting that seasonal changes in neurosteroid levels and aggression are melatonin-dependent. Male song sparrows also exhibit seasonal variation in neurosteroid concentrations within the social decision-making network, and these animals exhibit seasonal changes in the relationship between levels of sex steroids in circulation compared to brain tissue. Specifically, androgens (i.e., T, androstenedione and 5α-DHT) are only detectable in the brain during the breeding season, and androstenedione and 5α-DHT levels are up to 20-fold higher in specific brain regions than in blood. Conversely, non-breeding males have higher progesterone (PROG) concentrations in the POA, AH, VMH, and nucleus taeniae compared to breeding males, despite a lack of seasonal differences in circulating PROG (Jalabert et al., 2021). Although the behavioral implications of these results have not yet been investigated, these data demonstrate that neurosteroid levels can differ considerably from circulating steroid levels and that male song sparrows exhibit seasonal plasticity in neurosteroid concentrations, independent of circulating sex steroids.

The steroidal regulation of social behavior has also been examined outside of a seasonal context via LC-MS/MS. In zebra finches (*Taeniopygia guttata*), affiliative behavior is not correlated with plasma levels of androgens and progestins in males and females, despite both sexes having high concentrations of pregnenolone in circulation (Prior et al., 2016), which could suggest that local steroid synthesis may be responsible for mediating this behavior. Furthermore, female domestic chickens (*Gallus gallus*) show breed-specific behavioral and hormonal responses to acute stress, in which the domesticated White Leghorn breed generally displays less pronounced changes in exploratory, foraging, grooming, and anxiety-like behavior than the ancestral Red Junglefowl breed following restraint stress. The White Leghorn breed, however, shows a prolonged hormonal response to acute stress, including sustained reductions in circulating pregnenolone, DHEA, and androstenedione and elevated circulating corticosterone levels (Ericsson et al., 2014), suggesting that stress recovery in chickens has been altered by domestication. TQ LC-MS/MS has also been used to validate the *in vitro* inhibition of 11β-hydroxysteroid dehydrogenase (11β-HSD, an enzyme that converts 11β-hydroxytestosterone to 11-KT; Fig. 2) by the drug carbenoxolone, a pharmacological inhibitor that had previously only been used in mammals (Pradhan et al., 2014b; Webb et al., 2008). In an initial set of experiments, carbenoxolone abolished 11β-HSD activity in the brain and testes of male bluebanded gobies (*Lythrypnus dalli*). This result allowed for the subsequent use of carbenoxolone in an *in vivo* study to assess the role of neurally-produced 11-KT in controlling parenting behavior in male gobies, which exclusively display parenting behavior in this species, but also concurrently exhibit territorial aggression and courtship. ICV injections of carbenoxolone dramatically reduce parenting behavior in male gobies. This behavioral phenotype, however, is rescued by 11-KT treatment when paired with carbenoxolone, while cortisol treatment does not affect parenting (Pradhan et al., 2014b), suggesting that neural 11-KT regulates parenting behavior in this species via changes in 11β-HSD. Thus, LC-MS/MS approaches can be used to investigate steroidogenic enzyme pathways that have not been previously examined due to the unavailability of tritium-based precursors.

Conversely, no studies have examined the role of nonapeptides in controlling social behavior in a seasonal context. Some research, however, has used LC-MS/MS to characterize neuropeptides in non-traditional animal models and to assess how these hormones affect behavior (Table 1). Thus far, only one study has utilized TQ LC-MS/MS to investigate how nonapeptides control social behavior. Specifically, this study characterized how concentrations of AVT and isotocin within the forebrain, optic tectum, cerebellum, and brainstem relate to mutualistic behavior in females from four species of cleaner fishes with diverse behavioral strategies (family *Labridae*): two obligatory cleaners (*Labroides dimidiatus* and *Labroides bicolor*), which exhibit mutualistic behavior (i.e., cleaning behavior) throughout their entire lives; one facultative cleaner (*Labropsis australis*), in which only juveniles are cleaners; and one non-cleaner species (*Labrichthys unilineatus*). The obligatory cleaners *L. dimidiatus* and *L. bicolor* have higher AVT levels in the cerebellum, a brain region that has been implicated in associative learning and memory, than *L. australis* and *L. unilineatus*, whereas *L. unilineatus* has higher levels of isotocin in all of the brain regions examined relative to the obligate and facultative cleaner species (Kulczykowska et al., 2015). Thus, these results suggest a role for AVT in regulating associative learning and memory processes that are linked with mutualistic behavior in cleaner fishes.

In contrast to the sex steroid and nonapeptide LC-MS/MS studies described above, which used TQ and QTRAP LC-MS/MS to quantify hormone concentrations, neuropeptides have primarily been identified and measured using QTOF or matrix-assisted laser desorption/ionization tandem time-of-flight (MALDI-TOF) mass spectrometry (Table 1). In particular, these studies employed the HRAM capabilities of QTOF and MALDI-TOF MS to discover novel neuropeptides in invertebrates and investigate their potential roles in regulating social behavior. In the snail *Helix pomatia*, the concentrations of several peptides and polypeptides in the brain are affected by temperature, an environmental cue that induces hibernation in this species. More specifically, the intensity of eight neuropeptides is greater in active snails than in hibernating snails, while the intensity of six different neuropeptides is greater in hibernating snails than in active snails (Pirger et al., 2010), suggesting a potential role for neuropeptides in controlling hibernation behavior. Moreover, MALDI-TOF MS enabled researchers to identify and characterize the distribution and behavioral function of the vasopressin/oxytocin-type neuropeptide asterotocin in the common starfish (*Asterias rubens*). Asterotocin is expressed in the central nervous system, digestive system (including the cardiac stomach), body wall, and associated appendages, and *in vivo* administration of asterotocin triggers fictive feeding behavior, including eversion of the cardiac stomach and changes in body posture consistent with the extra-oral feeding behavior of starfish (Odekunle et al., 2019). Thus, these data indicate that asterotocin controls feeding behavior in starfish and suggest that vasopressin/oxytocin-type neuropeptides may have an evolutionarily ancient role in regulating feeding in animals. In further support of a role for neuropeptides in regulating social behavior in invertebrates, levels of the

neuropeptides AmTRP-5 and AST-1 (a tachykinin and an allatostatin peptide, respectively) within distinct brain regions are correlated with behaviors associated with division of labor in the eusocial Africanized honeybee (*Apis mellifera*). In young worker bees, who generally perform brood-rearing activities in the hive, AmTRP-5 and AST-1 are highest in the pedunculi of the mushroom bodies, a brain region that is involved in olfaction, learning, and memory. Conversely, in 20–25 day old worker bees, which are responsible for foraging and colony defense, AmTRP-5 and AST-1 concentrations are highest in brain regions associated with sensory input and movement (e.g., the antennal lobes, subesophageal ganglion, medulla, and lobula; Pratavieira et al., 2014), suggesting that neuropeptides regulate age-related division of labor in the honeybee brain. Taken together, these studies highlight a few of the potential applications of LC-MS/MS for behavioral neuroendocrinology studies, both in field and laboratory settings.

4.2. Current and future applications for LC-MS/MS in behavioral neuroendocrinology

Recent advances in technology that have facilitated the development of LC-MS/MS have also revolutionized many industries in applied biology. Within the last 20 years, LC-MS/MS has allowed for the creation of highly sophisticated drugs due to the ability of this technique to precisely quantify small biomolecules. Consequently, pharmacometabolomics, or the study of how differences in metabolites can be used to predict individual responses to a drug or medical intervention (reviewed in Corona et al., 2011; Kaddurah-Daouk et al., 2014), drug discovery (reviewed in Goodwin et al., 2020; Pacholarz et al., 2012), and forensic science (reviewed in Brown et al., 2020; Wood et al., 2006) have boomed, allowing for major profits. Other industries across applied biological fields that utilize LC-MS/MS analysis include agriculture and animal science (reviewed in Soares et al., 2012; Lippolis and Reinhardt, 2010), wildlife conservation (e.g., Charapata et al., 2018; Galligan et al., 2020; Legacki et al., 2020; Styrishave et al., 2017), neuropeptidomics, a bioanalytical strategy for identifying and characterizing neuropeptides present in the brain and central nervous system (reviewed in Vu et al., 2021; Yang et al., 2016), human medicine (reviewed in Jackson et al., 2000; Zhang et al., 2020), and toxicology (reviewed in Allen and McWhinney, 2019; Schulz et al., 2019).

Understanding mechanisms of phenotypic plasticity is a central question of behavioral neuroendocrinology. Historically, hormone measurements have been particularly challenging for researchers who study seasonal plasticity in neuroendocrine processes, as hormones and their synthetic enzymes are often present in minute concentrations in biological samples during certain times of the year and, thus, are non-detectable using traditional antibody-based techniques. Moreover, there is evidence that the precise steps involved in signal transduction in response to hormones may vary seasonally. Thus, LC-MS/MS will provide important insight into seasonal influences on brain-behavior interactions by enabling the detection of small changes in biological molecules across a wide range of sample matrices.

More broadly, this technology will allow behavioral neuroendocrinologists not only to identify novel hormones, but also to study how hormone levels vary with respect to other hormones that are present in the physiological landscape, providing a more comprehensive understanding of which hormones are important in regulating social behaviors. LC-MS/MS allows us to use a multi-dimensional approach, whereby we can identify and quantify not just the presence of multiple hormones, but also the mechanisms of hormone metabolism into more active forms that generate behavioral phenotypes. This technique also gives biochemists the opportunity to study pathways that were previously not possible, either due to technological limitations (see Section 1) or the inability to use radioactive samples in certain facilities. Finally, this tool can be used to understand the efficacy of pharmaceutical drugs (e.g., half-life) or breakdown of hormones in response to long-term or short-term drug manipulations. This application would be especially beneficial for behavioral neuroendocrinologists who use pharmacological manipulations to study the function of hormone receptors, carrier proteins, or biochemical pathways. While antibody-based assays need to be validated for each species being studied, neat standards from a mixture of biomolecules of interest only need to be separated initially for LC-MS/MS analysis.

5. Conclusions

Contrary to genomic approaches that provide information on the expression of genes, hormone detection via LC-MS/MS provides information about the phenome. The area of phenomics has flourished in areas such as human disease (Curcin, 2020), plant crop resilience to abiotic stressors (reviewed in Coppens et al., 2017; Tardieu et al., 2017), the knockout mouse project (Hasellmashhadi et al., 2020), economically-relevant livestock agriculture (reviewed in Baes and Schenkel, 2020), and pesticide toxicity in aquatic organisms (Hussain et al., 2020). It is clear from these examples that studies on natural systems and non-traditional animal models are extremely rare (reviewed in Thessen et al., 2020). Thus, additional resources are needed to establish partnerships in multidisciplinary scientific areas, both within and across institutions, to enable more widespread use of LC-MS/MS technology in the fields of behavioral neuroendocrinology and seasonal biology. We propose that institutions could provide better support for cross-disciplinary research through 'seed-funding' or programs for undergraduate and graduate students to better integrate the fields of biology and chemistry. Moreover, federal grant opportunities that foster cross-disciplinary collaborations will be essential for basic scientific discovery that utilizes LC-MS/MS. Collectively, accessibility and use of this cutting-edge technology will dramatically increase the scope of investigation of endogenous natural molecules underlying seasonal phenotypes.

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Declaration of competing interest

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