One cell’s trash is another cell’s treasure: The role of exosomes in stress physiology of the brown anole (*Anolis sagrei*)

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ABSTRACT

Exosomes are a type of extracellular vesicle that is produced by cells through an endosomal pathway and secreted into bodily fluids. Exosomes deliver bioactive cargo from their parent cells to target cells as a form of intercellular communication. While most research has studied exosomes in the context of disease, less is known about their role in normal physiology when disease is absent. Using the brown anole (*Anolis sagrei*) as a model organism, we investigated how stress exposure affects exosome concentrations in the plasma of anoles, and whether exosome release is necessary for the response to stress. We hypothesized that stress will increase exosome release in the brown anole. A population of brown anoles was maintained in captivity and exposed to various acute and chronic stressors. Exosomes were isolated from anole plasma using a commercial kit and quantified by measuring acetylcholinesterase (AChE) activity. We found that exosomes can be successfully isolated from brown anoles, and that AChE can be a reasonable measure of exosome concentrations. Our findings suggest that acute stress exposure may increase exosome release, while chronic stress exposure may decrease it over time. This was a novel and exploratory study that can be used as a starting point for future research on the role of exosomes in the normal stress response.

INTRODUCTION TO EXOSOMES

Exosomes are small extracellular vesicles of endosomal origin that are secreted by various types of cells under both healthy and diseased physiological states (Patel et al. 2019). These secreted vesicles can be found in a variety of bodily fluids, such as plasma, saliva, urine, seminal fluid, breast milk, and others (Beninson and Fleshner 2014; Fleshner and Crane 2017). It was previously believed that exosomes were simply products of a cellular waste (Schuh et al. 2019). However, it is now understood that exosomes are involved in intercellular communication (Schuh et al. 2019). They can deliver bioactive cargo from their parent cells to target cells in the body, and thus can change the composition and function of those recipient cells (Patel et al.

2019). Exosome composition and function has been observed to change in response to diseases, injuries, and infections (Beninson and Fleshner 2014). It is understood that exosomes are important inflammatory mediators that have immunological relevance, and they were even found to play a significant role in signaling pathways of cancer cells (Beninson and Fleshner 2014; Vistro et al. 2019). Exosomes are thus a current and very relevant topic of interest due to their potential role in long-distance cellular communication and in modifying the function of the cells that capture them (Vistro et al. 2019). While many studies have focused on expanding our knowledge on the immunological role of exosomes, less is known about their involvement in other aspects of stress physiology (Beninson and Fleshner 2014). It is possible that exosomes, being important inflammatory mediators, are involved in an organism's stress response when no disease is present (Beninson and Fleshner 2014).

Extracellular vesicles

Upon their discovery in 1946, extracellular vesicles from multicellular organisms were widely disregarded in research, being written off as nothing more than cellular waste and platelet dust (Beninson and Fleshner 2014). This changed in the 1980s, when the functional roles of these extracellular vesicles started being discovered, including their involvement in cellular communication and immunity regulation (Beninson and Fleshner 2014). Extracellular vesicles are secreted by all prokaryotic and eukaryotic cells during both normal and abnormal physiological conditions (Kalluri and LeBleu 2020). These vesicles are generated and released by cells into the extracellular space, and they serve as shuttles for delivering cellular contents between different cells of an organism (Simons and Raposo 2009). As a unique form of intercellular communication, extracellular vesicles enable highly specific transmission of signals directly related to cellular function (Chan et al. 2020). They allow for specific delivery of bioactive cargo from a donor cell that will alter the content and often the function of a recipient cell (Chan et al. 2020). The content of extracellular vesicles is variable and depends on the cells that they originate from (Simons and Raposo 2009). The bioactive cargo being delivered by these vesicles can include different lipids, proteins, and small noncoding RNAs (Chan et al. 2020).

While multiple types of extracellular vesicles have been named, these vesicles can be divided into two broad categories: ectosomes and exosomes (Kalluri and LeBleu 2020; Simons

and Raposo 2009). Ectosomes are vesicles with diameters within the range of ~50 nm to 1 μm that are formed by the outward budding of the plasma membrane where they directly pinch off the plasma membrane (Kalluri and LeBleu 2020). Exosomes are the other type of extracellular vesicle, which are of endosomal origin (Kalluri and LeBleu 2020). These nanosized vesicles originate in the endosomal pathway and have a diameter in the range of ~40 to 160 nm, with an average diameter of ~100 nm (Kalluri and LeBleu 2020; Nair and Salomon 2020). While larger extracellular vesicles have a closer resemblance to the composition of the cell they originate from, exosomes were found to consist of a distinctive subset of proteins, lipids, nucleic acids, and glycoconjugates derived from the donor cell (Pegtel and Gould 2019).

Exosome biogenesis

Exosomes are specialized membrane-bound vesicles generated from the endocytic compartments of a cell that get secreted into the extracellular space (Nair and Salomon 2020). They are produced by a variety of cells through the endosomal pathway and have cup-shaped structures (Jiang et al. 2020). Exosome formation involves two membrane inversion events, as shown in **Figure 1**. The first inversion is the endocytosis at the plasma membrane that leads to the formation of an endosome (Ge et al. 2012). The second inversion is the inward budding of the endosomal membrane, which encapsulates the cytoplasm contents of a cell (Ge et al. 2012).

Hence, exosome formation begins with the inward budding of a cell's plasma membrane, or endocytosis, which results in the formation of an early endosome (Aslan et al. 2021). A multivesicular body (MVB), or a late endosome, is then formed by the inward budding of the endosome (Zhang et al. 2019). Invagination of the endosomal membrane creates intraluminal vesicles (ILVs) within the larger MVB (Zhang et al. 2019). The ILVs that form from the invagination of endosomal membranes engulf cytosolic components, while the invaginating membrane incorporates certain proteins (Aslan et al. 2021). Fusion of MVBs with the cell's plasma membrane releases ILVs into the extracellular space, at which point they become proper "exosomes" (Aslan et al. 2021; Zhang et al. 2019). While fusing with the plasma membrane and releasing ILVs into the extracellular space is one fate of MVBs, an alternative route for MVBs is fusing with lysosomes where they get degraded (Minciacchi et al. 2016).

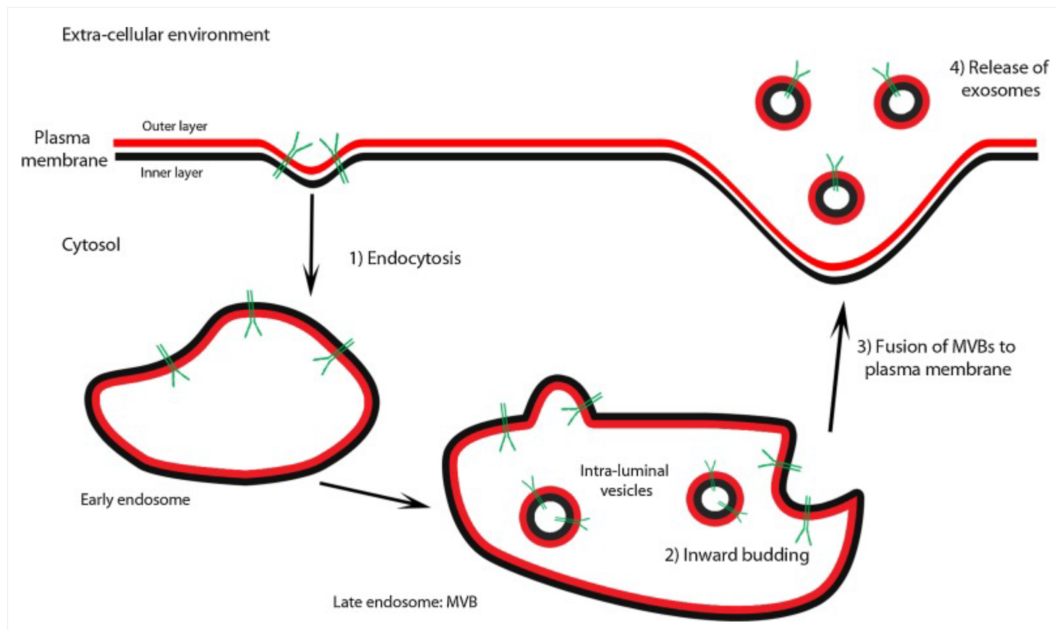

Figure 1. Exosomes originate from intraluminal vesicles (ILVs) that form through the inward budding of endosomal membranes and are released upon fusion of multivesicular bodies (MVBs) with the plasma membrane. Taken from Ge et al. (2012).

Exosome biogenesis depends on the sorting of bioactive cargo during ILV formation when the endosome membrane undergoes inward budding (Minciacchi et al. 2016). Research has revealed the endosomal-sorting complex required for transport (ESCRT) is essential for the formation of ILVs and the sorting of molecules being channeled into ILVs (Minciacchi et al. 2016). ESCRT is a protein machinery that consists of four main cytosolic protein complexes, ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III, which cooperatively facilitate the formation of MVBs, budding of vesicles, and sorting of protein cargo (Jiang et al. 2020; Zhang et al. 2019). The ESCRT machinery typically operates on the cytosol side of the endosomal membrane, where the different ESCRT complexes act sequentially to recognize and sort selected ubiquitinated proteins to ILVs (Jiang et al. 2020; Zhang et al. 2019). Protein ubiquitination is a post-translational modification that involves attaching ubiquitin, a small and highly conserved protein, to a target protein via covalent bonding using a series of enzymes (Qian et al. 2019). The ESCRT-dependent mechanism for exosome secretion begins with ubiquitinated proteins being recognized by the ubiquitin-binding subunits of ESCRT-0, which recruit them to specific domains on the endosomal membrane (Zhang et al. 2019). The membrane-embedded ubiquitinated proteins get bound by the ESCRT-I complex, which also activates the ESCRT-II complex (Jiang et al. 2020). The total complex then associates with the ESCRT-III complex,

which promotes the budding process of the endosomal membrane (Zhang et al. 2019). The vesicle formed by the inward budding of the membrane gets cleaved, which results in an ILV being formed inside an MVB (Zhang et al. 2019). The sorting protein Vps4 (an ATPase) provides the energy for the ESCRT-III complex to disassemble and detach from the MVB membrane (Jiang et al. 2020).

Exosome composition

Exosomes are comparable to cytosol that is captured within a phospholipid bilayer containing lipid rafts, sphingomyelin, ceramide, and phosphatidylserine (Beninson and Fleshner 2014). Glycerophospholipids, sphingolipids, diglycerides, phospholipids, cholesterol, and polyglycerophospholipids can all be found within the phospholipid bilayer membrane of exosomes (Jiang et al. 2020). A phospholipid bilayer that encapsulates the exosome contents serves as barrier that protects those internal molecules from degradation by proteases in the extracellular fluid (Jiang et al. 2020). Many different molecules have been identified inside of exosomes originating from various types of cells (Zhang et al. 2019). The large variety of bioactive elements contained in exosomes includes proteins, nucleotides, lipids, and certain other metabolites (Jiang et al. 2020; Patel et al. 2019). In fact, approximately 4400 proteins, 1639 messenger RNAs, 764 microRNAs, and 194 lipids have been detected in exosomes of different origin, which highlights the structural complexity of exosomes and points to their possible functional diversity (Zhang et al. 2019). Exosomes are sometimes described as miniature versions of their parental cells, and their content is strongly influenced by the status of the cell type they originate from (Zhang et al. 2019). While the health status of the parental cell and various environmental factors can affect the composition of exosomes, there are certain categories of biomolecules that are common to most exosomes (Patel et al. 2019).

MicroRNAs (miRNAs) are a universal component of exosomes (Beninson and Fleshner 2014). They are non-coding RNAs that can regulate messenger RNA (mRNA) by binding to its 3’ end and thus blocking the transcription of target genes post-transcriptionally (Beninson and Fleshner 2014). A single miRNA can regulate hundreds of different mRNAs, because the sequences of miRNA and the target mRNA do not have to be perfectly complementary for transcription to be repressed and protein expression to be modified (Beninson and Fleshner 2014). After being engulfed by exosomes, miRNAs can be transferred between cells

unidirectionally (Zhang et al. 2019). This network of intercellular trafficking results in phenotypic changes experienced by recipient cells, which can be transient or persistent (Zhang et al. 2019). Studies of exosome-based intercellular communication have identified the involvement of miRNA-214, miRNA-29a, miRNA-1, miRNA-126, and miRNA-320, which are miRNAs that participate in exocytosis, angiogenesis, hematopoiesis, and tumorigenesis (Zhang et al. 2019). MiRNA is not the only type of RNA found in exosomes (Zhang et al. 2019). Other types of RNA contained in exosomes, which are generally less abundant than miRNA, are ribosomal RNA, long non-coding RNA, piwi-interacting RNA, transfer RNA, small nuclear RNA, and circular RNA (Zhang et al. 2019). MiRNAs and long RNAs, specifically long non-coding RNAs and circular RNAs, were found to influence different biological processes (Zhang et al. 2019). It is thought that these RNAs transmit signals by operating together, which leads to an alteration or maintenance of local cellular environments (Zhang et al. 2019). They have also been linked to the development of tumors (Zhang et al. 2019).

The protein composition of exosomes is dependent on the cell type from which they originate, as well as their potential physiological function (Van Niel et al. 2006). Exosomes usually contain proteins that serve a variety of different functions. Proteins common to exosomes include tetraspanins, such as CD9, CD63, CD81, and CD82, which participate in the penetration, invasion, and fusion of cells; heat shock proteins, such as Hsp70 and Hsp90, which take part in antigen binding and presentation during the stress response; MVB formation proteins, such as Alix and Tsg101, which participate in exosome release; membrane transport and fusion proteins, such as annexins and Rab; and enzymes, such as acetylcholinesterase (AChE) (Zhang et al. 2019; Welch et al. 2017). Certain exosomal proteins can be considered common components, while other proteins are cell-type specific. Common protein components include the chaperones Hsp70 and Hsp90; G protein subunits; cytoskeletal proteins; ESCRT proteins like Alix and Tsg101; clathrin; transport and fusion proteins, such as Rab 2, Rab 7, and annexins; and even various enzymes and elongation factors (Van Niel et al. 2006). Some common protein components of exosomes are summarized in **Table 1**. While some of these common proteins are known to play a role in exosome biogenesis (such as Alix and Tsg101), the purpose of other common proteins (such as Rabs and annexins) is still not completely defined (Van Niel et al. 2006). As for proteins that are cell-type specific, some of them include MHC class I and II molecules from antigen presenting cells; transferrin receptor from reticulocytes; A33 antigen from intestinal epithelial

cells; CD3 from T-cells; as well as glutamate receptor subunits from neurons (Van Niel et al. 2006). Furthermore, certain adhesion molecules that are exposed at the surface of exosomes also seem to reflect their cell type origin, such as integrins, tetraspanins, and CAMs (Van Niel et al. 2006).

Nevertheless, while the protein and nucleic acid content of exosomes is similar to their cellular origin, there are certain common markers that are indicative of exosomes released from different cellular sources. It is common for most exosomes to contain annexins, rabs, and adhesion molecules that help exosomes to dock and fuse with a recipient cell (Beninson and Fleshner 2014). Another reliable marker of exosomes are tetraspanins, which are transmembrane domain proteins that are found most exosomes (Beninson and Fleshner 2014). Exosome tetraspanins include CD9, CD63, CD81, and CD82 (Beninson and Fleshner 2014). Other exosome markers include different immunological molecules that are found in exosomes, such as the T-cell co-receptor CD86 and major histocompatibility complex (MHC) (Beninson and Fleshner 2014). Such biomolecules that serve as exosome markers can be useful for identifying and even isolating exosomes (Beninson and Fleshner 2014).

Table 1. A summary of proteins that are commonly contained in exosomes. Taken from Zhang et al. (2019).

Protein category and description	Examples
Tetraspanins	CD9, CD63, CD81, CD82, CD37, CD53
Heat shock proteins (HSP)	HSP90, HSP70, HSP27, HSP60
Cell adhesion	Integrins, Lactadherin, Intercellular Adhesion Molecule 1
Antigen presentation	Human leukocyte antigen class I and II/peptide complexes
Multivesicular body Biogenesis	Tsg101, Alix, Vps, Rab proteins
Membrane transport	Lysosomal-associated membrane protein 1/2, CD13, PG regulatory-like protein
Signaling proteins	GTPase HRas, Ras-related protein, furloss, extracellular signal-regulated kinase, Src homology 2 domain phosphatase, GDP dissociation inhibitor, Syntenin-1, 14-3-3 Proteins, Transforming protein RhoA
Cytoskeleton components	Actins, Cofilin-1, Moesin, Myosin, Tubulins, Erzin, Radixin, Vimentin
Transcription and protein synthesis	Histone1, 2, 3, Ribosomal proteins, Ubiquitin, major vault protein, Complement factor 3
Metabolic enzymes	Fatty acid synthase Glyceraldehyde-3-phosphate dehydrogenase Phosphoglycerate kinase 1 Phosphoglycerate mutase 1 Pyruvate kinase isozymes M1/M2 ATP citrate lyase ATPase Glucose-6-phosphate isomerase Peroxiredoxin 1 Aspartate aminotransferase Aldehyde reductase
Trafficking and membrane fusion	Ras-related protein 5, 7 Annexins I, II, IV, V, VI Synaptosomal-associated protein Dynamin, Syntaxin-3
Antiapoptosis	Alix, Thioredoxine, Peroxidase
Growth factors and cytokine	Tumor Necrosis Factor (TNF)-α, TNF Receptors, Transforming growth factor-β
Death receptors	FasL, TNF-related apoptosis inducing ligand
Iron transport	Transferrin receptor

Control of Exosome Secretion

As was previously mentioned in the discussion of exosome biogenesis, there is support for the hypothesis that exosome release is an ESCRT regulated mechanism (Zhang et al. 2019). Exosomes isolated from different cell types were found to contain ESCRT components and ubiquitinated proteins (Zhang et al. 2019). It was also found that depleting certain protein members of the ESCRT machinery can modify the protein content in exosomes and the release rate of exosomes from cancer cells (Minciacchi et al. 2016). For example, it was reported that the typical exosomal protein Alix and the tumor susceptibility gene 101 (Tsg101) protein, both of which are associated with ESCRT, participate in the budding and cleaving of the endosomal membrane, otherwise described as the formation of ILVs that later become exosomes (Zhang et al. 2019). It was also found that Alix interacts with syndecans through syntenin, a cytosolic adaptor (Minciacchi et al. 2016). Through this interaction, Alix facilitates exosomal cargo selection and exosome formation in HeLa and MCCF-7 cells (Minciacchi et al. 2016; Zhang et al. 2019). When Alix is inhibited, muscle and dendritic cells were shown to exhibit a damaged ability to release CD63-enriched exosomes (Minciacchi et al. 2016). Aside from these findings, the Tsg101 and other proteins that are commonly involved in exosome biogenesis have been useful in serving as exosome markers in cancer models, which provides support for the likely involvement of these proteins in exosome formation in cancerous cells (Minciacchi et al. 2016).

An alternative mechanism for exosome secretion that does not involve the ESCRT machinery is referred to as the ESCRT-independent mechanism (Minciacchi et al. 2016). There is evidence of ESCRT-independent processes also being involved in exosome biogenesis and release by mediating the cargo sorting and formation of ILVs (Minciacchi et al. 2016). In the ESCRT-independent mechanism, it seems that the lateral segregation of cargo within endosomal membranes depends on lipid rafts, also known as lipid microdomains, which are located on the external side of plasma membranes and composed of cholesterol sphingolipids (Zhang et al. 2019). It is believed that these lipid rafts are enriched with sphingomyelinases, which are enzymes that catalyze the hydrolysis of sphingomyelin to ceramide and phosphorylcholine (Zhang et al. 2019). Thus, sphingomyelinases in these lipid microdomains help generate ceramides via hydrolytic removal of the phosphocholine moiety (Zhang et al. 2019). Involvement of sphingomyelinases in exosome biogenesis was originally reported in 2008, demonstrating that sphingomyelinase activity is associated with high ceramide levels within

purified exosomes, while inhibition of these enzymes is accompanied by a reduction of extracellular vesicle release (Minciacchi et al. 2016). Some known properties of ceramides are inducing lateral separation and joining of microdomains (Zhang et al. 2019). Also, their cone-shaped structure might contribute to the inward curvature of endosomal membranes and, thus, stimulate domain-induced budding (Zhang et al. 2019). This ceramide-dependent mechanism highlights the importance of exosomal lipids in exosome formation. In addition to lipids, proteins also play a role in exosome biogenesis and protein cargo loading, such as membrane proteins called tetraspanins (Zhang et al. 2019). Tetraspanin-enriched microdomains (TEMs) are membrane platforms for receptor compartmentalization and protein signaling in plasma membranes (Zhang et al. 2019). Findings show that a tertaspanin protein CD81 and TEMs are involved in the sorting of target receptors and cytosolic components to exosomes (Zhang et al. 2019). Essentially, exosome biogenesis and secretion, as well as the specific sorting of bioactive cargo into exosomes, can be accomplished by multiple specialized mechanisms, which can either involve the ESCRT machinery in ESCRT-dependent mechanisms or lipids and tetraspanins in ESCRT-independent mechanisms.

The role of exosomes in intercellular communication

The discovery of exosomes and the research conducted on these extracellular vesicles has revealed that they might comprise a novel mechanism of intercellular communication. It was previously known that communication between neighboring cells involves direct cell-to-cell contact through cell surface protein-to-protein interactions or gap junctions, while communication between distant cells involves secretion of soluble factors that enable signal transmission, such as hormones or cytokines (Zhang et al. 2019). However, exosomes containing cell-specific proteins, nucleic acids, and lipids are now recognized as a novel mechanism of communication between cells (Zhang et al. 2019). When exosomes are secreted by parental cells, they can interact with target cells and affect the behavior and phenotypic features of those cells (Zhang et al. 2019). There are several predictions for how exosomes interact with their target cells. Exosomes express surface adhesion molecules that potentially assist in the capture of exosomes by the target cells (Beninson and Fleshner 2014). Once exosomes bind to a target cell, they can either transfer their cargo to the cytosol of that cell or influence the cell through surface receptors (Beninson and Fleshner 2014). Delivery of bioactive molecules from exosomes to

recipient cells occurs through direct membrane fusion, internalization via endocytosis, or interactions of ligands and receptors (Zhang et al. 2019). Recipient cells can be impacted by the bioactive cargo contained in exosomes via the following three processes, as shown in **Figure 2**: (1) surface-bound ligands directly stimulating the recipient cells; (2) activated receptors being transferred to recipient cells; (3) and delivery of proteins, lipids, and nucleic acids causing epigenetic reprogramming of recipient cells. These exosome-mediated mechanisms enable communication between parental cells and specific neighboring or distant recipient cells (Zhang et al. 2019).

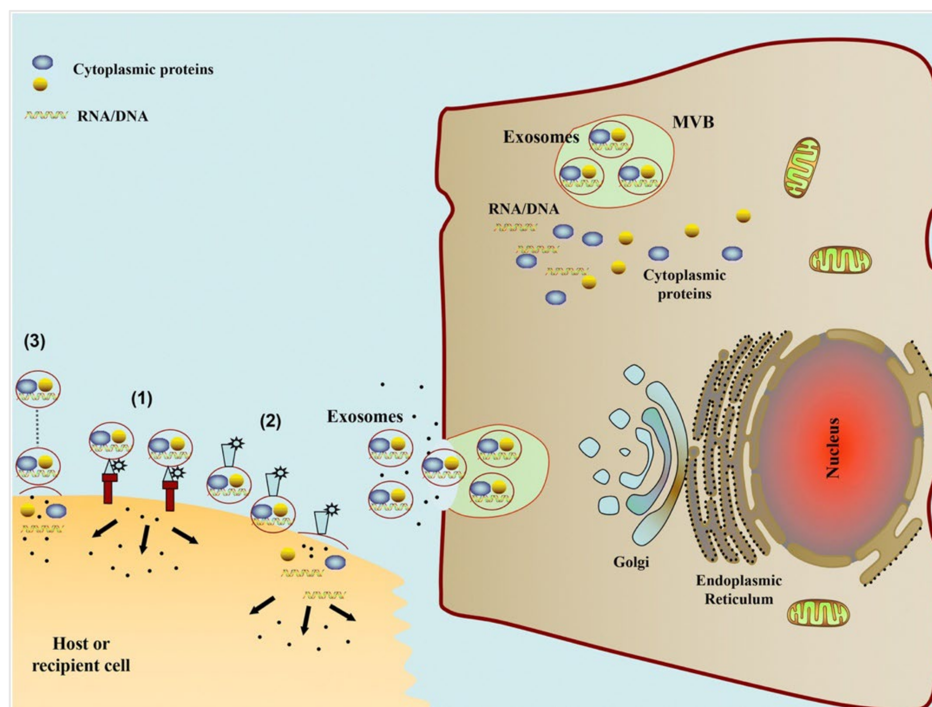

Figure 2. There are three pathways of exosome-mediated intercellular communication. (1) Surface-bound ligands directly stimulate the recipient cells. (2) Activated receptors are transferred to recipient cells. (3) Delivery of proteins, lipids, and nucleic acids causes epigenetic reprogramming of recipient cells. Taken from Zhang et al. (2019).

The exosome-mediated mechanism of intercellular communication has certain advantages over traditional communication mechanisms that involve soluble proteins. Firstly, the phospholipid bilayer membrane of exosomes serves as a protective barrier (Beninson and Fleshner 2014). Exosomes protect proteins and nucleic acids from degradation as they travel through the extracellular environment and mucosal membranes (Beninson and Fleshner 2014). As exosomes travel in a variety of bodily fluids, such as blood, urine, and bile, they protect the

bioactive cargo they carry from degradation and allow for long-distance transport of those molecules between distant cells (Patel et al. 2019). Secondly, exosomes serve as a single 'package' that contains multiple signals, which can activate the cell all at once (Beninson and Fleshner 2014). This is different from traditional intercellular communication, which relies on multiple separate signals to activate a cell (Beninson and Fleshner 2014). Exosomes facilitate simultaneous transfer of multiple miRNAs, which can then target several mRNAs in the target cell all at once (Beninson and Fleshner 2014). Finally, the docking and fusion of exosomal vesicles with recipient cells permits an efficient uptake of its contents by the cell (Beninson and Fleshner 2014). The advantages associated with exosome-mediated communication alleviate the need for cells to be in direct contact and enable effective long-distance intercellular communication (Beninson and Fleshner 2014).

The role of exosomes in immunoregulation

Exosomes play an important role in immunoregulation, with exosome-mediated intercellular communication being involved in immune activation, suppression, and tolerance, as well as in antigen presentation (Zhang et al. 2019). The role of exosomes in immunoregulation was first recognized when exosomes containing MHC and T-cell costimulatory molecules were isolated from dendritic cells and B-cells (Beninson and Fleshner 2014). It was also discovered that leukocytes, lymphocytes, injured cells, and tumor cells secrete exosomes that can stimulate inflammatory immune responses (Beninson and Fleshner 2014). It is important to note that the interaction of exosomes with recipient cells is not random. Adhesion molecules, such as tetraspanins and integrins, are highly expressed in exosomes, which can explain why exosomes exhibit specialized and selective cell homing (Zhang et al. 2019).

Exosomes can stimulate the innate immunity via different processes. For instance, when mammalian cells get infected with a pathogen, the exosomes derived from those cells can exhibit various inflammatory properties (Beninson and Fleshner 2014). Macrophages infected with bacteria were found to release exosomes that contain pathogenic molecules associated with the bacteria (Bhatnagar and Schorey 2007). Exosomes isolated from infected macrophages were able to induce a pro-inflammatory response in uninfected macrophages, which suggests that there is an exosome-mediated mechanism of immunoregulation associated with bacterial infections (Bhatnagar and Schorey 2007). It is likely that macrophages that become infected with a

pathogen produce exosomes that express the molecules associated with that pathogen, which then interact with uninfected immune cells via toll-like receptors (TLRs) (Beninson and Fleshner 2014). Furthermore, macrophages infected with pathogens were found to secrete exosomes that have higher surface expression of Hsp70, a highly stress-inducible heat shock protein that has the ability to stimulate a pro-inflammatory response by inducing macrophage TNF-α release and natural killer cell activity (Anand et al. 2010). In addition to infected macrophages releasing exosomes that have more Hsp70 on their surface, it was also found that they release a larger quantity of exosomes than uninfected macrophages (Anand et al. 2010). Regulation via miRNA is another mechanism by which exosomes can regulate innate immunity (Beninson and Fleshner 2014). Exosome-derived miRNA was found to enhance immunity by participating in pro-inflammatory expression of cytokines, differentiation of macrophages, inflammation mediated by TLRs, among other processes (Beninson and Fleshner 2014).

Exosomes and the stress response

It is now understood that exosomes are important inflammatory mediators that have immunological relevance (Vistro et al. 2019). Exosome composition and function changes in response to diseases, injuries, and infections, and they were even found to play a significant role in signaling pathways of cancer cells (Beninson and Fleshner 2014; Vistro et al. 2019). As a result, most current research focuses on the involvement of exosomes in the development of cancer, angiogenesis, metastasis, immune suppression, and other related processes (Patel et al. 2019). The use of exosomes as markers of an organism's disease status is being widely explored (Patel et al. 2019). Thus, while many studies have focused on expanding our knowledge on the immunological and disease-associated role of exosomes, less is known about their involvement in other aspects of stress physiology (Beninson and Fleshner 2014). It is possible that exosomes, being important inflammatory mediators, are also involved in an organism's response to stress exposure that is not directly associated with disease (Beninson and Fleshner 2014).

When exposed to a stressor, multicellular organisms rely on a variety of mechanisms to sustain physiological homeostasis (Beninson and Fleshner 2014). Upon exposure to an acute stressor, an organism initiates physiological changes that enhance its survival, known as the acute stress response (Fleshner and Crane 2017). This acute stress response consists of activating the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS),

which results in physiological changes, such as glycogen breakdown, increased heart rate and blood pressure, redirection of oxygen and nutrients, piloerection, and pupil dilation (Beninson and Fleshner 2014). The acute stress response allows the organism to utilize its energy stores, thus increasing its chance of survival through stimulated movement and alertness (Beninson and Fleshner 2014).

The HPA axis is a neuroendocrine mechanism that involves the hypothalamus, anterior pituitary gland, and adrenal gland (Sheng et al. 2021). Exposure to a stressor activates the HPA axis by stimulating a cascade of pathways that respond to negative feedback loops associated with those three endocrine glands (Sheng et al. 2021). When an organism experiences stress, the hypothalamus secretes corticotropin-releasing hormone (CRH), which in turn stimulates the anterior pituitary gland to secrete adrenocorticotropic hormone (ACTH) into the bloodstream (Fleshner and Crane 2017; Nicolaides et al. 2015). In response to ACTH, the adrenal cortex releases glucocorticoids, such as cortisol or corticosterone, which are anti-inflammatory agents (Fleshner and Crane 2017; Nicolaides et al. 2015). Low levels of circulating glucocorticoids stimulate the innate immune system (Yada and Tort 2016). In instances of chronic stress, glucocorticoids can reach high concentrations and modulate the immune response by inhibiting inflammation (Yada and Tort 2016). As glucocorticoid release is stimulated by stress, circulating glucocorticoids can be used as a measure of stress and their release may be stimulated by artificial administration of ACTH (Gong et al. 2015; Sung et al. 2021). There are few direct associations between stress and exosomes. However, recent findings suggest that extracellular vesicles derived from immune cells released into plasma carry receptors for CRH and thus participate in intercellular communication during the immune response (Hagiwara et al. 2019). Another recent *in vitro* study found that macrophage-derived exosomes participate in the regulation of adrenal steroidogenesis and corticosterone biosynthesis during LPS-induced septic shock, which further points to the involvement of exosomes in the HPA axis and stress physiology (Ye et al. 2021).

Activation of the SNS during the acute stress response involves the production of catecholamines, such as epinephrine and norepinephrine, which results in enhanced immunity (Fleshner and Crane 2017). Catecholamines can stimulate the release of danger-associated molecular patterns (DAMPs), which regulate the innate immune system and behave like signals that work towards stimulating an organism’s survival (Beninson and Fleshner 2014; Fleshner and

Crane 2017). It was found that Hsp72, a DAMP that stimulates inflammation, is able to associate with exosomes (Beninson et al. 2014; Faught et al. 2017). While Hsp72, just like other heat shock proteins, is known to be immunologically active in the presence of disease, this specific heat shock protein was also found to be elevated in circulation during exposure to a stressor when no disease is present (Beninson and Fleshner 2014). Elevated levels of Hsp72 induced by stressors like heat, exercise, fear of predation, and heat shock suggest that this protein participates in the normal physiological stress response (Beninson and Fleshner 2014). Furthermore, studies on human plasma and tumor cells discovered an elevated expression of Hsp72 on the surface of exosomes upon exposure to a heat stressor (Chen et al. 2011; Lancaster and Febbraio 2005). It is possible that Hsp72 associated with exosomes contributes to immunoregulation that can be induced by stress (Beninson and Fleshner 2014). Moreover, it is unlikely that Hsp72 release occurs via a classical secretion pathway due to its very rapid elevation in plasma upon exposure to a stressor, among other reasons (Beninson and Fleshner 2014). It is thus possible that exosomes offer a secretory pathway for Hsp72 release during an organism's stress response (Beninson and Fleshner 2014). This example highlights the potential role that exosomes play in the acute stress response.

It is also possible that exosomes are involved in an organism's stress response through stress-modified miRNAs. While miRNA modulation is known to occur in the presence of disease, it was also found that miRNA modification can be caused by exposure to stressors when no injury or disease is impacting the organism (Beninson and Fleshner 2014). Stress-modified miRNAs exhibit various immunological properties, such as involvement in the inflammation and healing processes (Beninson and Fleshner 2014). Since exosomes are involved in miRNA transport, stress-modified miRNAs may be another way that exosomes are involved in the physiological response to stress exposure (Beninson and Fleshner 2014). In fact, exposure to an acute stressor has been linked to not only increases in exosomal DAMPs like Hsp70, but also to reductions in exosomal miRNAs that inhibit immunity (Fleshner and Crane 2017). In fact, there is a greater likelihood of plasma-derived exosomal miRNA being modified by stressor exposure than miRNA that is not associated with exosomes (Fleshner and Crane 2017). Such reductions in immune-inhibitory exosomal miRNA upon stressor exposure also correlate with an increase in inflammatory proteins, which gives support to stress-modified exosomes participating in

inflammation modulation and immune stimulation during the stress response (Fleshner and Crane 2017).

The role of exosomes in the stress response is not as well-studied as their role in processes associated with disease (Beninson and Fleshner 2014). Nevertheless, there is some evidence that points to the involvement of exosomes in the normal stress response in the absence of disease or injury. It is possible that exposure to acute stressors modifies the miRNA and protein cargo contained within exosomes (Beninson and Fleshner 2014). The protective barrier of exosomes allows for a stable circulation of those miRNAs and proteins over long distances without the need for direct cell-to-cell contact (Jiang et al. 2020). The known immunomodulating properties of exosomes and their associated compounds, such as miRNAs and Hsp72, suggest that secretion of modified exosomes can occur during exposure to a stressor and lead to enhanced innate immunity (Beninson and Fleshner 2014).

MODEL SPECIES: THE BROWN ANOLE

This research used the brown anole (*Anolis sagrei*) as the model organism for studying the role of exosomes in the physiological stress response. While the brown anole is native in the Caribbean, it became an invasive and abundant species in several regions of the USA, which include Florida, Louisiana, California, and Hawaii (Fisher et al. 2020). There are several reasons for why *A. sagrei* was a great model species to be used in this research. Since this study was conducted in Winter Park, Florida where this invasive species is abundant, *A. sagrei* served as an accessible model organism that could be easily collected from nearby habitats without significantly reducing its overall population. Also, it is not difficult to maintain a colony of this species in captivity. Finally, past research has looked at the physiology and endocrinology of *A. sagrei* that relates to the stress response, including the HPA axis, which provided useful framework for designing and conducting this study (Himmelstein et al. 2021; Fokidis and Brock 2020).

Exosomes in reptiles

Past research has more commonly focused on exosomes isolated from mammalian cells (Chen and Holt 2021; Schuh et al. 2019). While mammalian cells and bodily fluids are considered the “conventional” sources for producing and isolating exosomes, there is a growing

collection of research that investigates other sources of exosomes. The various sources of exosomes that have been identified include all mammals, as well as reptiles, fish, insects, birds, plants, and even microbes (Askenase 2021; Schuh et al. 2019). While reptiles have not been the focus of most exosome-related research, some studies have identified the presence of exosomes in fluids of different reptiles. Research on Chinese soft-shelled turtles (*Pelodiscus sinensis*) found that their oviduct epithelium and glands are involved in the secretion of exosomes into the lumen (Waqas et al. 2017). For the same turtle species, their bile was also found to release exosomes, with these biliary exosomes potentially being released by hepatocytes and travelling with bile into the gallbladder (Zhu et al. 2019). The presence of exosomes was also identified in the venom of snakes, which is secreted from their salivary-related venom glands (Schuh et al. 2019). While research on the presence of exosomes in bodily fluids of reptiles is limited, a handful of past findings suggest that it could be possible to detect exosomes in *A. sagrei*.

Exosome-mediated intercellular communication is not confined to human or mammalian cells (Schuh et al. 2019). The ability to isolate and study exosomes from non-mammalian sources can allow us to learn more about the role of exosomes in physiology and provide more accessible alternatives for research (Schuh et al. 2019). Using the abundant *A. sagrei* as the model organism for this study allowed us to investigate the role of exosomes in the physiological stress response and to determine whether it is possible to study exosomes in this species.

RESEARCH QUESTION, GOALS, AND HYPOTHESES

There is emerging evidence of exosomes serving as a novel mechanism of intracellular communication and responding to physiological stressors, and thus there is value in examining their involvement in the physiological stress response. This study explores whether exosome concentrations in the blood plasma of *A. sagrei* are affected by stress exposure, and if exosome release is needed to reach the stressed phenotype. The first goal of this research was to develop and refine an exosome extraction protocol for plasma samples of our model organism, *A. sagrei*. Once we confirmed that exosomes from the plasma of *A. sagrei* can be isolated and quantified, we proceeded with stress studies using our optimal exosome isolation method. We hypothesized that exposure to stress will result in higher exosome concentrations and that blocking exosome release would produce a less pronounced stress response.

METHODS

Anole capture and husbandry

All *A. sagrei* research subjects were captured in Winter Park, Florida on the Rollins College campus. A total of 27 adult brown anoles were caught using a fishing pole with a loop made out of a fishing line. Following capture, each anole was taken to the colony room in Bush, weighed, and housed in a separate tank containing a carpet, PVC pipe, hammock, and a live plant. The tanks were separated by partitions and exposed to identical environmental conditions (27 °C; even light exposure; photoperiod set to 14 hours of light and 10 hours of darkness). The cages and live plants were sprayed with water every two days. The anoles were each fed with two crickets coated in calcium and multivitamin supplements every three days. The cages were cleaned monthly.

Attempts to isolate exosomes with ultracentrifugation

Frozen reserves of anole plasma obtained from studies in 2017 were pooled to test exosome isolation methods using a standard ultracentrifugation protocol (Lässer et al. 2012). Briefly, **(1)** plasma was centrifuged at 300 x g at 4 °C for 10 minutes; **(2)** supernatant was collected and centrifuged at 16,500 x g at 4 °C for 20 minutes; **(3)** supernatant was collected again, diluted with 5 mL of PBS (1X, pH 7.4), and filtered through a 0.2 µm filter; **(4)** the filtrate was then ultracentrifuged at 120,000 x g at 4 °C for 70 minutes; **(5)** and a crude exosome pellet was obtained by discarding the supernatant.

Another variation of this protocol was then attempted, which involved the use of a sucrose density gradient to achieve greater purification. In this variation, the supernatant obtained in step **(2)** above was added to a sucrose gradient (50%, 40%, 35%, 30%, 20%, and 10% solutions), and the gradient was then ultracentrifuged as in step **(4)**. It was predicted that exosomes would concentrate in the 35% to 40% layer of the gradient (Chiou et al. 2018).

All attempts to isolate exosomes using ultracentrifugation were unsuccessful. While we altered the protocol multiple times and used different volumes of plasma, the success of exosome isolation was ultimately prohibited by equipment limitations. Performing filtration in step **(3)** was inefficient and unfeasible. Moreover, the ultracentrifuge was not working properly and destroyed our samples at each attempt.

Exosome isolation using a commercial kit

After numerous unsuccessful ultracentrifugation attempts, we used the commercial Invitrogen Total Exosome Isolation Kit for plasma (Product No. 4484450, ThermoFisher Scientific, Waltham, MA, USA). As validations, pooled reserves of both anole and also red-eared slider (*Trachemys scripta elegans*) plasma were used at different volumes of plasma (25, 50, 100, and 500 µL of anole plasma; 100 µL of slider plasma). We followed the exosome isolation protocol provided by the kit, which included the following steps: **(1)** 0.5 volume of PBS (1X, pH 7.4) was added to each plasma sample and vortexed thoroughly; **(2)** 0.2 volume of the Exosome Precipitation Reagent was added to the samples with PBS and vortexed thoroughly; **(3)** each sample was incubated at room temperature for 10 minutes; **(4)** each sample was then centrifuged at 10,000 x g at room temperature for 5 minutes; **(5)** the supernatant was discarded, and each pellet containing exosomes was stored in a freezer until further processing.

Quantifying exosomes via an acetylcholinesterase assay

To quantify exosome concentrations, we measured acetylcholinesterase (AChE) activity of isolated pellets, which is thought to correlate with exosomes, using the commercial colorimetric kit (ab138871 AChE Assay Abcam Inc., Boston, MA, USA). We followed the manufacturer's instructions. Briefly, **(1)** we first prepared the acetylthiocholine reaction mixture (4.5 mL of assay buffer; 250 µL of 20X DTNB stock solution; 250 µL of 20X Acetylthiocholine stock solution); **(2)** then used a 1000 mU/mL acetylcholinesterase standard solution and assay buffer to perform serial dilutions (200, 100, 50, 10, 2, 1, and 0 mU/mL) for the standard curve; **(3)** then we added 100 µL of assay buffer to each isolated exosome pellet; **(4)** then loaded 50 µL of each standard and sample into a 96-well plate; **(5)** then added 50 µL of the acetylthiocholine reaction mixture to each well; **(6)** incubated the reaction at room temperature protected from light; **(7)** and finally read the absorbance at 410 nm at both the 10 and 30 minute mark.

Study 1: The baseline concentration of exosomes in brown anoles

To determine the degree of individual variation in exosome concentrations and provide a basis for future comparison, blood samples were collected from anoles (n=16) within two minutes of capture from their tanks. Blood was sampled from the post-orbital sinus using a heparinized capillary tube and stored on ice until plasma was isolated by centrifugation at 2,000

x g at room temperature for 5 minutes. Extracted plasma was stored in a freezer until exosomes were isolated and quantified as outlined above.

Study 2: The effect of acute stress on exosome concentrations

As in study 1, we collected baseline blood samples from anoles (n=16) from the post-orbital sinus. After 20 minutes, a period sufficient for anoles to experience an acute physiological response to the stress of handling, a second blood sample was collected from each anole, and samples were stored on ice until plasma was isolated by centrifugation and then stored in a freezer until further processing.

Study 3: The effect of exogenous ACTH on exosome concentrations

To determine if exosome concentrations change with stress, and specifically with the release of ACTH, which stimulates glucocorticoid release, we administered intraperitoneal (i.p.) injections of either ACTH (0.325 IU in 50 µL of saline, n=6 anoles) or saline (50 µL, n=6 anoles). At 45 minutes post-injection, blood samples were collected as above, and the isolated plasma was kept in a freezer until assayed.

Study 4: The effect of an exosome blocker on exosome and corticosterone concentrations

To determine if exosomes are necessary for mounting a stress response, anoles were injected with either the exosome biogenesis inhibitor GW4869 (Tocris Inc. Bio-Techne Inc. at a dose of 12 µg/anole in 100 µL of saline, n=5) or saline (100 µL, n=4). At 30 minutes post-injection, blood samples were collected as above to measure the stress hormone corticosterone, as well as exosomes, and the isolated plasma was stored in a freezer until being processed.

Enzyme-linked immunoassay for corticosterone

We used the DetectX Corticosterone Enzyme Immunoassay Kit (Arbor Assays Inc., Ann Arbor, MI, USA) to quantify plasma corticosterone. We followed the assay protocol provided by the kit. Briefly: **(1)** 5 µL of the dissociation reagent and 490 µL of assay buffer were first added to 5 µL of each plasma sample; **(2)** then a 100,000 pg/mL corticosterone stock solution was serially diluted with assay buffer at 10,000, 5,000, 2,500, 1,250, 625, 312.5, 156.25, and 78.125 pg/mL concentrations; **(3)** then 50 µL of each sample and standard, 50 µL of assay buffer (to

determine maximum antibody binding), and 75 μL of assay buffer (to determine non-specific binding) were loaded into a 96-well plate; **(4)** then 25 μL of corticosterone conjugate were added to each well, followed by 25 μL of corticosterone antibody added to all wells, except for the non-specific binding well; **(5)** then the plate was placed on a shaker at room temperature for 30 minutes; **(6)** then aspirated and washed with wash buffer; **(7)** then 100 μL of TMB substrate were added to each well and **(8)** incubated at room temperature for 30 minutes; **(9)** then lastly 50 μL of stop solution were added to each well and **(10)** the plate was read at an absorbance of 450 nm.

Data analysis

For both the AChE activity and corticosterone concentration assays, the absorbance values from known concentrations were used to calculate a standard curve. The standard curve was then used to obtain the activity or concentration values for our unknown samples. For all studies, we calculated the mean AChE activity and standard error for each group of samples. For study 4, we also calculated the mean corticosterone concentration and standard error for each group of samples. We used paired t-tests to compare the baseline and post-stress AChE activity of study 1 and to compare the baseline AChE activity of studies 1 and 2. We used two-sample t-tests for all other comparisons. Significant results were those with $p < 0.05$.

RESULTS

Preliminary data

To validate whether we can isolate exosomes from plasma, we tested different volumes of plasma derived from common pools of two species, the brown anole and the red-eared slider. AChE activity was detected in all isolated exosome pellets, and it increased as the volume of plasma used for isolation increased, confirming that we can successfully isolate exosomes from blood plasma (**Figure 3**). Interestingly, AChE activity was higher in plasma from red-eared sliders than from that of the brown anole (**Figure 4**).

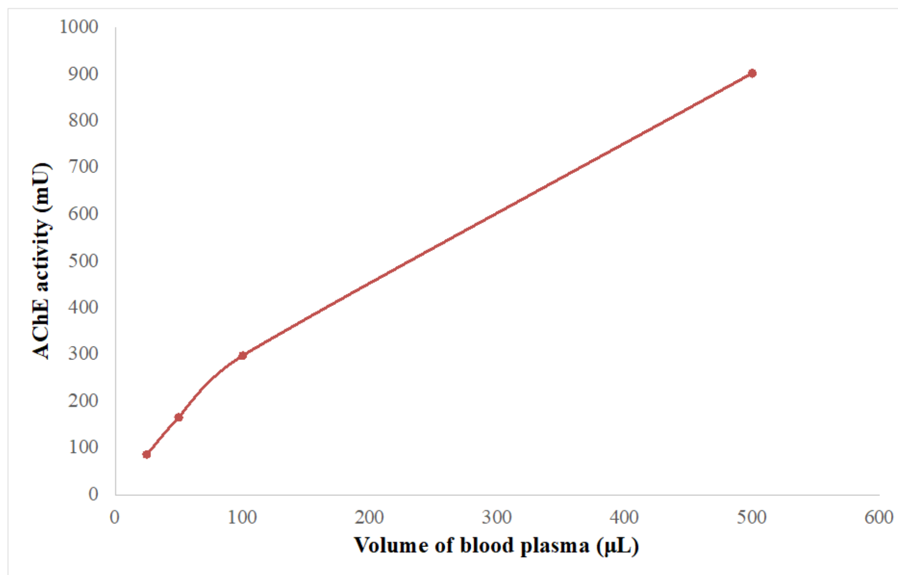

Figure 3. AChE activity increased as the volume of brown anole blood plasma used for exosome isolation increased. Blood plasma reserves of brown anoles (*Anolis sagrei*) were pooled. Exosomes were isolated from different volumes (25, 50, 100, and 500 μL) of pooled plasma via a commercial kit. Acetylcholinesterase (AChE) activity of isolated exosome pellets was measured using a commercial colorimetric kit.

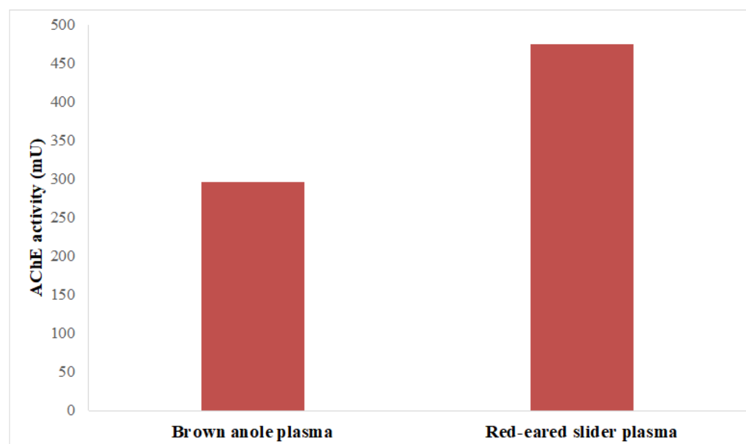

Figure 4. Blood plasma of red-eared sliders had higher AChE activity than that of brown anoles. Blood plasma reserves of brown anoles (*Anolis sagrei*) and red-eared sliders (*Trachemys scripta elegans*) were pooled. Exosomes were isolated from 100 μL of anole and slider plasma via a commercial kit. Acetylcholinesterase (AChE) activity of isolated exosome pellets was measured using a commercial colorimetric kit.

To test whether repeated freezing and thawing of plasma alters AChE activity, plasma samples that were frozen and then thawed multiple times over several weeks were compared to samples that were only thawed once immediately prior to exosome isolation. AChE activity was comparable across the samples, suggesting that repeated thawing of anole plasma does not impact AChE activity (**Figure 5**).

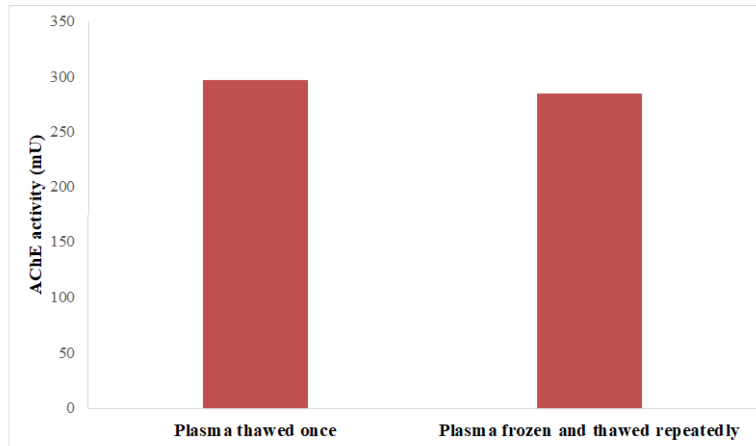

Figure 5. Repeated freezing and thawing of brown anole blood plasma did not affect measured AChE activity. Blood plasma reserves of brown anoles (*Anolis sagrei*) were pooled. Exosomes were isolated from 100 µL of a plasma sample that was repeatedly frozen and thawed and one that was thawed once. Acetylcholinesterase (AChE) activity of isolated exosome pellets was measured using a commercial colorimetric kit.

Study 2: Acute handling stress increased exosome concentrations in brown anoles

Acute handling stress increased the mean AChE activity, with AChE activity being significantly higher in the stress samples compared to the baseline samples ($t = -4.21$, $df = 15$, $p = 0.0008$; **Figure 6**).

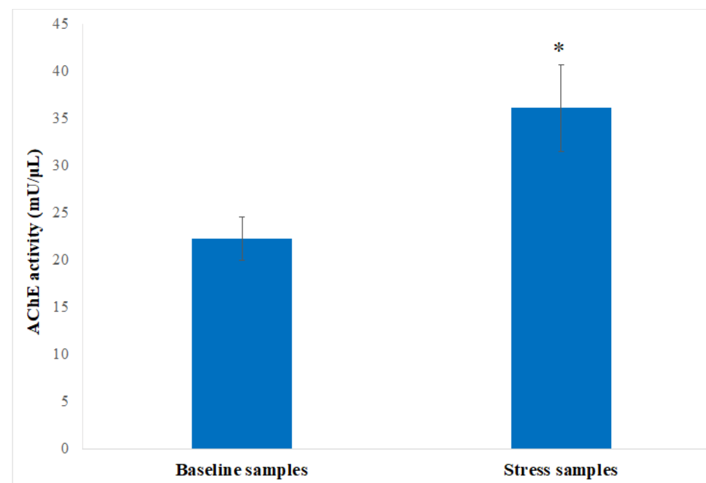

Figure 6. Exposure to an acute stressor (handling stress) caused an increase in AChE activity in brown anoles. Two sets of blood plasma samples were collected from the post-orbital sinus of brown anoles (*Anolis sagrei*; n=16) in captivity, one consisted of baseline samples and the other of stress samples collected 20 minutes later, after which anoles experienced an acute physiological response to the stress of handling. Exosomes were isolated from the samples via a commercial kit. Acetylcholinesterase (AChE) activity of isolated exosome pellets was measured using a commercial colorimetric kit. The mean AChE activity of each sample group was plotted with standard error. Statistically significant difference ($p < 0.05$) was indicated with an asterisk (*).

Study 3: Exogenous ACTH decreased AChE activity in brown anoles

Exogenous ACTH decreased the mean AChE activity, with activity being significantly higher in the saline controls compared to the ACTH treatment group ($t = -3.07$, $df = 10$, $p = 0.0118$; **Figure 7**).

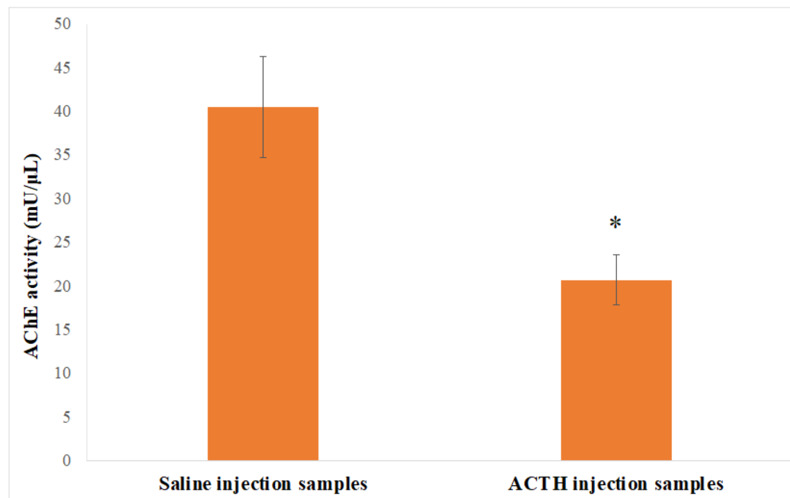

Figure 7. Exogenous ACTH caused a decrease in AChE activity in brown anoles. Intraperitoneal injections of either adrenocorticotropic hormone (ACTH) (0.325 IU in 50 µL of saline, n=6) or saline (50 µL, n=6) were administered to brown anoles (*Anolis sagrei*) in captivity. Blood plasma samples were collected from the post-orbital sinus of the anoles at 45 minutes post-injection. Exosomes were isolated from the samples via a commercial kit. Acetylcholinesterase (AChE) activity of isolated exosome pellets was measured using a commercial colorimetric kit. The mean AChE activity of each sample group was plotted with standard error. Statistically significant difference ($p < 0.05$) was indicated with an asterisk (*).

Prolonged captivity decreased exosome concentrations in brown anoles

To test how prolonged captivity (i.e., a chronic stressor) alters exosome concentrations, we compared the AChE activity of baseline samples collected during study 1 to those collected one week later as part of study 2. Prolonged captivity decreased the mean AChE activity, with AChE activity being significantly higher in the first set of baseline samples compared to the second set ($t = 2.59$, $df = 15$, $p = 0.0204$; **Figure 8**).

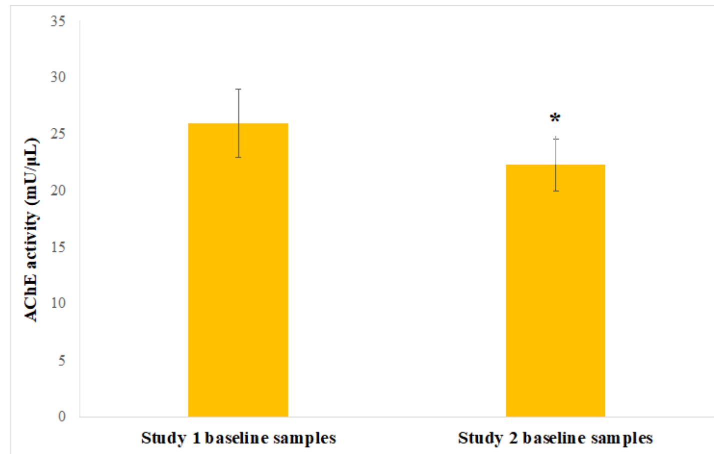

Figure 8. Prolonged captivity caused a decrease in AChE activity in brown anoles. Two sets of baseline blood plasma samples were collected one week apart from the post-orbital sinus of brown anoles (*Anolis sagrei*; n=16) in captivity. Exosomes were isolated from the samples via a commercial kit. Acetylcholinesterase (AChE) activity of isolated exosome pellets was measured using a commercial colorimetric kit. The mean AChE activity of each sample group was plotted with standard error. Statistically significant difference ($p < 0.05$) was indicated with an asterisk (*).

To further test the effects of prolonged captivity on exosome concentrations, we compared the AChE activity between our saline injection samples from study 3 and 4, which were collected 3 weeks apart. Prolonged captivity decreased AChE activity, with the mean AChE activity of study 3 samples being significantly higher than those from study 4 ($t = 4.56$, $df = 10$, $p = 0.0011$; **Figure 9**).

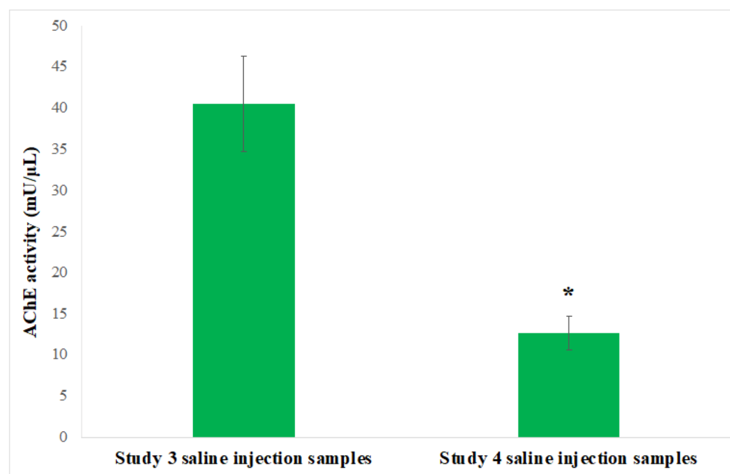

Figure 9. Prolonged captivity caused a decrease in AChE activity in brown anoles injected with saline. Two sets of blood plasma samples (n=6) were collected 3 weeks apart from the post-orbital sinus of brown anoles (*Anolis sagrei*) in captivity that were injected with saline. Exosomes were isolated from the samples via a commercial kit. Acetylcholinesterase (AChE) activity of isolated exosome pellets was measured using a commercial colorimetric kit. The mean AChE activity of each sample group was plotted with standard error. Statistically significant difference ($p < 0.05$) was indicated with an asterisk (*).

Study 4: An exosome blocker injection increased AChE activity in brown anoles

Administering an exosome blocker caused an unexpected increase in the mean AChE activity compared to the control group ($t = 3.45$, $df = 10$, $p = 0.00624$; **Figure 10**).

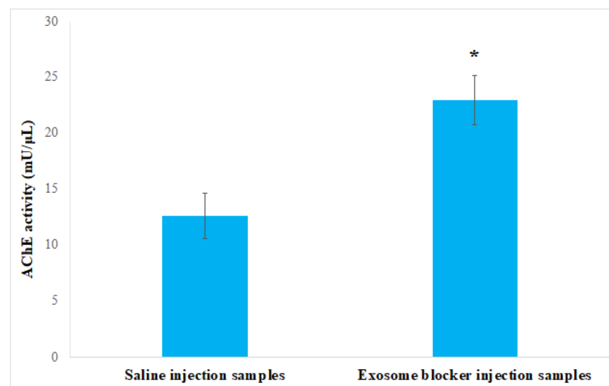

Figure 10. An exosome blocker injection caused an increase in AChE activity in brown anoles. Intraperitoneal injections of either an exosome biogenesis inhibitor GW4869 (12 µg/anole in 100 µL of saline, n=5) or saline (100 µL, n=4) were administered to brown anoles (*Anolis sagrei*) in captivity. Blood plasma samples were collected from the post-orbital sinus of the anoles at 30 minutes post-injection. Exosomes were isolated from the samples via a commercial kit. Acetylcholinesterase (AChE) activity of isolated exosome pellets was measured using a commercial colorimetric kit. The mean AChE activity of each sample group was plotted with standard error. Statistically significant difference ($p < 0.05$) was indicated with an asterisk (*).

Injection with the exosome blocker produced lower concentrations of corticosterone compared to the saline injection group, however this was not statistically significant ($t = -1.07$, $df = 10$, $p = 0.156$; **Figure 11**).

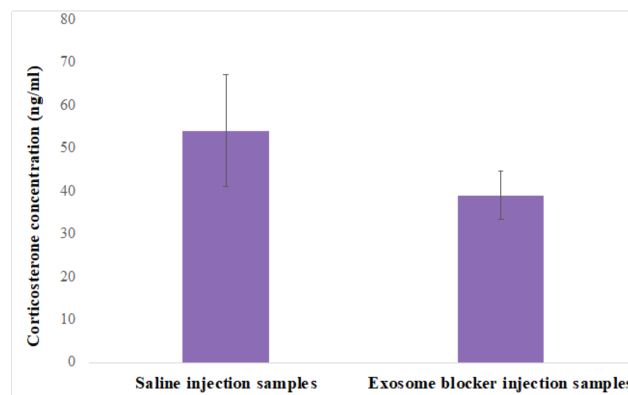

Figure 11. An exosome blocker injection did not cause a significant difference in plasma corticosterone levels in brown anoles. Intraperitoneal injections of either an exosome biogenesis inhibitor GW4869 (12 µg/anole in 100 µL of saline, n=5) or saline (100 µL, n=4) were administered to brown anoles (*Anolis sagrei*) in captivity. Blood plasma samples were collected from the post-orbital sinus of the anoles at 30 minutes post-injection. Corticosterone concentrations of the samples were measured using a commercial immunoassay kit. The mean corticosterone

concentration of each sample group was plotted with standard error. Statistically significant difference ($p < 0.05$), if any, was indicated with an asterisk (*).

DISCUSSION

While exosomes are recognized as a novel mechanism of intercellular communication, most research focuses on the involvement of exosomes with the diseased physiological state, and less is known about the role of exosomes in normal aspects of physiology that are not associated with disease (Beninson and Fleshner 2014; Patel et al. 2019; Zhang et al. 2019). This study aimed to contribute to the limited knowledge on the intrinsic role of exosomes in the physiological stress response. Exosomes were successfully isolated from the blood plasma of both the brown anole and red-eared slider turtle using a commercial kit. Exosome concentrations in plasma varied across our studies. Interestingly, while exposure to acute handling stress increased exosome concentrations as hypothesized, chronic stress associated with captivity decreased exosome concentrations over time. Additionally, exogenous ACTH, which stimulates glucocorticoid release, caused a decrease in AChE activity. Finally, my hypothesis that blocking exosome release would result in a less pronounced stressed response was not supported. Together this set of studies suggest that exosome release is impacted by stress, but in complex ways that demand further study.

Exosomes can be successfully isolated from the blood plasma of brown anoles

Our research showed that exosomes are present in non-mammalian species, which can potentially make exosome-focused research more accessible. While mammalian cells and bodily fluids have served as conventional sources for studying exosomes *in vitro*, our research suggests that future exosome studies can also consider using non-mammalian species. The presence of exosomes has been detected *in vitro* not only in mammals, but also in various other living organisms, such as reptiles, birds, insects, fish, plants, and microbes (Askenase 2021; Fokidis et al. 2015; Schuh et al. 2019). Our preliminary findings indicate that plasma reserves of brown anoles had lower exosome concentrations than observed in red-eared sliders. Thus, it could be interesting to explore how exosome concentrations vary between different species in future research.

Ultracentrifugation turned out to be a time-consuming and unreliable method of isolating exosomes. Even with successful isolation, there are other drawbacks of ultracentrifugation,

including only being able to process six samples at a time, allotting much of the research time towards exosome isolation, and risking loss of samples and incomplete isolation (Zeringer et al. 2015). A commercial exosome isolation kit offered efficiency and consistency with exosome isolation. The kit used in this study precipitates exosomes using a classified polymer, and it operates by tying up molecules of water and forcing less-soluble components out of solution. Detecting AChE activity in all exosome pellets isolated from plasma reserves using the commercial kit allowed us to validate the use of this kit for future exosome isolation from anole (and slider) plasma.

AChE activity is a reasonable measure of exosome concentrations

This study relied on measuring AChE activity as a way of quantifying exosomes in the collected plasma samples. AChE is an enzyme that is enriched in most exosomes, which gets incorporated into exosomes during exosome formation (Welch et al. 2017). Enzymatic activity of AChE is thus frequently used as an exosome marker (Welch et al. 2017). While AChE activity is not an exact measure of exosome concentrations, it is still a promising and accessible method of studying exosomes. Indeed, Matsumoto et al. (2016) found that there is a positive correlation between exosome concentrations quantified by AChE activity and by nanoparticle tracking analysis, which is another more direct method of quantifying exosomes. These results suggest that measuring AChE activity is a reasonable method of quantifying exosomes (Matsumoto et al. 2016).

In our study, AChE activity increased as the volume of plasma used for exosome isolation increased. Assuming that greater volumes of plasma contain more exosomes, our results suggest that more exosomes are associated with higher AChE activity, which validates the use of the AChE assay as an accessible method for studying exosome concentrations in exploratory studies. Our results also suggest that repeated freezing and thawing of plasma does not affect measured AChE activity, which further validates the use of this method for exosome quantification in long-term research. However, it is possible that the abundance of AChE varies across individual exosomes and that AChE activity and exosome concentrations do not have a perfectly positive correlation. Therefore, the relationship between AChE activity and exosome concentrations needs to be studied more extensively to further confirm the validity of this exosome quantification method.

Acute stress exposure may increase exosome release, while chronic stress exposure may decrease it over time

The conducted stress studies reveal several significant and interesting results. Firstly, our results suggest that exposure to an acute stressor increases exosome release in brown anoles. After experiencing a physiological response to the stress of handling, which is an acute stressor, exosome concentrations in anole plasma increased, as was indicated by a significant increase in AChE activity. Discovering that acute stress may potentially cause more exosomes to be released into blood plasma is an important finding that aligns with certain observations of other studies. Emerging research findings reveal that the composition, biogenesis, and secretion of exosomes can be affected by different stress conditions, such as oxidative stress, heat shock, chemotherapy, hypothermia, and others (Vulpis et al. 2019). In fact, past studies found that stress stimuli can provoke an increased release of exosomes from cancer cells (Vulpis et al. 2019). Infected macrophages were found to release more exosomes than uninfected macrophages (Anand et al. 2010). It was also found that Hsp72, a DAMP that promotes inflammation and can associate with exosomes, is elevated in circulating plasma during exposure to stressors like heat, exercise, fear of predation, and heat shock (Beninson and Fleshner 2014; Beninson et al. 2014; Faught et al. 2017). This is a relevant finding, because evidence suggests that exosomes serve as a secretory pathway for Hsp72 release during stress exposure (Beninson and Fleshner 2014). Another study also found that endoplasmic reticulum stress leads to the release of extracellular vesicles that carry pro-inflammatory DAMPs (Collett et al. 2018). Not only does this evidence support the idea of exosomes being involved in the physiological stress response, but it also suggests that stress exposure may provoke increased exosome release.

While handling stress caused an increase in exosome concentrations in our study, we observed opposing effects with other forms of stress experienced by the anoles. Our results suggest that exposure to a chronic stressor reduces exosome release in anoles over time. After conducting the stress studies, we decided to look at how AChE activity changed over time in plasma samples that were collected under similar circumstances. By comparing two sets of baseline plasma samples that were collected 1 week apart, we found that the first set of samples had significantly higher AChE activity that the second set, indicating that exosome concentrations in anoles decreased with prolonged captivity. Similarly, by comparing two sets of plasma samples that were collected from anoles injected with saline 3 weeks apart, we found that

the first set of samples had significantly higher AChE activity that the second set, again indicating that exosome concentrations in anoles decreased with prolonged captivity. So, while exposure to acute stress may have caused exosome concentrations in anole plasma to increase, chronic stress seemed to have caused a decrease in exosome concentrations over time. These opposing responses to acute and chronic stress provide potentially valuable insight into the role of exosomes in stress physiology and intercellular communication. While activation of the acute stress response initiates inflammatory processes, exposure to chronic stress can result in the exhaustion of the stress response and have detrimental effects on an organism's physiology (Fleshner and Crane 2017). The opposite changes in exosome concentrations we observed with acute versus chronic stress possibly reveal that the two types of stress responses rely on different quantities of circulating exosomes. Perhaps, the physiological response to short-term and acute stress involves increased exosome release, while long-term and chronic stress causes the opposite. Since past research on exosomes has mostly focused on the diseased physiological state and measured exosome composition more often than exosome release, out study reveals novel findings about exosome release during acute and chronic stress responses that are not associated with disease.

Exogenous ACTH unexpectedly increased AChE activity

In addition to testing the effects of handling stress and prolonged captivity on exosome concentrations in anoles, we also tested how exosome release is affected by exogenous ACTH. As was explained earlier, stress activates the HPA axis by causing CRH to be secreted by the hypothalamus and, in turn, ACTH to be secreted by the anterior pituitary gland (Fleshner and Crane 2017; Nicolaides et al. 2015). Circulating ACTH then stimulates the adrenal cortex to release glucocorticoids, such as corticosterone (Fleshner and Crane 2017; Nicolaides et al. 2015). This study administered exogenous ACTH to anoles as an attempt to overstimulate their HPA axis, which is involved in the physiological stress response. While we expected exogenous ACTH to act as an acute stressor and increase exosome release, the opposite effect was observed. Exogenous ACTH caused a significant decrease in the mean AChE activity, indicating that it decreased exosome release in anoles. While unexpected, this result could have been observed for several reasons. Firstly, the artificially administered ACTH could have caused unpredicted effects on other physiological processes within anoles that resulted in lower exosome release. It

is also possible that exogenous ACTH did not stimulate the HPA axis any further. Perhaps, not enough time was given for exogenous ACTH to cause an additional effect on the HPA axis. Otherwise, it is possible that the accumulated physiological effects of prolonged captivity overshadowed the potential effects of administered ACTH on exosome release. In turn, the measured difference in exosome concentrations could be a result of individual variation caused by small sample size.

The administered exosome blocker was not successful at inhibiting exosome release

In addition to testing how stress exposure affects exosome release in anoles, we also attempted to test whether exosome release is needed for reaching the stressed phenotype. This study administered an exosome biogenesis inhibitor to anoles to test whether blocking exosome release prevents them from achieving their normal stress response. Since corticosterone release is stimulated by stress via the HPA axis, we used plasma corticosterone concentrations as a measure of stress in the anoles (Fleshner and Crane 2017). Unexpectedly, administering the exosome blocker caused a significant increase in the mean AChE activity, indicating that exosome release increased. This observation suggests that the administered exosome blocker did not inhibit exosome release. It is possible that the chosen blocker is not effective in our model organism or that not enough time was given for the blocker to inhibit exosome release. The measured difference in exosome concentrations could again be the result of individual variation caused by small sample size.

Anoles injected with the exosome blocker had a lower mean corticosterone concentration compared to the control group, however this was not a statistically significant result. The ineffectiveness of the exosome blocker injection prevented us from observing how corticosterone concentrations are affected by inhibited exosome biogenesis and from testing whether exosome release is needed for reaching the stressed phenotype. It is known that the acute stress response involves low levels of circulating glucocorticoids, and that chronic stress results in higher glucocorticoid concentrations (Yada and Tort 2016). It was also found that macrophage-derived exosomes participate in the regulation of corticosterone biosynthesis during LPS-induced septic shock (Ye et al. 2021). Thus, future research that studies exosome concentrations during acute and chronic stress could benefit from measuring glucocorticoid concentrations.

Future directions

To confirm that prolonged captivity causes exosome concentrations in plasma to decrease, future studies can collect samples from anoles in the field first, and then sample the same anoles in captivity several times. The potential effects of prolonged captivity on exosome concentrations should be controlled for in studies on acute stress by reducing the time that subjects spend in captivity. Other forms of acute and chronic stress should also be tested, as well as other species and bodily fluids, to gain a broader knowledge on the effect of stress on exosome release. With access to more advanced equipment, future studies should also test how exposure to stress affects the composition of exosomes in the absence of disease. Finally, other exosome blockers and measures of stress should be used to test whether reaching the stressed phenotype is dependent on exosome release.

CONCLUSION

While exosomes are no longer disregarded from research or considered to be cellular waste, a lot is still unknown about the role of exosomes in physiology. This was a novel and exploratory study that attempted to gain more insight into the role of exosomes in stress physiology. Past studies on exosomes have mostly focused on expanding our knowledge on the immunological and disease-associated role of exosomes. Currently, very little is known about the involvement of exosomes in other aspects of stress physiology. Our research revealed that, at the very least, exosomes are involved in the normal physiological stress response. By using this study as a starting point for conducting future research on the relationship between exosomes and stress, we can gain a deeper understanding of the role of exosomes in our physiology as a whole.

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