Ethobehavioral Studies of Fear, Sex, and Biological Rhythms

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Abstract

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All animals, to varying degrees, must make decisions regarding when and how to balance the need to acquire resources ("foraging") and the need to avoid threats ("avoidance"). The neurobiological and psychological processes supporting strategy selection have evolved not just within specific ecological contexts, but they have also evolved to be plastic and integrate information across contexts and motivational states to promote survival and reproductive fitness. The research methods commonly used among behavioral neuroscientists are inadequate to understand the dynamics of neurobiological and psychological processes as integrated functions of space and time. The studies described in this dissertation explore a closed behavioral system which attempts to hold context constant in order to measure the temporal dynamics and functions of behavior. The first study demonstrates that cyclic experiences of fear can entrain circadian rhythms and reverse the "natural" active period of rats. The next study examines sex differences

in behavioral decisions regarding approach-avoidance conflict in the closed behavioral system and in a novel foraging task containing an artificial predatory threat, and compares these results to traditional Pavlovian fear conditioning. Finally, the influence of female's reproductive cycle on risky foraging behaviors is analyzed, demonstrating that a phase-specific increase in risky-taking behavior is not met with greater reward.

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Chapter 1. BACKGROUND AND INTRODUCTION

1.1 HISTORICAL CONTEXT

Philosophers and scientists have long pondered the nature of the psyche and the relationship between "rational", intellectual cognition and "irrational", passionate emotion. Plato conceived of the human mind as consisting of a constant conflict between reason and emotion which drives the will, a perspective that persisted up until the 17th and 18th centuries, when early empiricists conjectured the opposite, as David Hume proclaimed: "Reason is, and ought only to be the slave of the passions…." While philosophers debated the metaphysical and moral essence of emotions, and economic philosophy continued operating under assumptions of rational agency well into the 20th century, it was in the early 19th century that a scientific understanding of emotions began to form. In 1806, physiologist Sir Charles Bell meticulously mapped the muscles involved in the expression of human emotions, and in 1876, Charles Darwin was among the first to consider what the *functions* of emotions were, in humans as well as other animals, which was made possible by his formalization of a spatiotemporal context for understanding function: the causes and consequences of differential reproduction, i.e., evolution by means of natural selection.

Darwin's theory forms the basis of modern ethology – the study of the natural expression and function of behavior – which was pioneered by Konrad Lorenz, Karl von Frisch, and Nikolaas Tinbergen. Tinbergen (1963) proposed four questions that ethologists should consider to help organize the analysis of behavior, two addressing "ultimate", or evolutionary, functions of behavior, and two addressing "proximate", developmental and mechanistic functions, as paraphrased and briefly elaborated upon below:

1. Why did the behavior evolve in the way it did, i.e., what is the phylogenetic history of the behavior? (Phylogeny)
2. Why does the behavior persist, i.e., what are the fitness costs and benefits of the behavioral trait in the present environment? (Adaptation)
3. How does the behavior develop, i.e., what environmental factors, epigenetic changes, critical stages, or learning must occur to generate the behavior? (Ontogeny)
4. How does the behavior occur, i.e., what are the molecular, cellular, and psychological causes of the behavior? (Causation)

While many early ethologists were naturalists and zoologists, the importance of understanding the connection between proximate and ultimate causes has not escaped psychologists and behavioral neuroscientists. Almost 30 years prior to Tinbergen's Four Questions, psychologists Edward C. Tolman and Egon Brunswik outlined a framework, which they called *probabilistic functionalism,* for understanding the relationship between external cues, what they ultimately served to indicate, and the mechanisms of perceiving these cues and responding to them in way that achieved proximate and ultimate goals (Tolman & Brunswik, 1935). For a simple example, a predator odor is a proximal stimulus – one of many potential proximal stimuli, including the visual features of the predator or the sounds it makes – that elicits a defensive response, which achieves the proximal goal of safety (negative reinforcement). Furthermore, the predator odor is tied to the ultimate need to sustain life until reproduction, which is the ultimate goal achieved, in part, by avoidance behavior. In Brunswik's Lens Model (Fig. 1.1), central processes, such as fear or cognitive maps, evolved as a means of parsing biologically-relevant information from an environment filled with "noise" or uncertainty and selecting the response most likely to be adaptive (Brunswik, 1952; Petrinovich, 1979).

This dissertation is primarily concerned with understanding the functions of a particular class of emotions – fear and anxiety – and addressing issues regarding the methodology used toward this goal. This first chapter will focus on reviewing previous research toward understanding fear, its neurobiological underpinnings, and its functions; and biological sex – being necessary for a comprehensive understanding of the function of fear – its evolutionary importance, biological characteristics, and influence on behavior. The second chapter will introduce the experimental system used throughout the dissertation and present findings that the cyclic fear interacts with circadian (daily) rhythms to adapt behavior to the risk of shock in a way that supersedes traditional associative learning mechanisms. The third chapter will discuss a study examining sex differences and estrous cycle influences in fear, anxiety, and foraging behavior, and their correspondence with other paradigms used to study fear, and argues that males and females have different functional interests. The final chapter will provide a summary of the findings and present the general conclusions regarding the utility of ethobehavioral studies.

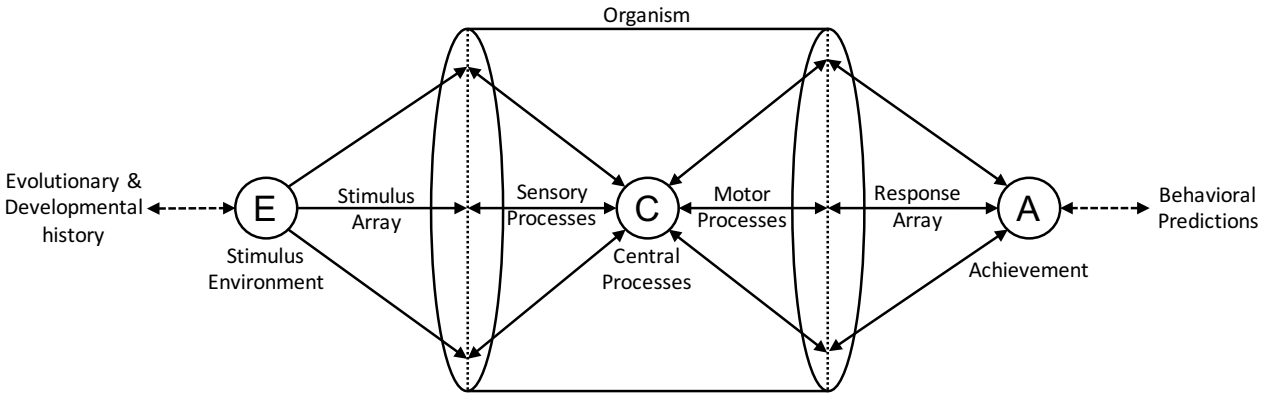

Figure 1.1. The Lens Model of organismic function. Cues from the environment (E), which are probabilistically associated with vital eco-evolutionary factors (ultimate needs), are detected and accumulated by organisms' sensory organs, processed centrally (C), and a response is selected from a hierarchy of behavioral responses probabilistically associated with achieving immediate goals (A), which serve as (fallible) predictions of what is evolutionarily adaptive. Two-way arrows represent feedback mechanisms such as sensory sensitization/habituation and reinforcement learning. (Adapted from Petrinovich, 1979).

1.2 What Can Ethobehavioral Studies Tell Us about the Brain's Fear System?

"No real-life predator is going to present cues before it attacks… [or give] enough trials for the necessary learning to occur…What keeps [foraging] animals alive in the wild is that they have very effective innate defensive reactions which occur when they encounter any kind of new or sudden stimulus". Robert Bolles (1970)

1.2.1 *The Nature of Fear*

Fear is a defensive mechanism that rapidly activates coordinated bodily and behavioral responses to environmental stimuli that the brain, as a result of genetics and experience, has come to recognize as potentially dangerous. The fear system likely evolved because animals that successfully evade predatory threats while foraging for resources (e.g., food, water, mate, shelter) have a reproductive advantage over those that do not (Darwin, 1872a). The brain's ability to instinctively recognize and respond accordingly to certain dangers and undergo experience-dependent plasticity to new threats is thus predicated by the evolutionary pressure associated with each species' interactions within its ecological niche (Fig. 1.1). For example, the main defensive behavior displayed by the woodland-living deermouse, *Peromyscus maniculatus austerus*, is freezing, which provides a stealth function against its natural predators (e.g., the weasel's sensitivity to the prey's movement), whereas the vertical leap response of the arid region-residing deermouse, *Peromyscus maniculatus gambeli*, is adaptive against its natural predators (e.g., the gopher snake's strike) (Hirsch & Bolles, 1980). Likewise, each species' biological history predisposes fear learning to certain stimuli and not others. A canonical example of this is the discovery that laboratory rats can easily learn, via Pavlovian conditioning, to associate sound/light

with footshock (fear conditioning) and tastes with emetic agents (conditioned taste aversion) but cannot associate sound/light with emetics and tastes with footshock (Garcia & Koelling, 1966). Presumably, rats have an evolutionary history of encountering the temporal coincidence of sound/visual (but not taste) cues with cutaneous pain of predatory attack and experiencing the delayed temporal overlap of taste (but not sound/visual) cues with gustatory pain of consuming poisonous food. In contrast, birds rely on visual acuity for searching for food and thus easily associate visual cues with emetic food (Darwin, 1872b), such as the monarch butterfly's wing pattern and its cardenolides poison. Thus, the rodent brain's capacities to rapidly and lastingly associate auditory and ocular inputs with cutaneous pain-inducing stimuli (including artificial footshock) and taste inputs with gustatory illness-inducing stimuli have evolved as a genetic trait.

Consistent with this notion, a recent study found that different populations of neurons dispersed in the basolateral nucleus of the amygdala (BLA) become activated to either context-footshock or saccharin-LiCl conditioning, but not to both (Chung, Barot, Kim, & Bernstein, 2011), providing evidence of biologically predisposed learning at the cellular level within the same brain region. Similarly, a subset of distributed neurons in the dorsal pedal ganglion of the marine mollusk *Tritonia* are predetermined to develop into memory networks (Hill, Vasireddi, Wang, Bruno, & Frost, 2015). Such distinct neuronal information processing likely enables the same brain structures to perform diverse functions effectively.

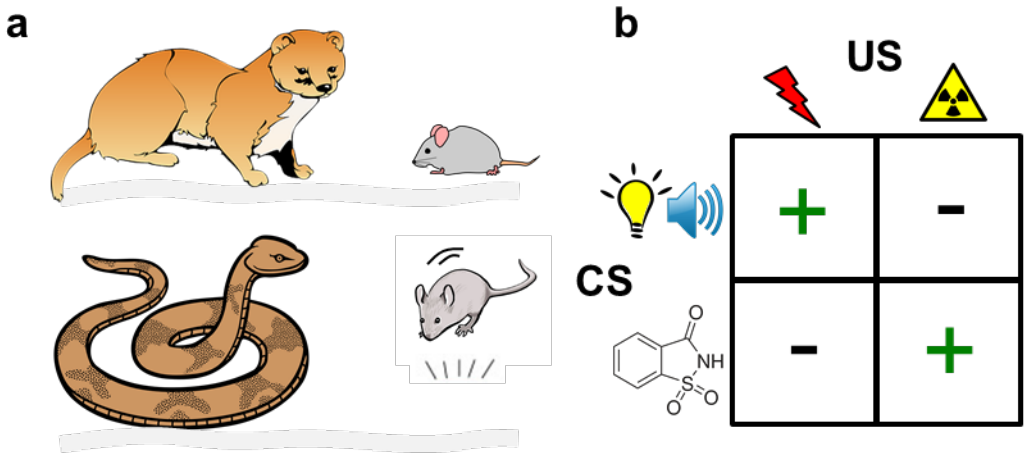

Figure 1.2. Evolutionary influences on innate and learned fear. (a) Predatory history shapes prey's innate fear responses as illustrated by *P. m. austerus* deermouse's freezing to weasel and *P. m. gambeli* deermouse's jump (Jan Gillbank, "Drawing of a grey mouse" October 27, 2012 via Wikimedia, Creative Commons Attribution 3.0 License) to gopher snake (Hirsch & Bolles, 1980). (b) Ecological history predisposes fear learning. A classic study by John Garcia (1966) found that rats easily acquired conditioned fear to bright/noisy CS paired to footshock US and conditioned taste aversion to saccharin taste CS paired to X-rays (or LiCl) US. However, rats showed lack of conditioning to bright/noisy-X-ray (or LiCl) and saccharin-footshock pairings.

1.2.2 *The Neurobiology of Fear*

Compared to CS information pathways to the amygdala (particularly auditory information) (Fig.1.3), much less is known about how US information reaches the amygdala. Because fear conditioning is only one of several operations of the amygdala (Chang et al., 2015; Everitt, Cardinal, Parkinson, & Robbins, 2003; Moscarello & LeDoux, 2013; Zeeb & Winstanley, 2013), those neurons that are essential to the formation of a conditioned fear memory should be distinct from other, non-fear-functioning neurons by receiving (or being capable of rewiring to receive) both CS and US afferents in order to undergo associative synaptic plasticity. The PAG and the parabrachial nucleus (PBN), two components of the ascending pain transmission pathway (Almeida, Roizenblatt, & Tufik, 2004), are postulated as providing footshock-US inputs to the amygdala. Electrical stimulation of the dorsal PAG, which produces strong activity bursts (jumping and running), have been shown to be a sufficient surrogate US to support auditory and contextual fear conditioning in rats (Kim et al., 2013). Correspondingly, pharmacological inactivation of PAG neurons attenuated periorbital shock US-evoked response in LA neurons and decreased acquisition of auditory fear conditioning (Johansen, Tarpley, LeDoux, & Blair, 2010; but see Kim, Rison, & Fanselow, 1993). This study (Johansen et al., 2010) further showed that PAG neurons are initially responsive to the US, but as the conditioned fear developed, the US-evoked responses in the PAG decreased via the increasing amygdala-PAG pathway-mediated analgesia. In essence, this negative feedback characteristic of the amygdala-PAG circuit represents a biological analog of the Rescorla-Wagner model (Rescorla & Wagner, 1972) of Pavlovian fear conditioning (Fanselow & Wassum, 2016). Another recent study showed that optogenetic silencing of the calcitonin gene-related peptide (CGRP)-expressing neurons in the PBN, or their terminals in the CEA, blocked the footshock-US's ability to support contextual and auditory fear

conditioning (Han, Soleiman, Soden, Zweifel, & Palmiter, 2015). Conversely, optogenetic stimulation of these originating and terminal regions served as an effective US in fear conditioning. Given the monosynaptic dPAG-PBN projection (Krout, Jansen, & Loewy, 1998), it appears that the dPAG and PBN constitute an overlapping US pathway to the amygdala, and the finding that silencing PBN neurons blocked fear conditioning to a footshock-US suggests that the PAG's indirect pathways to the BLA and reciprocal connections with the CEA are not necessary for fear conditioning to footshock-US. If the dPAG and PBN are indeed both sufficient and necessary in relaying the footshock-US information to the amygdala, a straightforward prediction would be that, in previously fear conditioned animals, subsequent silencing/lesioning of neurons in these structures with continued CS-US presentations should lead to a gradual extinction because the amygdalar neurons would only be receiving unreinforced CS information. This "litmus test" de rigueur for confirming the essential US pathway (McCormick, Steinmetz, & Thompson, 1985) has yet to be performed. Future studies will also need to investigate non-pain US pathways to the amygdala that support fear conditioning, such as loud noise (Watson & Rayner, 1920) and predatory odor (Takahashi, Chan, & Pilar, 2008). Most likely, a network of amygdalar neurons that represents a conditioned fear memory must receive both CS and US inputs, and thus characterizing the projection topography of different USs on amygdalar nuclei is relevant to optogenetic, genetic ablation and cellular imaging studies.

While many fear responses require the amygdala in order to be expressed, some studies suggest other circuitry can mediate fear responses in the absence of the amygdala. For example, patients with bilateral amygdala lesions due to Urbach-Wiethe disease do not exhibit fear responses to a variety of normally fear-eliciting stimuli and are unable to acquire fear through conditioning (Feinstein et al., 2013). However, inhalation of CO_2 is still capable of eliciting panic-

like responses in these patients. The circuits mediating such responses in the absence of the amygdala remain uncertain, but potential candidates have been identified. The PAG may be part of the circuit, as previous research has shown that stimulation the PAG in humans induces sensations of panic (Carrive & Morgan, 2004), but hypothalamic regions may be sufficient for fear responses and learning as well (Kunwar et al., 2015). Thus, it appears that different circuits mediate different forms of fear responses, which are likely to be differentially activated depending on the situation.

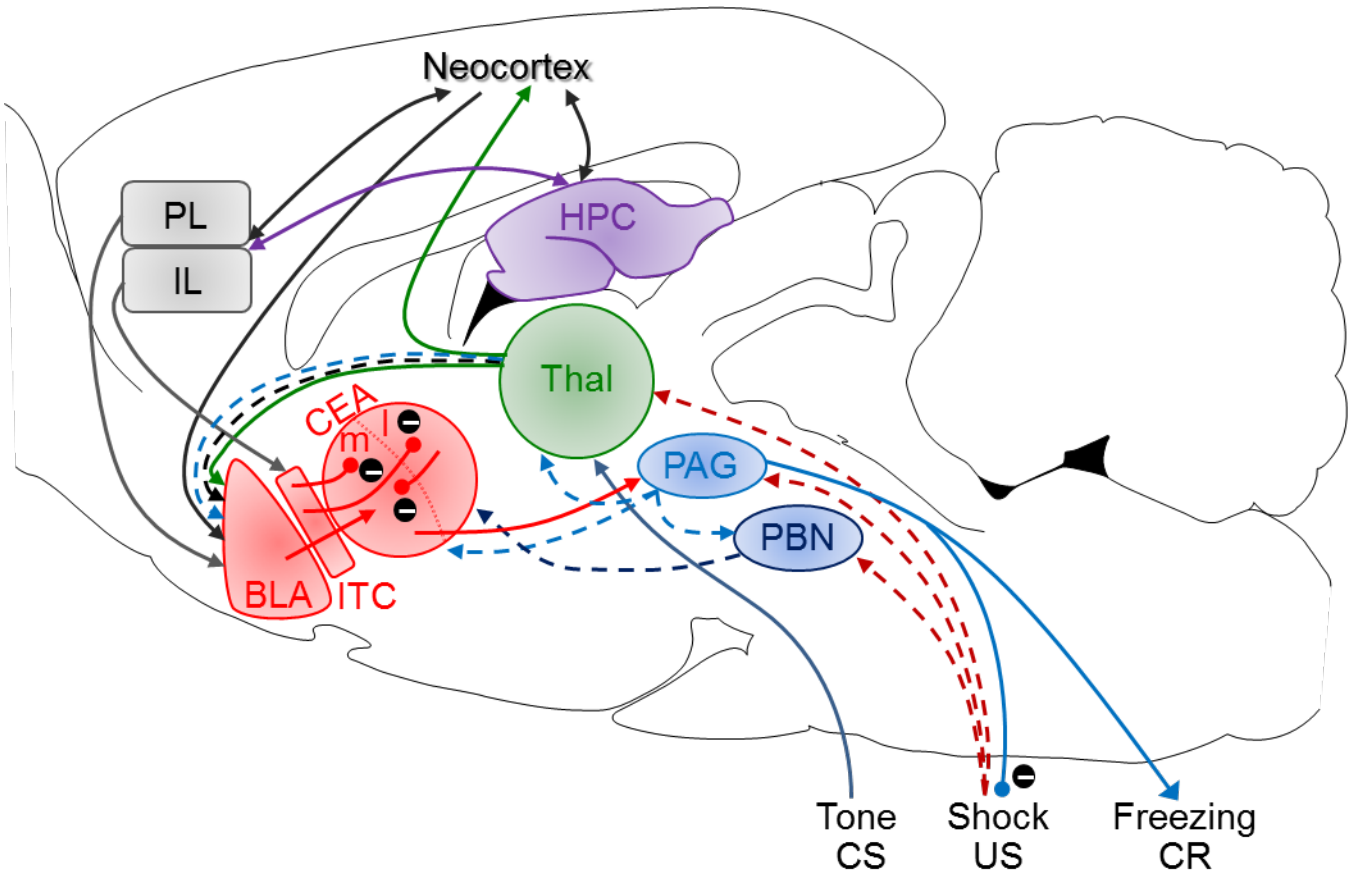

Figure 1.3. A putative fear conditioning circuit. The auditory CS information reaches the amygdala via the direct thalamic pathway and indirect cortical pathway. The footshock US information is relayed to the amygdala via the ascending pain pathways. The CS-US association formation is thought to occur in specific subnuclei via associative LTP-like mechanism that strengthens the CS-amygdala synapses. BLA, basolateral complex of the amygdala; CEA, central nucleus of the amygdala; ITC, intercalated cells of the amygdala; PL, prelimbic cortex; IL, infralimbic cortex; HTP, hippocampus; Thal, thalamus; PAG, periaqueductal gray; PBN, parabrachial nucleus. Inhibitory pathways are represented by encircled minus symbols.

Given that the amygdala (including LA) is also implicated in appetitive/reward learning, attention, memory modulation, aggression, sex, decision-making, and social behavior (Chang et al., 2015; Everitt et al., 2003; Moscarello & LeDoux, 2013; Zeeb & Winstanley, 2013), it is vital to understand how its neurons participate in fear conditioning while integrating other behavioral functions. As mentioned in the opening, one possibility is that there are different subsets of amygdalar neurons that are prewired for different functions, consistent with the notion of biologically predisposed learning (Fig. 1.1) (Chung et al., 2011; Garcia & Koelling, 1966; Hill et al., 2015). The other possibility is that amygdalar neurons (or a subset of neurons) have pleiotropic mnemonic functions and that those with relatively elevated intracellular protein signals related to neuronal plasticity become preferentially engaged during any amygdala-dependent experience. Consistent with the latter view are findings that the **c**yclic adenosine monophosphate-**r**esponsive **e**lement **b**inding protein (CREB) is crucial for the formation of contextual fear memories (Kida et al., 2002), that LA neurons with relatively higher CREB activity are preferentially recruited into an auditory fear memory (Han et al., 2007), and that post-training selective ablations, via an inducible diphtheria toxin (DT), of ~15% sparsely-distributed LA neurons overexpressing CREB, 'tagged' via replication-defective herpes simplex viral (HSV) vectors, permanently abolished auditory fear memory (Han et al., 2009; Josselyn, Köhler, & Frankland, 2015). Following the DT-induced loss of memory retrieval, the animals were able to relearn the conditioned fear response, suggesting that amongst the remaining neurons, those with relatively higher CREB activity were successors to encoding this auditory fear memory. However, whether these CREB-overexpressing neurons receive both CS and US inputs, a crucial requirement for encoding fear memory, has not been confirmed. Recent cellular compartment analysis of temporal activity by fluorescence in situ hybridization (catFISH) studies reported that only about 4% of neurons in the LA/BLA showed

coincident *Arc/Arg3.1* (an immediate early gene expressed in glutamatergic neurons) mRNA activation to both CS and US events (Barot, Chung, Kim, & Bernstein, 2009). If a small fraction of dispersed LA neurons receive convergent CS and US inputs, and it is unlikely that the HSV vectors would selectively target those neurons in advance of fear conditioning, then the post-training induced ablation likely included nonspecific cell death that indirectly affected fear CRs rather than excised the fear engram per se. Similarly, opto/chemo-genetic activation studies of fear conditioning do not demonstrate that all virally-infected neurons with the same promotor are fear-functioning neurons. In my view, it is more likely that broad stimulation of the amygdala triggers fear behaviors because these output circuits would have the lowest thresholds for activation if the brain is wired to err on the side of survival. Future studies will need to address whether fear memories are encoded in the amygdala via biologically predisposed neurons or via pleiotropic-functioning neurons based on their relative CREB activity. One possible avenue of research is to stimulate the US pathways (dPAG and PBN) to overexpress CREB selectively in US-responsive amygdalar neurons, and quantify and ablate those neurons *pre hoc* to see if fear conditioning can be permanently prevented (Kim et al., 2013).

1.2.3 *Ethobehavioral Approaches to Studying Fear*

Despite the advances made, fear conditioning research likely provides an incomplete picture of the brain's fear system because it is based on assessing the magnitude of a specific response (e.g., freezing) in a small experimental chamber that restricts the animal's repertoire of behavior. While learning about threats in the environment is an important function for survival, the generalizability of fear conditioning studies has been called into question (Bolles, 1970). The primary issue is that instinctive fear to sudden stimuli (auditory, visual, olfactory, etc.) would provide a competitive advantage over the time-consuming and hazardous process of trial-and-error

learning. In the case of predator-prey interactions, there is a constant arms-race: Predators will benefit by minimizing their detectability/predictability and prey will benefit by increasing their sensitivity, and consequently their false alarm rate. In nature, foraging distance and duration correlate with the likelihood of encountering predatory (and other) threats (Fig. 1.4), and fear serves two general functions—an immediate defensive response when facing predators and an enduring influence on foraging strategy as a function of experience (Boissy, 1995; Fanselow & Lester, 1988; Ydenberg & Dill, 1986). Ethologically-relevant paradigms provide a greater match to the real-world threat situations that the brain's fear system evolved to solve, and thus present means to critically evaluate the utility of fear conditioning models. Whereas fear conditioning research typically emphasizes the processes underlying fear learning specifically, ethological studies can provide greater control over the degree to which innate and learned fear interact, allowing researchers to study the functional relationships between innate and learned fear in situations that simulate contingencies animals are likely to encounter in the wild. Several studies have employed such paradigms to study fear in rodents, and demonstrate the significance of innate fear in evading predation.

Many species instinctively respond to simple yet reliable indicators of threat. For example, "looming stimuli", simple cues that expand rapidly in the visual field, mimicking advancing predators, elicit avoidance responses in diverse species (Sun & Frost, 1998). Recently, Yilmaz and Meister (2013) showed that overhead visual display of a rapidly expanding dark disc, akin to a shadow cast by an approaching aerial predator, triggers immediate flight and freezing behavior in mice. Münch and colleagues (2009) found that a subset of "OFF" retinal ganglion cells in mice respond selectively to stimuli approaching or increasing in size compared to laterally-moving or shrinking stimuli, perhaps allowing rapid responding to imminent threats without the need for

cortical processing. Indeed, it has been shown that several types of motion-sensitive retinal ganglion cells directly innervate the superior colliculus (Huberman et al., 2009), and that neurons in the medial intermediate layers of the superior colliculus, which project to the BLA via the lateral posterior thalamus, are necessary for the defensive responses to looming stimuli (Wei et al., 2015).

Other studies (Amir, Lee, Headley, Herzallah, & Pare, 2015; Choi & Kim, 2010; Cianca, Bartolini, Porfiri, & Macrì, 2013; Kim et al., 2015) have utilized predator-like robots to examine naturalistic fear behavior within laboratory settings. For example, Choi and Kim (2010) placed rats in a naturalistic 'approach food-avoid predator' situation to study how rats integrate appetitive and defensive motivations to make risky foraging decisions, and found that rats form a distance gradient of fear near the source of threat. Furthermore, the amygdala was necessary for responding defensively to the robot (Choi & Kim, 2010), and amygdalar stimulation was sufficient to elicit the same defensive behavior in foraging rats without external threat (Kim et al., 2013). Interestingly, dPAG stimulation produced stronger defensive behavior than amygdalar stimulation, and the effect of dPAG stimulation was abolished by lesioning/suppressing the amygdala while the amygdalar stimulation effect remained intact with PAG lesions, suggesting that, in contrast to fear conditioning-based models (Fig. 1.2), the amygdala may act downstream of the dPAG in responding to predators (Kim et al., 2013). Very recently, Amir and colleagues (2015) have used the same paradigm to characterize amygdala neuronal activity during risky foraging. When all projection neurons recorded in the BLA were analyzed, about 69% decreased while 7% increased their firing rate when rats began foraging regardless of whether the robot or the food were present. These findings suggest that rather than signaling the presence of threat and activating defensive behavior, as concluded from amygdala recordings in Pavlovian fear conditioning studies (Maren & Quirk, 2004), BLA activity is closely associated with actual

behavioral output regardless of threat risk (Amir et al., 2015). However, given previous research that shows BLA stimulation can produce multiple forms of defensive behaviors (i.e., freezing and escape) (Kim et al., 2013) and that distinct threats activate distinct amygdalar neurons (Barot et al., 2009), more research will be needed to differentiate whether the BLA gates locomotor behavior as opposed to playing a motivational role. A similar artificial predator approach has been used effectively in a different animal model system to demonstrate that a biomimetic robotic predatory fish elicits robust and consistent defensive behaviors in zebrafish (*Danio rerio*) (Cianca et al., 2013). The precise control achieved by using robotic threats provides an opportunity to understand the parameters of defensive behaviors and provide insight into the naturalistic workings of neural circuits.

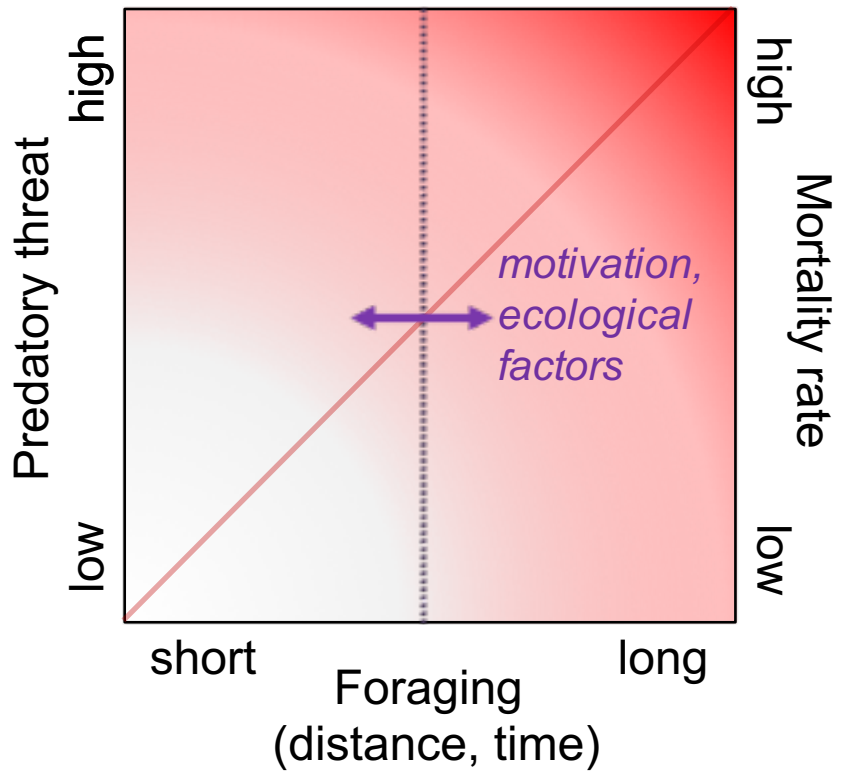

Figure 1.4. Foraging and risk of predation. Foraging distance and time away from the safety of a nest are positively correlated with the risk of meeting predators, which can result in injury or death. Motivational factors, such as hunger, reproductive and parental state, and ecological factors, such as food availability and predator density, influence foraging behavior (represented by a horizontal arrow) and thus predation risk. Fear elicits immediate species-specific defense reactions upon meeting a predator and exerts enduring influences on foraging strategy.

There are likely many other stimuli that evoke innate defensive responses, such as olfactory and auditory cues associated with predators. Olfactory cues associated with predators, such as cat fur/saliva (Papes, Logan, & Stowers, 2010), bobcat, weasel, ferret, and fox urine/feces (Ferrero et al., 2011; Rosen, Asok, & Chakraborty, 2015), have also been reported to evoke avoidance, freezing, and stretch-attend posturing and other risk-assessment behaviors. Different predator odors seem to share some common features (e.g., sulfated chemical signatures) and substrates (e.g., olfactory receptors and the Grueneberg ganglion), and the lateral habenula and the laterodorsal tegmentum appear to be important for regulating fear responses to predator odors coordinated by hypothalamic nuclei (Yang et al., 2016). Recent work by Zanette and colleagues (Suraci, Clinchy, Dill, Roberts, & Zanette, 2016; Zanette, White, Allen, & Clinchy, 2011) used playbacks of predator vocalizations to effectively manipulate fear in songbirds (*Melospiza melodia*) and wild raccoons (*Procyon lotor*), and demonstrated that innate fear alone has significant effects not only on the natural foraging decisions of diverse animals, but that it also influences reproduction and parental behavior, the quality of offspring, and the structure of the ecosystem. Thus, utilizing ethological approaches in both laboratory and field settings to understand the neural circuitry underlying innate fear signals and their roles in risky foraging decisions will undoubtedly prove fruitful.

Ethobehavioral studies highlight how spatial factors influence 'cost-benefit' decisions while attaining resources and avoiding threats. While the hippocampus is well-known for encoding spatial information necessary for navigation (Buzsáki & Moser, 2013; T. Hartley, Lever, Burgess, & O'Keefe, 2014), initial fear studies examined its function in static fear conditioning contexts (Kim & Fanselow, 1992; Phillips & LeDoux, 1992). Subsequent research has shown that, after fear conditioning, hippocampal place cells exhibit conditioned responses to the CS interval (Moita,

Rosis, Zhou, LeDoux, & Blair, 2003) and altered firing locations in fear-conditioned, but not control, chambers (Moita, Rosis, Zhou, LeDoux, & Blair, 2004). Remapping of place fields has also been observed following electrical stimulation of the amygdala in rats foraging for food on an open-field platform (Kim, Kim, Park, Cho, & Kim, 2012).

The precise behavioral functions of such changes are harder to understand, and will likely benefit from ethological approaches. For example, in rats placed in an 'approach food-avoid predator' situation, hippocampal place cells that had exhibited place fields located farthest from the 'safe' area before being exposed to the predatory robot remapped after encountering the robot (Kim et al., 2015). In contrast, place fields inside and near the nest regions were unaffected by the predatory robot. These data suggest that place cells can delineate the boundaries of threat and safety as a function of distance. Similarly, a recent study (Telensky et al., 2011) utilized an 'enemy avoidance' task in which rats foraged on a circular platform for food while attempting to avoid a moving robotic threat; if the rats were within 25 cm of the robot, they received a footshock. Inactivation of the dorsal hippocampus with tetrodotoxin significantly impaired avoidance of the moving robot. These studies support the notion that fear guides and shapes foraging behaviors in fluid risky foraging situations.

Ethobehavioral approaches are also successfully implemented to understand how humans perceive and process fear across spatial gradients. Mobbs and colleagues (Mobbs et al., 2007) utilized functional magnetic resonance imaging (fMRI) and an "Active Escape Paradigm" (AEP) wherein a virtual predator chases a virtual representation of the participant, and upon being 'caught', the participants receive an electric shock. These studies showed that as the threat moves from a distal location toward imminent contact with the participant, neural activity shifts from the ventromedial PFC and amygdala toward the PAG. In another study (Mobbs et al., 2010), a live

tarantula was moved closer to participant's feet while they were restrained in an fMRI machine, and this produced similar patterns of neural activity to the AEP experiments and corresponds to similar findings of experiments in rodents (Mobbs & Kim, 2015). Research on "defensive peripersonal space", an area in which defensive behaviors scale with distance from the body (Graziano & Cooke, 2006), in human and non-human primates (Ferri, Tajadura-Jiménez, Väljamäe, Vastano, & Costantini, 2015; Sambo & Iannetti, 2013) suggests that parieto-frontal interactions also play a role in enhancing defensive reflexes as threats come closer to the body. Incorporating ethological stimuli and situations into human studies will provide a functional understanding of fear across spatial dimensions.

Predation risk and threat imminence also vary as a function of time, and thus fear is likely to influence decisions about the timing and duration of foraging activity. For example, Fenn and MacDonald (1995) discovered a population of wild Norway rats (*Rattus norvegicus*), which are typically nocturnal, that exhibited diurnal rhythms of activity, presumably as a consequence of the presence of nocturnal red foxes (*Vulpes vulpes*) in the environment. A sample group of the rats was brought into a safe enclosure, and they reverted to being nocturnal, suggesting that the diurnality effect was not due to direct predation but was instead an avoidance response to perceived risk. Laboratory research has shown that fear conditioning can disrupt entrainment of circadian rhythms in rodents (Amir & Stewart, 1998), and clinical research demonstrates that stress-related disorders such as post traumatic stress disorder are associated with disruptions to circadian rhythms in humans (Wulff, Gatti, Wettstein, & Foster, 2010), suggesting emotional states can interact with the circadian system and have important real-word consequences. Pellman et al. (2015), presented in Chapter 2, directly tests whether nocturnal threat can override the light-dark cycle to entrain circadian rhythms and shift the foraging time of rats away from the time of threat.

1.3 What Can Ethobehavioral Studies Tell Us about Sex Differences in Behavior?

"It is time for researchers, editors and funding bodies to consign sex-biased animal studies to medical history." – Irving Zucker and Annaliese K. Beery, 2010

1.3.1 *Biological Basis of Sex*

There is no scientific consensus on the evolutionary origins of sex, although the most prominent theories suggest that sexual reproduction and sex specialization (i.e., distinct "male" and "female" forms) evolved as a consequence of reducing the accumulation of deleterious genetic mutations that tends to occur in asexually-reproducing populations as well as speeding up the rate of evolution via increasing the heritability of adaptive alleles (Kodric-Brown & Brown, 1987; McDonald, Rice, & Desai, 2016). The asymmetry in the gamete characteristics between males (small, numerous, and containing sparse resources) and females (large, few, and resource rich) is thought to be fundamental to the asymmetry in the differential allocation of resources between mating effort (to which males tend to allocate more resources) and parenting effort (to which females allocate more resources) (Kodric-Brown & Brown, 1987). Such asymmetries demonstrate that males and females have fundamentally different evolutionary interests, and are likely to drive sexual dimorphism in biological and behavioral phenotypes (Gray & Buffery, 1970; Kodric-Brown & Brown, 1987; Shine, 1989). For example, males are thought to be more tolerant of risk than females, particularly when the potential gains or rewards are high (Jolles, Boogert, & Bos, 2015).

Mammals (with the exception of monotremes, such as the platypus) have an XY sex-determination system, where sex is largely determined by the presence of the Y chromosome, or

more specifically the sex-determining region Y (SRY) gene. Thus, sex is determined by the father's sperm - whether it contains an X or a Y chromosome – as the ovum will always contain an X chromosome. The SRY gene then activates (and/or deactivates) a cascade of signals that leads to the differentiation of male versus female gonads (testes and ovaries, respectively), which will produce different ratios of steroid hormones: androgens (including testosterone) are produced in much greater concentrations from the testes while estrogens are produced in much greater concentrations from the ovaries. These hormones will have organizational effects on the morphology of diverse biological systems throughout development, and will also continue to have activational effects into adulthood (Beatty, 1979; Bell & Zucker, 1971).

While the reproductive strategies may differ between the sexes, both males and females have the shared ultimate goal of reproducing. Rodent females, spontaneously ovulate on a regular cycle, called the estrous cycle (Fig. 1.5), which is typically divided into four primary "stages" (although it is a continuous process): proestrus, estrus, metestrus, and diestrus. During proestrus, estradiol levels reach their peak as a consequence of the development of ovarian follicles, which leads to a surge in progesterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) just before the estrus phase (Spornitz, Socin, & Dravid, 1999). The hormones lead to ovulation during estrus, during which time female rats become receptive to copulation. Following estrus, the corpora lutea grow during the metestrus stage, and in the absence of pregnancy, the rat enters the diestrus stage, progesterone peaks again, and the corpora lutea retract as the cycle starts anew (Marcondes, Bianchi, & Tanno, 2002; Spornitz et al., 1999).

The fact that females are fertile during a short period of time, whereas males may reproduce at any time, may underlie differences in propensity to take risks. For example, males are thought to be more tolerant of risk than females in general (Jolles et al., 2015). However, it has also been

shown that females exhibit a decrease in fear and anxiety just before the estrus phase, which may be related to the need to find potential mates and copulate despite the potential violence commonly accompanying mating behavior in many animals (Eberhard, 2009; West-Eberhard, 2014). Indeed, the presence of males, or just their odors, has been shown to elicit a stress response in rats (Shors, Tobón, DiFeo, Durham, & Chang, 2016; Sorge et al., 2014). The role of sex and gonadal hormones in resolving approach-avoidance conflict is discussed further in Chapter 3.

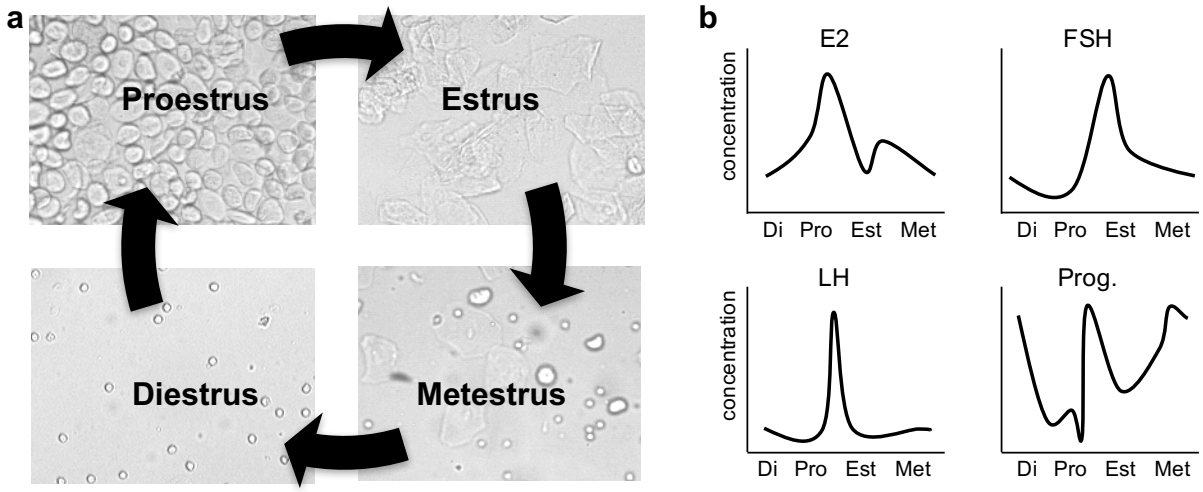

Figure 1.5. Estrous cycle and related hormone fluctuations. (*a*) Diagram of the estrous cycle and photographs of phase-typical cytological composition. During proestrus, epithelial (larger round cells) predominate. During estrus, cornified epithelial cells (large, irregular cells) predominate. During metestrus, there is roughly equal proportions of epithelial, cornified epithelial, and leukocytes (smaller round cells). During diestrus, leukocytes predominate. (*b*) Example plots of ovarian hormone concentration fluctuations throughout the estrous cycle (adapted from Spornitz, Socin, & Dravid, 1999). Abbreviations: E2 = Estradiol; FSH = Follicle stimulating hormone; LH = Luteinizing hormone; Prog. = Progesterone.

1.3.2 *The Need for Ethological Studies of Sex Differences*

There has been, and continues to be, a bias against the use of female animals in preclinical and biomedical sciences (Prendergast, Onishi, & Zucker, 2014; Zucker & Beery, 2010). In an effort to address this bias, the National Institutes of Health (NIH), has recently mandated that as of January 25, 2016, all NIH-funded research must include balanced samples of male and female animals, but the initiative does little to address the *reasons* why researchers have avoided or ignored including female animals in their studies.

While some reasons may be more valid than others, common rationales include sampling and testing issues related to greater variability among female animals and the difficulty or high-cost of controlling for periodic fluctuations in gonadal hormones and their effects on behavior. On the one hand, some researchers believe that a group of females will exhibit greater variability in a single test due to different females being in different stages of their estrous cycle (inter-individual variability), while also displaying greater intra-individual variability across tests. Thus, in order to control for effects of estrous phase, or fluctuations in gonadal hormones, one needs to measure the phase of estrous a female animal is in and ensure testing occurs in the same phase for all female animals. Estrous phase determination requires either direct sampling of gonadal hormone concentrations via immunological assay of blood samples taken from female animals, or by vaginal lavage or swab and histological characterization of cell concentrations. Both of these procedures introduce a potential confound by adding a stressor to the study, so such procedures need to be performed, or simulated adequately, in male counterparts. This stress is more readily balanced when taking blood samples from each sex, but it is less clear that handling and swabbing male genitalia is an adequate homologue to inserting a swab into the vagina. Repeated vaginal lavage or swabbing has been shown to interfere with normal estrous cycle-related modulation of

motor activity, reduce cocaine-stimulated activity of females in an open field test, and also condition a place *preference* if performed immediately before conditioning (Walker, Nelson, Smith, & Kuhn, 2002). It has also been reported that vaginal swabs occasionally induce pseudo-pregnancy, wherein female rats stop their normal estrous cycle (Goldman, Murr, & Cooper, 2007). Thus, there is an apparent need to develop methods for examining sex differences and the potential influence of gonadal hormones on naturalistic behavior that do not involve invasive procedures and potentially uncontrollable effects.

Some studies have met these challenges and studied how learning and memory differ between the sexes, however. In classical eyeblink conditioning, females acquire the conditioned response faster than males, and exhibit better timed eyeblink responses to the CS, and while removal of ovarian hormones eliminates the sex differences in these studies, specific phases of the estrous cycle do not appear to modulate eyeblink conditioning (Dalla & Shors, 2009). In operant tasks, males rats acquire lever pressing for food more efficiently and respond at higher rates than females, although they perform worse than females when response timing is important, which appears to be primarily related to levels of testosterone (van Haaren, van Hest, & Heinsbroek, 1990). In rodents, males have been shown to have an advantage in learning spatial tasks such as the Morris water maze and the radial arm maze (Jonasson, 2005). Spatial memory, as well as declarative memory, is largely dependent on the hippocampus, which contains receptors for estrogens and androgens, and plasticity with the hippocampus has been shown to be modulated by such hormones (McEwen, 2010). For example, ovariectomy impairs hippocampal-dependent memory in female rats and women, and ovarian hormone fluctuations influence the excitability and rate of synaptic turnover of neurons in the hippocampus (McEwen, 2010). However, few studies, if any, have examined sex differences in tasks where multiple behavioral responses are

possible, instead focusing on tests that restrict the repertoires of behavior so as to measure one particular response, and thus important processes involved in selecting appropriate behaviors in more naturalistic conditions may be obscured. Chapter 3 discusses in detail the functional roles of fear, anxiety, and foraging behaviors, and sex differences therein, and presents a series of experiments to address such issues utilizing an environment in which approach and avoidance motivations are pitted against each other and animals must decide how to resolve such conflict.

Chapter 2. TIME-SPECIFIC FEAR ACTS AS A NON-PHOTIC ENTRAINING STIMULUS OF CIRCADIAN RHYTHMS IN RATS

2.1 INTRODUCTION

Most animals exhibit rhythmic patterns of activity that are restricted to specific periods of the daily cycle, such as the daytime (diurnal), nighttime (nocturnal), or dawn-and-dusk times (crepuscular) (Florez & Takahashi, 1995; Stephan & Zucker, 1972; Takahashi & Zatz, 1982). Such circadian rhythms are generated by endogenous molecular clocks that oscillate with approximately 24-h periods, but because these periods are usually slightly shorter or longer within individuals, these clocks must be "entrained" by external cues (*zeitgebers* or "time-givers") to remain environmentally relevant (Florez & Takahashi, 1995; Reppert & Weaver, 2002; Stephan & Zucker, 1972; Takahashi, Hong, Ko, & McDearmon, 2008; Takahashi & Zatz, 1982). These functions are thought to be critical in crafting an ecological niche by coordinating psychophysiological functions to balance optimal times for exploring resources (e.g., food, water, and mates) and avoiding predatory threats in the environment (Castillo-Ruiz, Paul, & Schwartz, 2012; Fenn & MacDonald, 1995; Hut, Kronfeld-Schor, van der Vinne, & De la Iglesia, 2012; Lima & Bednekoff, 1999; Mobbs et al., 2007; Stephens & Krebs, 1986).

The principal zeitgeber is an organism's local light-dark (LD) cycle (Florez & Takahashi, 1995; Reppert & Weaver, 2002; Takahashi et al., 2008; Takahashi & Zatz, 1982). Via projections from light-sensitive retinal ganglion cells, the LD cycle entrains the "master" circadian clock located within the suprachiasmatic nucleus (SCN) in mammals (Buhr & Takahashi, 2013;

Golombek & Rosenstein, 2010; Stephan & Zucker, 1972; Takahashi & Zatz, 1982; Zucker, Rusak, & King, 1976). This master clock then drives secondary "slave" clocks in other brain regions or peripheral organs to coordinate daily physiological and behavioral rhythms (Buhr & Takahashi, 2013; Mohawk, Green, & Takahashi, 2012; Moore, 2013). Cyclic stimuli other than light (non-photic cues), such as ambient temperature and time-restricted feeding schedules, have also been found to entrain circadian rhythms (Buhr, Yoo, & Takahashi, 2010; Gritton, Stasiak, Sarter, & Lee, 2013; Mistlberger, 1992, 2011).

In humans, circadian rhythms are vital to mental health as they are often disturbed in psychopathologies (Germain & Kupfer, 2008; von Zerssen et al., 1985; Wulff et al., 2010). While clinical and experimental studies have shown that emotional states, such as fear, anxiety, and depression, can disrupt circadian rhythms (Amir & Stewart, 1998; Germain & Kupfer, 2008; Gorka, Moryl, & Papp, 1996; Meerlo, Hoofdakker, Koolhaas, & Daan, 1997; von Zerssen et al., 1985; Wulff et al., 2010), it remains unknown whether they can serve entraining functions. Fear is a crucial, highly-conserved mechanism of survival that guides behaviors that help organisms minimize exposure to threats in their habitat (Bolles & Fanselow, 1980; Choi & Kim, 2010; M. Fanselow, 1994; LeDoux, 2012; Lima & Bednekoff, 1999; Lima & Dill, 1990), and it is conceivable that cyclic daily threats may act as entraining environmental stimuli to the circadian system.

The present study investigated the significance of emotions, specifically fear, on circadian rhythms in rats under naturalistic conditions where defensive and appetitive behaviors were all a meaningful, integrated part of the animals' lives. Rats lived for extended periods in "closed economy" chambers (Fanselow, Lester, & Helmstetter, 1988; Helmstetter & Fanselow, 1993; Hursh, 1980; Kim et al., 2014), comprised of a safe, bedded nest and a risky foraging area that had

to be entered to obtain food and water (Fig. 2.1a). The foraging zone was rendered dangerous by administering either signaled or unsignaled footshocks only during the dark phase of the LD cycle (Fig. 2.1b), which is typically the natural active phase for rats. A closed economy paradigm was chosen to allow experimental animals to have control over their own appetitive and defensive behaviors and reflect a more natural foraging situation. In response to unsignaled nocturnal shock, animals switched their natural feeding and activity from the dark phase to the light phase. This fear-induced diurnal behavior persisted (free-ran) when the light cues and footshocks were removed (Fig. 2.1c), and the phase of the free-running rhythm approximated the phase when the recurring threat was present, confirming that daily cyclic fear can act as a zeitgeber. Additionally, the expression of this fear-entrained circadian rhythm was dependent on an intact amygdala and SCN. The finding that amygala-coded fear can reprogram SCN-directed circadian behavior suggests that the amygdala is a part of the circadian oscillator network that temporally organizes behavior.

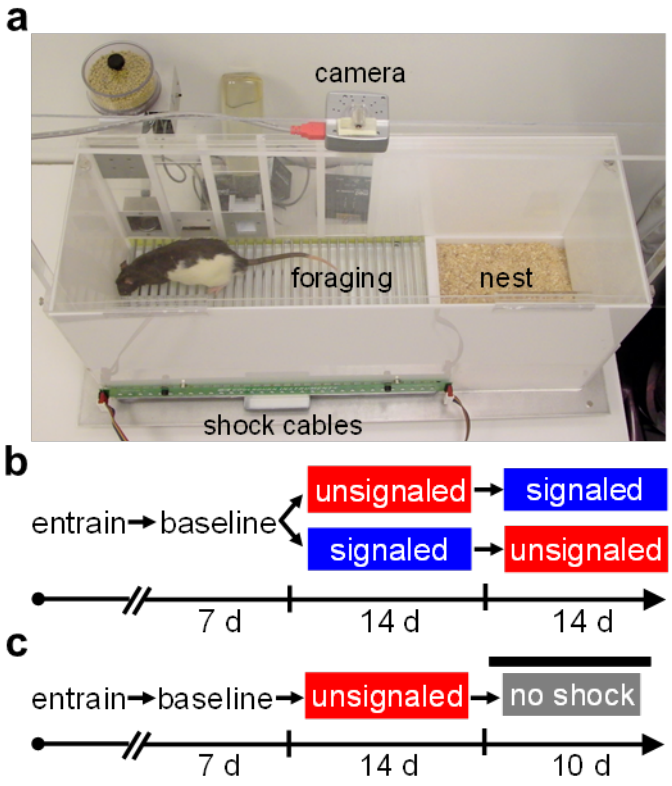

Figure 2.1. Experimental apparatus and design. (*a*) Photograph of the closed economy apparatus. Diagram of the experimental designs for (*b*) the primary experiment and (*c*) the free-running experiment.

2.2 METHOD

2.2.1 *Animals and Apparatus*

Naïve, male Charles River Long-Evans rats initially weighing 275-300 g were individually housed in eight closed economy chambers (Fig. 2.1a) on a 12 h/12 h LD cycle (Kim et al., 2014). The closed economy dimensions were 74.3 cm x 25.4 cm x 33 cm (length x width x height) and consisted of a 'nest' (20.3 x 25.4 cm) and a 'foraging' arena (54 x 25.4 cm). The nest floor was covered with sawdust, while the floor of the foraging arena was composed of 32 stainless steel rods (4.5 mm diameter) wired to a precision animal shocker (Coulbourn Instruments, Allentown, PA) for delivery of footshocks. A camera (Fire-I B/W Board camera; Unibrain Inc., San Ramon, CA) was mounted above each closed economy chamber and connected to a computer for tracking animal activity via ANY-maze software (Stoelting, Wood Dale, IL), which also measured activation of the food levers and dispensers (Med Associates, Inc., Georgia, VT) and the shock generator connected to an ANY-maze Interface (AMi; Stoelting, Wood Dale, IL). Forty-five-mg grain-based pellets for rodents were used in all experiments (#F0165, Bio-Serv, Flemington, NJ). The total number of pellets dispensed was chosen as the primary *foraging* variable due to the necessity of the animal to be in the foraging area and actively pressing the lever in order to obtain and consume the food pellets. Importantly, all pellets obtained by each animal were consumed. The total distanced traveled (m) among both the foraging and nest areas, rather than within one specific area, was used as the primary *activity* variable for analyzing circadian rhythms as this would include but not be confounded by avoidance behavior. White noise (70 dB) generated by the ANY-maze software was continuously played through computer speakers throughout the experiment to obscure external noises. The actual times corresponding to the onset of the dark and

shock phases (ZT12) occurred between 10 a.m. and 3 p.m. (varied by cohort) so any human work-related vibrations would be poorly associated with the experimental cycles.

All animal experiments were conducted in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were reviewed and approved by the University of Washington Institutional Animal Care and Use Committee.

2.2.2 *Procedures*

After arrival, animals were given 10-14 d to acclimatize to the chamber (e.g., lever press for food pellet on a continuous reinforcement schedule) and entrain to the LD cycle (as confirmed by an actogram). After 7 baseline days, half of the animals were exposed to 14 d of unsignaled, pseudo-random footshocks (0.8 mA; ~2 shocks/h in the dark phase only) followed by 14 d of signaled, pseudo-random footshocks (9-s light cue preceding the shock in the dark phase only). This was counterbalanced by the other half of the animals (assignment randomized; Fig. 2.1b). If the animal was in the foraging area when ANY-maze triggered the footshock, the shock continued for up to 10 s or until the animal escaped to the nest. In the signaled footshock condition, if the animal moved to the nest within 9 s of a light cue (i.e., avoidance response), then the light (Med Associates, Inc., Georgia, VT) promptly terminated and no shock occurred. If the animal failed to enter the nest within 9 s of the light, the footshock ensued and both stayed on for up to 10 s or until the animal escaped to the nest. If the animal was in the nest at the time the program generated the light or the footshock, they both terminated instantaneously. Since only the unsignaled nocturnal footshocks caused animals to switch the majority of their feeding behavior to the light phase, a signaled footshock condition was not included in the free-running or lesion experiments.

Animals were removed from the closed economy chambers during the last hour of the light phase (ZT11-ZT12) every 2-3 days so the chambers could be cleaned, the food and water

replenished, and animals weighed. Animals were returned to the experimental chambers at the beginning of the dark phase (ZT12). This was the extent of experimenter-animal interaction. In the free-running experiment (Fig. 2.1c), animals were undisturbed for 3 d to assess free-running under constant dark conditions. In the lesion experiment, animals were only exposed to 14 d of unsignaled nocturnal shock, except for the SCN lesion and sham animals, which experienced an additional 14 d of diurnal (light phase only) shock to further examine the SCN lesion effect on arrhythmia.

2.2.3 *Surgery*

Under anesthesia (30 mg/kg ketamine and 2.5 mg/kg xylazine, i.p.), rats were randomly assigned to receive either bilateral electrolytic lesions to their amygdalae (AMYX group, n = 7; from bregma: AP -2.5; ML +4.2/5.0; DV -8.4/8.6 mm) (Paxinos & Watson, 1997), the SCN (SCNX group, n = 8; from bregma: AP –1.3; ML + 0.3; DV –9.1) (Paxinos & Watson, 1997) or had lesion electrodes inserted 1 mm above the amygdala or the SCN, except current was not delivered (CON, n = 7; sham groups combined because there were no statistical differences). For the AMYX group, lesions were made by passing constant current at 1.0 mA for 10 s (Grass Medical Instrument, Quincy, MA) through epoxy-coated insect pins (#00, ~0.75 mm tip exposed) (Kim et al., 2014). For the SCNX group, lesions were made by passing constant current at 1.75 mA for 17.5 s (Grass Medical Instrument, Quincy, MA) through epoxy-coated insect pins (#00, ~.25 mm tip exposed).

2.2.4 *Histology*

At the completion of the experiment, all rats were overdosed with Beuthanasia and perfused intracardially with 0.9% saline followed by 10% buffered formalin. The brains were

removed and stored in 10% formalin overnight and then kept in 30% sucrose solution until they sank. Transverse sections (50 μm) through amygdalar and SCN lesions were taken, mounted on gelatin-coated slides, and stained with cresyl violet and Prussian blue dyes for confirmation of electrode placement and lesion accuracy.

2.2.5 *Statistical Analysis*

All data are presented as mean ± SEM. Group sizes were selected based on power analyses performed using G*Power 3.1 software with estimates of effect sizes obtained from previously-published findings in the lab (Kim et al., 2014). The behavioral data (with the exception of the free-running experiment, see below) was analyzed using mixed factorial ANOVAs on the daily total number of pellets dispensed and total distance traveled (in m) with the within-subject factors of time (day or 10-min time-bin), light phase, and experimental condition (baseline, signaled, or unsignaled shock). In cases where the assumption of sphericity was violated (Mauchly's test), Greenhouse-Geisser corrected degrees of freedom were used. In cases where Levene's Test for Equality of Variance was significant, the degrees of freedom were corrected using the Welch-Satterthwaite method. Bonferonni-adjusted, two-tailed, paired-samples t-tests or independent t-tests were used for post hoc tests where appropriate. Free running rhythms were analyzed first by visual inspection of actograms, then a Sokolov-Bushel periodogram on the days of free-running was used to determine the circadian period of each animal. The period for each animal was then used to construct waveforms of the successive days of feeding and activity. Each of these two waveforms was analyzed by a one-way ANOVA to determine a significant effect of time (Acosta-Galvan et al., 2011). Two animals did not show significant periods for feeding, and were thus not included in the mean feeding free-running analysis. All animals showed significant periods for the measure of activity. Time-series analysis was done with ElTemps software (A. Díez Noguera,

University of Barcelona, Spain). Statistics were performed using SPSS (version 18.0). Five animals were excluded from the analysis due to incomplete, misplaced, or unilateral lesions to the amygdala (four) or SCN (one), and one CON animal was excluded due to health complications following surgery. Three animals were excluded for failing to entrain to the LD cycle during the initial acclimation period.

2.3 RESULTS

Animals living in the closed economy chamber and maintained on a 12-h/12-h LD cycle quickly learned to press a lever to procure food pellets (a continuous reinforcement schedule) in the foraging area. As expected, rats preferred to forage during the dark phase during baseline (7 d), as measured by feeding and total locomotion [mixed-model ANOVA, see Methods; pellets: $F_{1, 14} = 112.69$, $p < 0.001$; activity: $F_{1, 14} = 81.51$, $p < 0.001$] (Fig. 2.2). When exposed to the risk of unsignaled nocturnal shocks, rats increased their locomotor and feeding behavior during the light phase and decreased locomotor and feeding behavior during the dark phase [phase × day: pellets, $F_{13, 182} = 9.03$, $p < 0.001$; activity, $F_{13, 182} = 11.87$, $p < 0.001$] (Fig. 2.2). Although rats remained more active during the dark phase than the light phase overall [$F_{1, 14} = 45.68$, $p < 0.001$], they preferred to obtain food during the light phase by the end of the unsignaled shock period [last 2 d, L vs. D: dark: 203.44 ± 24.58 pellets; light: 335.41 ± 34.15 pellets; $t_{15} = 2.60$, $p = 0.02$]. The effects of signaled nocturnal shock varied depending on the order in which it was experienced. For the rats that experienced unsignaled shock first (Fig. 2.2a-d), diurnal behavior persisted into the signaled footshock period because the avoidance of footshocks (i.e., not foraging during the dark phase) served as negative reinforcement (Mowrer, 1939) [phase × day × order: feeding, $F_{4.055, 56.776} = 3.05$, $p = 0.023$; activity, $F_{4.865, 68.108} = 2.50$, $p = 0.040$; *d.f.* adjusted].

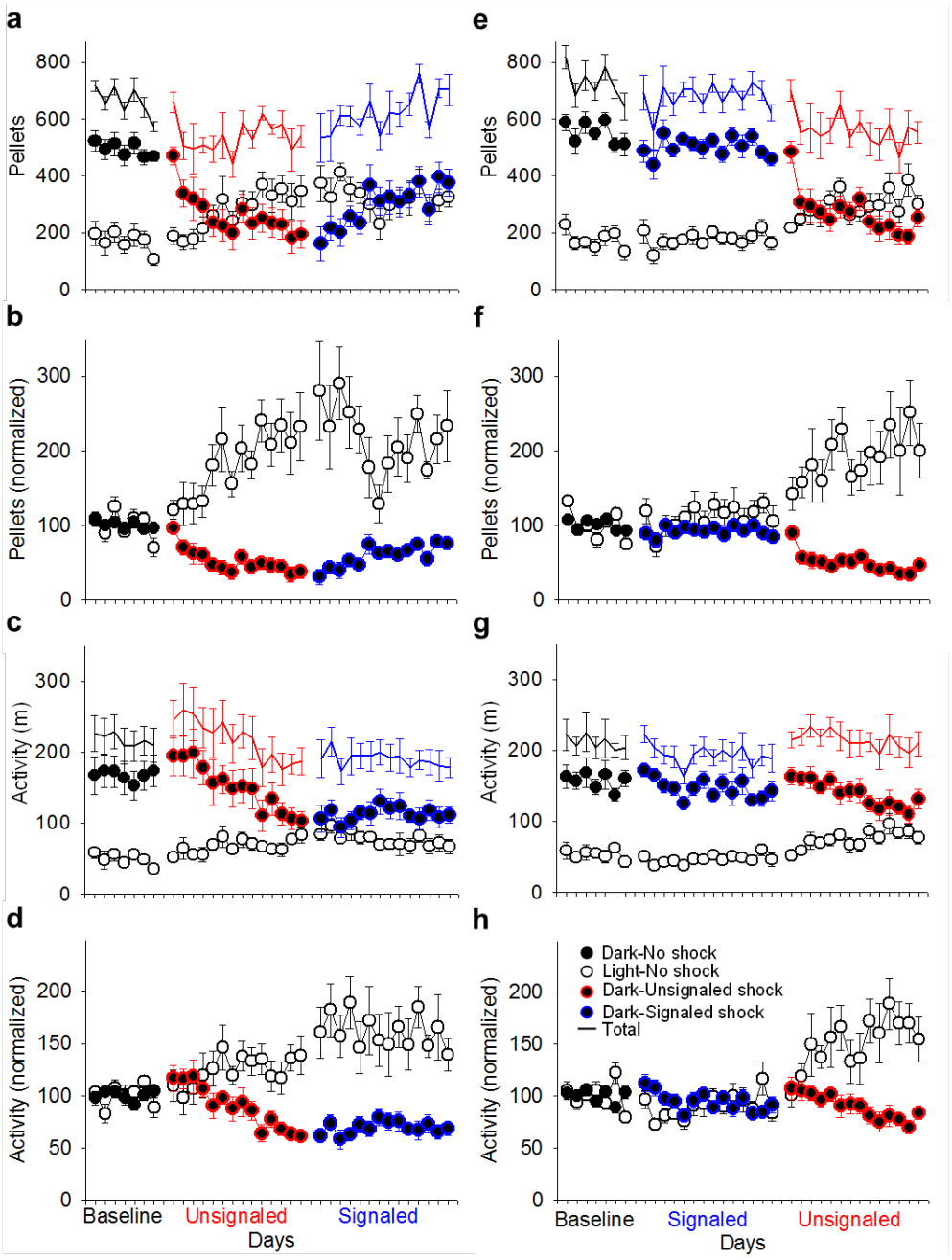

Figure 2.2. Effects of nocturnal shock on foraging and activity patterns. (*a*) Raw number of pellets obtained, (*b*) number of pellets obtained normalized to baseline (black) average, (*c*) raw activity (distance traveled in m), and (*d*) activity normalized to baseline average of rats that experienced unsignaled (red) nocturnal footshocks before signaled (blue) nocturnal footshocks (n = 8). (*e*) Raw number of pellets obtained, (*f*) number of pellets obtained normalized to baseline (black) average, (*g*) raw activity (distance traveled in m), and (*h*) activity normalized to baseline average of rats that experienced signaled (blue) nocturnal footshocks before unsignaled (red) nocturnal footshocks (n = 8). When exposed to unsignaled shock, rats shift from natural nocturnal behaviors to diurnal behaviors. During signaled shock, behavior depended on whether unsignaled shock was already experienced or not. All error bars represent SEM.

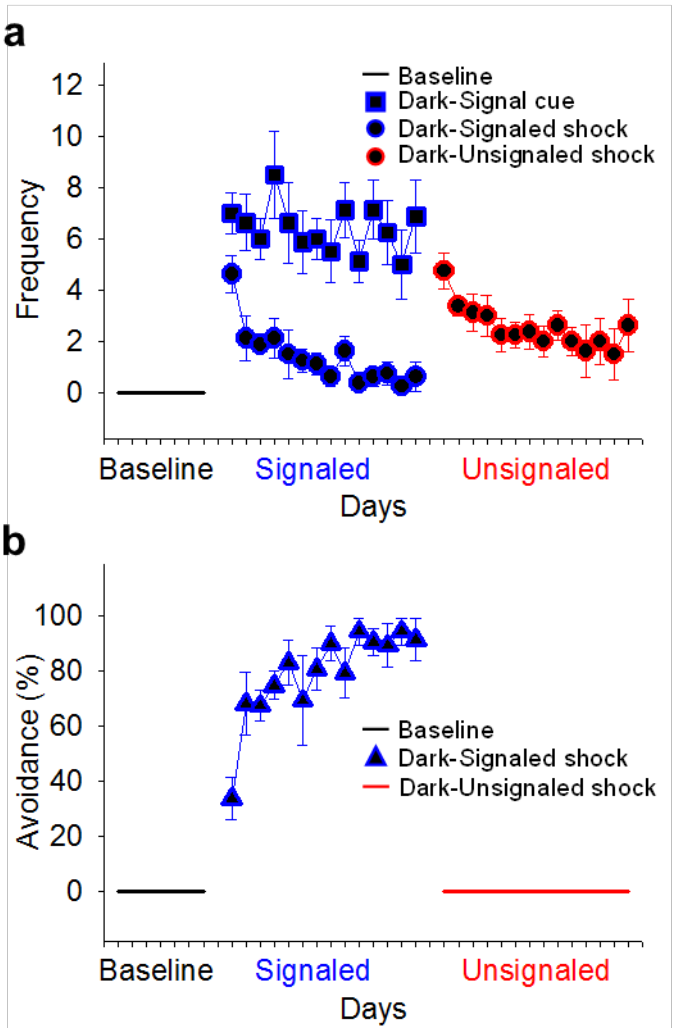

Figure 2.3. Light cue, footshock, and avoidance during nocturnal shock. (*a*) Mean number of light cue presentations (squares) and mean number of shocks (circles) animals (n = 8) received during signaled shock and unsignaled shock conditions. (*b*) Mean percentage of avoidance responses made (i.e., moving from foraging area to the nest) during the signaled shock (< 9 s of 10 s light cue). All error bars indicate SEM.

In contrast, the rats that were exposed to the risk of signaled nocturnal footshocks at the outset (Fig. 2.2e-h) quickly learned to avoid the footshock and thereby maintained their baseline circadian behavior, only to become diurnal when subsequently switched to unsignaled nocturnal footshock condition (Fig. 2.3). Thus, the rats that had been exposed to unsignaled shock first were significantly less active [114.26 ± 11.65 m; $t_{14} = 2.68$, $p = 0.018$] and ate fewer pellets [295.80 ± 35.67; $t_{14} = 5.10$, $p < 0.001$] in the dark phase and were significantly more active [77.01 ± 5.37 m; $t_{14} = 4.29$, $p < 0.001$] and ate more pellets [325.74 ± 34.41; $t_{10.44} = 3.86$, $p = 0.003$; *d.f.* adjusted] in the light phase compared to the rats that experienced signaled shock first (dark: 150.69 ± 7.04 m, 502.29 ± 19.12 pellets; light: 46.91 ± 4.53 m, 176.65 ± 17.64 pellets).

It is interesting to note that during the unsignaled nocturnal shock condition, animals began to increase their activity and feeding before the switch from the dark to the light phase (Fig. 2.4; red lines). To analyze this behavior, we compared the mean activity and feeding behavior of the rats across the last 5 d of baseline and the unsignaled shock condition in 10-min time bins during the last 4 h of the dark phase (Zeitgeber Time (ZT) 20-24/0, with ZT0/24 being the time of lights on and ZT12 being the time of lights off; see Methods). Feeding behavior (Fig. 2.4a; left side) and activity (Fig. 4a; right side) significantly increased before the onset of the light phase (ZT0) in the unsignaled nocturnal shock condition relative to baseline [condition × time: activity, $F_{23, 92} = 2.85$, $p < 0.001$; feeding, $F_{23, 92} = 3.35$, $p < 0.001$]. This *anticipatory* behavior is incompatible with the possibility that the light simply served as a safety cue for the animals to start foraging (i.e., not a Pavlovian response). In contrast, animals experiencing signaled nocturnal shock after baseline did not show a significant increase in feeding or activity during this time [condition × time: activity, $F_{23, 92} = 0.95$, $p = 0.543$; feeding, $F_{23, 92} = 1.48$, $p = 0.099$] (Fig. 2.4b) relative to baseline.

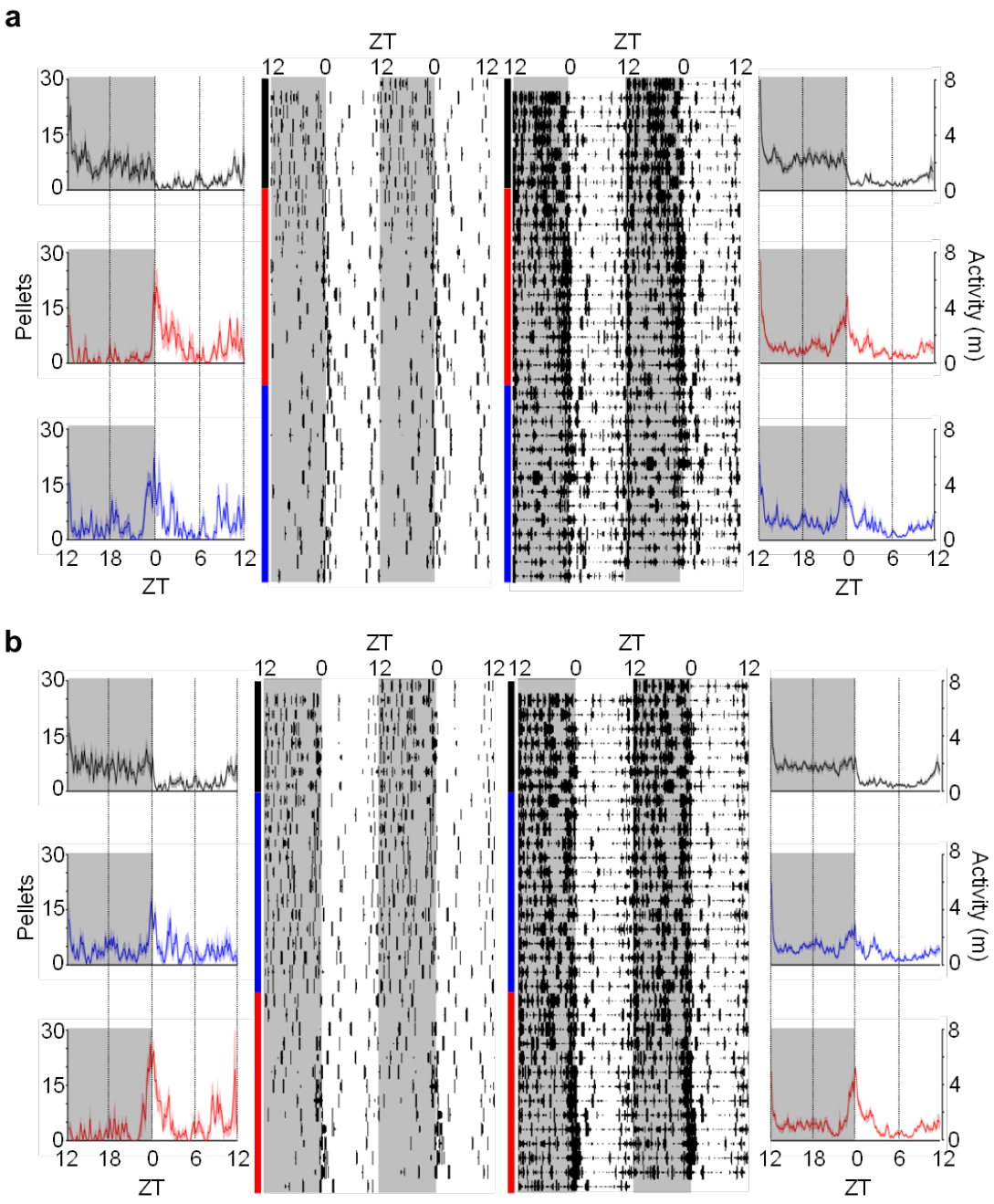

Figure 2.4. Effects of nocturnal shock on circadian rhythms. 24-h waveforms (outside) and raster plots (inside) of feeding (number of pellets obtained; left) and activity (distanced traveled in m; right) through baseline (black), unsignaled (red) and signaled shock (blue) conditions in rats that (*a*) experienced unsignaled shock first (n = 8) and (*b*) experienced signaled shock first (n = 8). Waveforms show mean feeding/activity over 24 h (bold lines), in 10-min time-bins, averaged over the last 5 d of each condition. Raster plots are from a representative animal from each group. Gray shaded areas (ZT12-24/0) indicate dark/shock phase. Behavior during nocturnal shock conditions increases 4 h leading up to the dark-to-light transition (ZT0). SEM is represented by shaded areas above and below the bold lines.

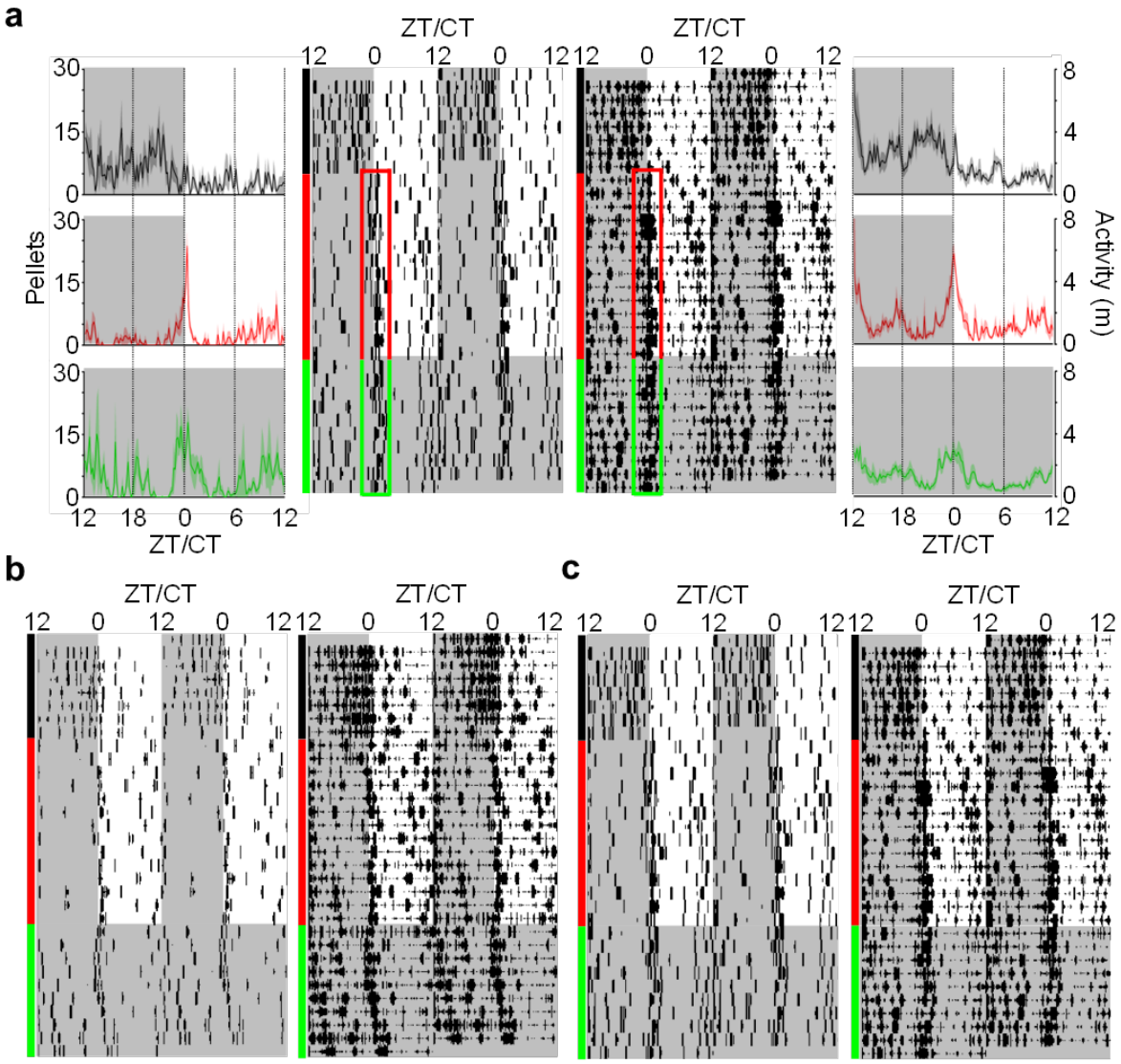

Figure 2.5. Fear-entrained anticipatory circadian feeding and activity. (*a*) 24-h waveforms and raster plots of feeding (number of pellets obtained; left) and activity (distanced traveled in m; right) through baseline (black), unsignaled shock (red), and constant dark conditions (green) demonstrate fear-induced, anticipatory circadian rhythms. Waveforms show mean (n = 5) feeding/activity over 24 h (bold lines) averaged over the last 5 d of each condition. Mean waveforms of feeding (bottom left; n = 3) and activity (bottom right; n = 5) under constant dark conditions demonstrate free-running rhythms. Phases are aligned according to free-running onset of feeding or activity respectively. SEM is shown as the shaded areas above and below the bold lines. The raster plots are from a representative animal. The red outline highlights the anticipatory circadian rhythm generated under unsignaled nocturnal shock conditions, which continues as a free-running rhythm (green outline) under constant dark conditions. Raster plots of feeding (left) and activity (right) from representative animals with free-running periods that were (*b*) greater than 24 h and (*c*) less than 24 h. Gray shaded areas represent the dark phase. Circadian times (CT) are extrapolated from the LD cycle, with CT12 being the extrapolated time of lights off (ZT12).

To test the hypothesis that fear produced by the unsignaled nocturnal shock can reprogram circadian rhythms, a group of rats was entrained and exposed to the same conditions as in the first experiment, except that after the 14 d of unsignaled nocturnal shock they underwent 10 d of constant darkness conditions without shock (Fig. 2.1c; see Methods). If the observed rhythmic anticipatory and diurnal foraging behavior is indeed a function of an endogenous circadian oscillator, then it should persist even when all external cyclic stimuli are removed from the environment (Golombek & Rosenstein, 2010). These rats continued to display the same time-restricted feeding [waveform: $F_{95, 192} = 1.51$, $p = 0.0084$] and activity [waveform: $F_{95, 384} = 4.78$, $p < 0.0001$] throughout the free-running portion of the experiment, with a free-running phase that could be extrapolated from the phase before the release into constant conditions (Fig. 2.5), indicating that changes in the timing of foraging behavior are sustained by an endogenous circadian clock that is entrained by the nocturnal presentation of footshocks.

In order to examine whether these changes in feeding and activity were dependent on known circadian timing- and fear-related brain structures, the effects of unsignaled nocturnal footshocks on circadian feeding and activity were examined in SCN- or amygdala-lesioned rats (Fig. 2.6). During baseline, amygdala-lesioned (AMYX; Fig. 7a-d) rats were significantly biased toward the dark phase, as measured by feeding [dark: 454.69 ± 41.02 pellets, light: 168.27 ± 29.58 pellets] (Fig. 2.7a, b) and activity [dark: 193.81 ± 11.20 m; light: 72.63 ± 4.64 m] (Fig. 2.7c, d). This dark phase preference did not change when unsignaled nocturnal shocks were presented [feeding: condition × phase, $F_{1, 6} = 1.98$, $p = 0.21$; activity: condition × phase, $F_{1, 6} = 0.001$, $p = 0.98$], and feeding even increased overall during the unsignaled shock condition [$F_{1, 6} = 7.38$, $p = 0.035$].

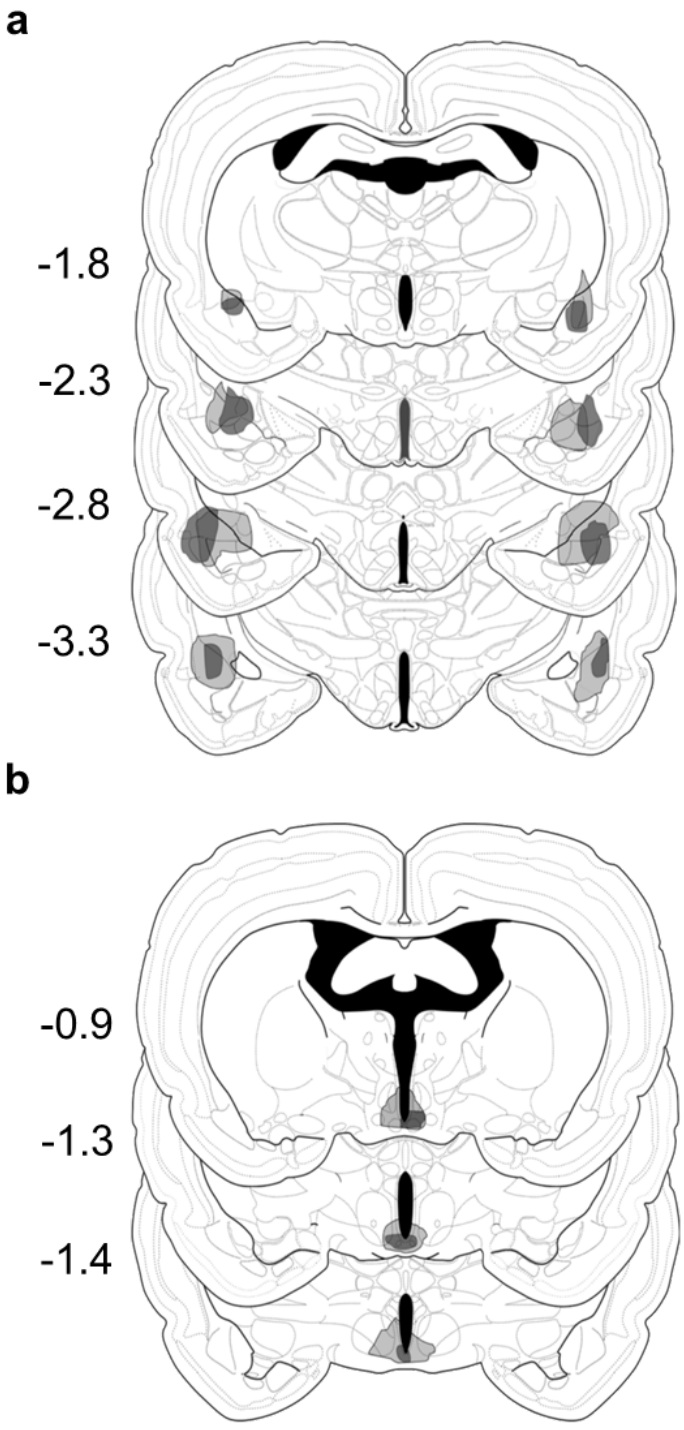

Figure 2.6. Amygdala and SCN lesions. Histological reconstruction of the smallest (dark-shaded) and largest (light-shaded) lesions of the (*a*) amygdala and (*b*) SCN. Numbers indicate mm posterior to bregma. Adapted from (Swanson, 2004), which is licensed under the Creative Commons Attribution-Noncommercial 4.0 International Public License.

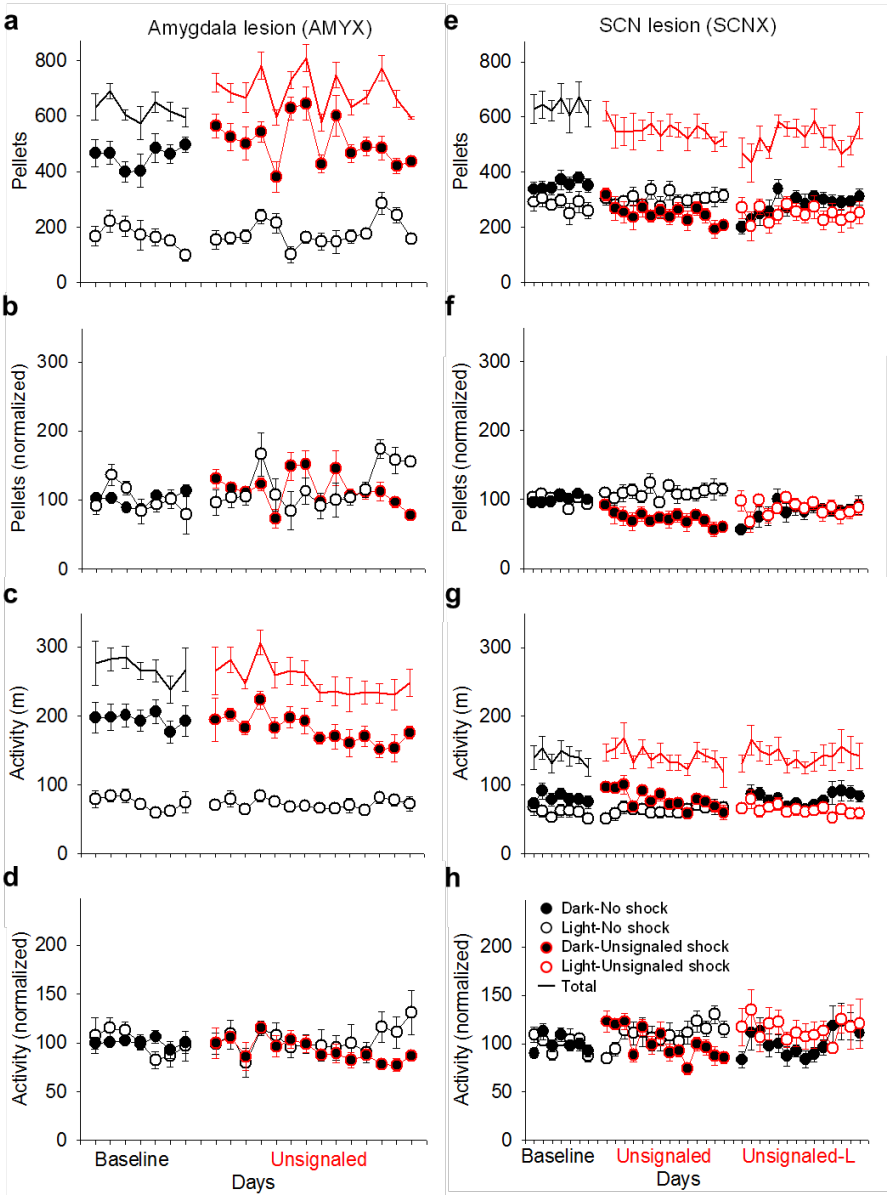

Figure 2.7. Effects of lesions on shock-induced changes to foraging and activity patterns. (*a*) Raw number of pellets obtained, (*b*) number of pellets obtained normalized to baseline average, (*c*) raw activity (distance traveled in m), and (*d*) activity normalized to baseline average of amygdala-lesioned (AMYX; $n = 7$) rats exposed to baseline (black) and unsignaled nocturnal shock (red) conditions. AMYX animals maintained their nocturnal feeding/activity behavior during unsignaled nocturnal shock. (*e*) Raw number of pellets obtained, (*f*) number of pellets obtained normalized to baseline (black) average, (*g*) raw activity (distance traveled in m), and (*h*) activity normalized to baseline average of SCN-lesioned (SCNX; $n = 8$) rats exposed to baseline (black), unsignaled nocturnal shock (black circles with red outline), and unsignaled diurnal shock (open circles with red outline) conditions. SCNX animals slightly preferred feeding in the dark phase during baseline, which abolished during unsignaled nocturnal shock. When exposed to unsignaled diurnal shock, SCNX rats' feeding behavior remained arrhythmic. Error bars indicate SEM.

On the other hand, while arrhythmic SCN-lesioned (SCNX; Fig. 2.7e-h) rats were still slightly biased toward the dark phase in feeding [dark: 353.86 ± 22.14 pellets, light: 281.93 ± 27.05 pellets; $F_{1, 7} = 20.49$, $p = 0.003$] (Fig. 2.7e, f) and activity [dark: 80.51 ± 6.50 m, light: 60.02 ± 6.90 m; $F_{1, 7} = 32.49$, $p = 0.001$] (Fig. 2.7g, h) during baseline the introduction of unsignaled nocturnal shock abolished this dark phase preference for feeding [$F_{1, 7} = 4.26$, $p = 0.078$]. Activity remained slightly biased toward the dark phase [$F_{1, 6} = 12.74$, $p = 0.012$], though dark phase activity decreased over time [phase × day: $F_{13, 78} = 4.26$, $p < 0.001$]. Because SCNX rats still showed, to some extent, greater nocturnal activity and feeding during baseline, unsignaled shock was then switched to occur only during the light phase to examine whether this would augment their dark phase bias. Despite diurnal unsignaled shock, both feeding [phase: $F_{1, 7} = 0.43$, $p = 0.53$] and activity remained arrhythmic [phase: $F_{1, 7} = 2.63$, $p = 0.15$]. The mean feeding and activity behavior for each group are summarized in Figure 2.8a and 2.8b, respectively. Unlike intact animals, neither AMYX nor SCNX rats showed anticipatory feeding [AMYX: condition × time, $F_{23, 92} = 1.339$, $p = 0.165$; SCNX: $F_{23, 92} = 0.940$, $p = 0.548$] or activity [AMYX: condition × time, $F_{23, 92} = 0.746$, $p = 0.786$; SCNX: $F_{23, 92} = 1.131$, $p = 0.329$] before the LD transition (Fig. 2.8c, d). These results indicate that both the SCN and amygdala are necessary to shift circadian foraging rhythms away from the time of threat and generate anticipatory activity organized around safe periods.

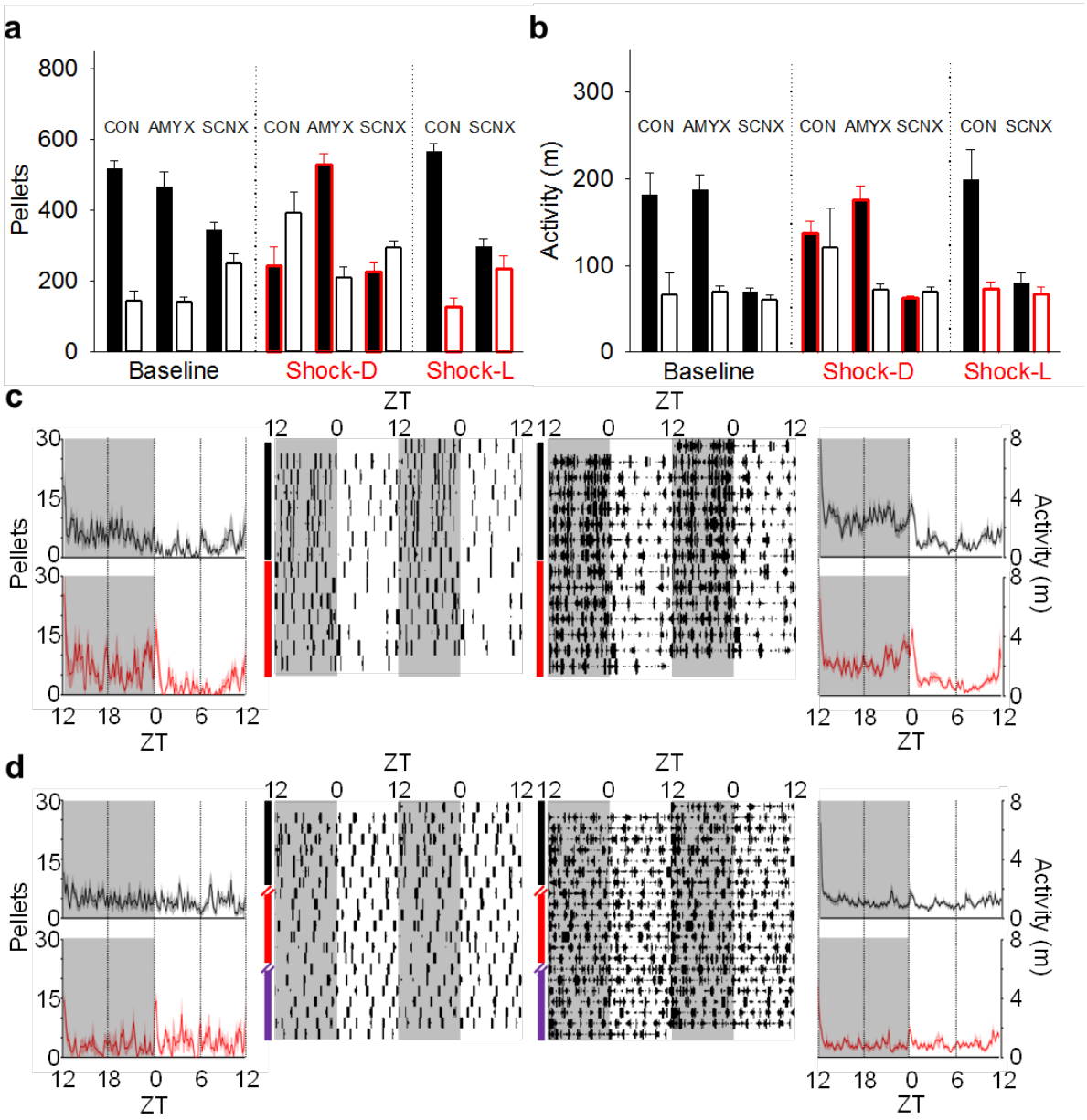

Figure 2.8. Effects of lesions on shock-induced changes to circadian rhythms. (*a*) Mean number of pellets and (*b*) mean activity (distanced travelled in m) in the dark (filled bars) and light (open bars) phases of AMYX (n = 7), SCNX (n = 8), and sham (CON; n = 7) animals during the last 5 d of baseline (left), unsignaled nocturnal (center) and unsignaled diurnal shock (right; SCNX and CON only). Red highlights indicate shock period. Error bars represent SEM. (*c*) 24-h waveforms (outside) and raster plots (inside) of feeding (left) and activity (right) from amygdala-lesioned rats (n = 7; baseline and unsignaled shock conditions represented by black and red, respectively). (d) 24-h waveforms (outside) and raster plots (inside) of feeding (left) and activity (right) from SCN-lesioned animals (n = 8). Waveforms show the mean feeding and activity over 24 h (bold lines), in 10-min time-bins, of all animals in each group averaged over the last 5 d of each experimental condition. SEM is shown as the shaded areas above and below the bold lines.

2.4 DISCUSSION

The significance of environmental threats to daily behavior is apparent from naturalistic studies that have reported that increased predation risk or hunting by humans is associated with profound changes in the activity patterns of mammalian prey (Castillo-Ruiz et al., 2012; Lima, 1998; Lima & Bednekoff, 1999; Lima & Dill, 1990). For instance, one study (Fenn & MacDonald, 1995) observed a population of wild rats (*Rattus norvegicus*) exhibiting diurnal activity that shared a habitat with red foxes, a nocturnal predator. After a subset of the diurnal rats were captured and kept in a safe enclosure, they reverted to being nocturnal, and it was concluded that the rats were diurnal in order to avoid predation (Fenn & MacDonald, 1995). Another study examining the temporal and spatial activity of wild boar (*Sus scrofa* L.) as a function of hunting pressure found that areas with increased diurnal hunting by humans were associated with a reduction in the boars' diurnal activity (Keuling, Stier, & Roth, 2008). However, it has remained unclear whether such shifts in activity represent simple conditioned fear responses to predatory stimuli or reprogramming of circadian rhythms. The present study employed an ethologically-relevant foraging setting that simulates the environment in which circadian rhythms likely evolved and demonstrates that time-specific fear can serve as a non-photic zeitgeber entraining a circadian oscillator that times foraging behavior to a non-threatening time of the day. The discovery that a dark phase-associated unsignaled threat leads to endogenous persistent rhythmic activity just before and during the safe light period provides further evidence against a strictly Pavlovian interpretation in which the LD cycles as a conditioned stimulus.

Previous studies using hamsters and Wistar rats have reported that the strength of conditioned place preference (CPP) and avoidance (CPA) is modulated by the time of day of training (Cain, Chou, & Ralph, 2004; Cain, Ko, Chalmers, & Ralph, 2004). That is, the expression

of the CPP or CPA was strongest when tested 24 and 48 h after training but was not exhibited at 32 or 40 h intervals. These effects were present even with lesions to the SCN, suggesting the mechanism of this "time-stamped" learning was not dependent on circadian oscillators in the SCN (Cain & Ralph, 2009). It is important to note that the effects observed in the CPP experiments may have involved food-entrainable oscillators as the animals were food restricted and also that Long-Evans rats, unlike Wistar rats, did not exhibit time-dependent expression of CPA (Cain, Ko, et al., 2004; Cain & Ralph, 2009). Our experiments used Long-Evans rats that were not food-restricted, and the anticipatory behavior we observed depended on an intact SCN. Thus, the effects were likely due to an SCN-dependent mechanism rather than the time-stamped learning suggested by Ralph and colleagues (Cain & Ralph, 2009). These shifts in circadian feeding and activity may also be context-dependent, as other studies have reported a lack of phase-shifting following exposure to stressors presented in a different context than where circadian behavior was measured (Meerlo et al., 1997; Meerlo, Sgoifo, & Turek, 2002).

The present effect is also distinct from proposed "cognitive oscillators" that shift behavior toward periods requiring heightened attention (Gritton et al., 2013) in that the fear-induced oscillator shifts behavior *away* from the time associated with unpredictable threat, which may represent a period requiring greater attentional demand. Furthermore, the dependence of this fear-induced oscillator on an intact amygdala indicates that the circadian neural network that temporally orchestrates complex behaviors likely includes limbic centers that encode aversive stimuli and coordinate fear and anxiety responses. It is important to note that, while there is some debate regarding the use of the term "fear" with regard to non-human animals (LeDoux, 2014, 2012), we use "fear" here to refer to the central defensive-motivational state activated by aversive stimuli (Bindra, 1969; Bolles & Fanselow, 1980; Fanselow, 1994; Kim & Jung, 2006).

Similarly to the food-entrainable oscillator, which times activity to a restricted feeding schedule, the fear-entrainable oscillator we describe overrides entrainment of rhythmic foraging and activity by the LD cycle (Golombek & Rosenstein, 2010). However, unlike the food-entrainable oscillator, which does not depend on an intact SCN, the fear-entrainable oscillator depends both on an intact SCN and intact amygdala. Thus, the fear-entrainable oscillator could reside within the SCN itself or, alternatively, it could be an extra-SCN oscillator to which the SCN relays information on the phase of the LD cycle. Alternatively, cyclic fear could modulate the phase relationship between the SCN pacemaker and downstream oscillators that time behavior. However, unless the change in this phase relationship involves the entrainment of a circadian oscillator, the original phase relationship should be restored upon removal of the cyclic fear, which was not the result we obtained. Finally, the cyclic fear stimulus could change the phase of entrainment of the SCN to the LD cycle, but we do not favor this interpretation. Although it has been reported that an aversive stimulus can inhibit photic phase-shifting when paired with light pulses (Amir & Stewart, 1998), we are unaware of cyclic non-photic stimuli that can change the steady-state phase of entrainment of the master circadian clock to the LD cycle.

If the shift in feeding and locomotor activity involves an anatomically identifiable fear-entrainable clock, what could be the site of this oscillator? The central and basolateral nuclei of the amygdala exhibit 24-h rhythmic expression of *Per2* that is apparently dependent on intact SCN oscillations (Lamont, Robinson, Stewart, & Amir, 2005; Segall, Perrin, Walker, Stewart, & Amir, 2006). Clock genes are not only critical components of the transcription-translation feedback loop that constitutes circadian oscillators but they are also part of complex gene networks that transduce physiological and behavioral conditions including metabolic, nutritional and emotional state (Zhang & Kay, 2010). It is therefore plausible that an oscillator in the amygdala that is sensitive

to threatening stimuli works synergistically with the SCN to gate behavior to a time of the LD cycle when the threat of harm or predation is not present. Indeed, direct projections from the SCN to the central amygdala have been shown, but the function of this projection remains unknown (Sofroniew, 1980; Watts, Swanson, & Sanchez-Watts, 1987). Similarly, the dorsomedial nucleus of the hypothalamus and the SCN have been proposed to participate in a multi-oscillatory system to regulate food anticipatory activity after restricted feeding schedules (Acosta-Galvan et al., 2011).

It remains to be determined whether cyclic threat entrainment of foraging and feeding behaviors also involves reprograming of other circadian rhythms. For instance, could fear-entrained oscillator(s) change the timing of endocrine rhythms, such as the evening peak of glucocorticoid release in nocturnal rodents (Leliavski, Dumbell, Ott, & Oster, 2015)? Recent studies have shown that when mice have to "work" to obtain food they switch to a diurnal pattern of activity, and this switch can be explained by the beneficial effects that the diurnal pattern of activity has, as it lowers energy expenditure (Hut, Pilorz, Boerema, Strijkstra, & Daan, 2011; van der Vinne et al., 2014). This work-for-food switch in the activity pattern is associated with changes in the daily release of corticosterone as well as the phase of some peripheral circadian oscillators. Future studies could determine the extent of the circadian reprograming that can be induced by cyclic threat.

In summary, the present study found that amygdala-coded fear can reprogram circadian behavior to override behavioral outputs of LD-entrained oscillators. Our observations suggest an intriguing possibility that the amygdala and the SCN interact as a fear-entrained oscillator to adapt to cyclic predatory threats by predicting times of danger and safety and organizing circadian behaviors accordingly.

Chapter 3. SEX DIFFERENCES AND ESTROUS CYCLE EFFECTS IN RESOLVING APPROACH-AVOIDANCE CONFLICT

3.1 INTRODUCTION

It is well-known that women are more susceptible to anxiety-related disorders than men. In general, women experience specific phobias (particularly of other animals such as snakes, spiders, and dogs), social phobias, agoraphobia, generalized anxiety disorder, and separation anxiety disorder (as children) more often and typically with greater severity than men even when considering discrepancies in rates of referral and reporting (Donner & Lowry, 2013; Mackinaw-Koons & Vasey, 2000; McLean & Anderson, 2009). In studies of non-clinical anxiety and fear, men exhibit greater reactivity to fear conditioning and are slower to habituate to nocuous stimuli (Maeng & Milad, 2015). Given that, as a whole, anxiety disorders are one of the most common mental disorders, with a lifetime prevalence of about 16% (Maeng & Milad, 2015), and that women are particularly affected, it is vital to ensure sex differences and their naturalistic functions are adequately studied and modeled. As rodents are the most commonly-used research subjects in translational and preclinical research, it is also important to understand what characteristics can and cannot be generalized from rodents to humans. Because overt behaviors are species-specific, it is important to understand the natural functions of behavior, as general principles gleaned from studying the underlying spatiotemporal dynamics and bio-economic trade-offs involved may be more generalizable across species.

The definitions of "fear" and "anxiety" remain disputed among researchers, and are often defined ambiguously and without a clear relationship to external factors (see Perusini & Fanselow, 2015 for a review). In the present study, fear and anxiety will be defined functionally within the framework of Predatory Imminence Theory (Fanselow & Lester, 1988). In this model, fear and anxiety operate along a continuum determined by the spatiotemporal distribution of threat, with fear (or panic) behaviors being those that are activated by more imminent threats (or cues predicting such) and anxiety behaviors being those that are activated by "diffuse" or less imminent/probable threats. For example, panic (or "circa-strike") behaviors, such as escape or defensive fighting, are elicited when contact with a threat is most imminent, and serve as a last resort for survival. As contact with a threat becomes less likely, fear (or "post-encounter") behaviors, such as freezing, are activated to reduce the probability of detection by a potential threat. Anxiety (or "pre-encounter") behaviors are those, such meal pattern reorganization or heightened vigilance, activated in situations that convey a minimal but heightened vulnerability (such as lighted conditions, for a rat) and serve to preemptively minimize the risk of encounter (Fanselow & Lester, 1988; Perusini & Fanselow, 2015). While such functional definitions provide a useful organization of naturalistic behaviors, observed defensive behaviors and their eliciting stimuli are species-specific and may therefore have limited generalizability to human populations.

Several lines of research suggest that there are sex differences in fear and anxiety behaviors in rodents. However, the findings from these studies often do not provide a cohesive picture of sex differences, and species and strain-differences in the direction of the male-female difference are common (Donner & Lowry, 2013). The following discussion will focus primarily on rats, the species used in the present research. Research examining sex differences

in basic sensory and perceptual thresholds have shown that females startle less than males to a noxious acoustic stimulus (Gulinello, Orman, & Smith, 2003), but they have lower thresholds for shock (even after controlling for weight) and exhibit faster shock escape latencies (Archer, 1975; Beatty & Fessler, 1976). Females tend to demonstrate greater potentiation of startle to a loud stimulus when it is presented under lighted conditions or during presentation of a previously-conditioned fear CS (de Jongh, Geyer, Olivier, & Groenink, 2005). Traditional Pavlovian fear conditioning studies using rats tend to demonstrate that, while acquisition of conditioned fear to discrete stimuli and contexts is similar between males and females, contextual fear memories are more persistent in males as indicated by stronger freezing and slower extinction rates compared to females (Graham, Yoon, Lee, & Kim, 2009; Gupta, Sen, Diepenhorst, Rudick, & Maren, 2001; Maren, De Oca, & Fanselow, 1994; Pryce, Lehmann, & Feldon, 1999), while males and freely-cycling females exhibit similar levels of conditioned fear and extinction to a discrete CS. Female rats fear conditioned to a discrete CS during proestrus show reduced fear memory and faster and more stable fear extinction learning compared to females conditioned during other phases of the estrous cycle (Markus & Zecevic, 2013; Milad, Igoe, Lebron-Milad, & Novales, 2009). Furthermore, injection of both estrogen and progesterone in metestrus females generated similar reductions in freezing during recall tests to proestrus females (when estrogen and progesterone levels are high), and blockage of estrogen and progesterone in proestrus females caused a similar decrease in the rate of fear extinction as metestrus females (Milad et al., 2009). Milad et al. also showed that sex differences are eliminated when fear conditioning occurs among freely-cycling females (i.e., conditioning occurs irrespective of estrous phase). Importantly, they did not observe greater variability among the females than in males.

In common tests of anxiety such as open field tests and elevated-plus mazes, which measure animals' innate aversion to areas that are relatively more exposed or illuminated, females typically exhibit less anxiety than males, as measured by less defecation, increased activity in the open field, and more time spent in and entries to the open-arms of the elevated-plus maze (Blizard, Lippman, & Chen, 1975; Frye, Petralia, & Rhodes, 2000; Johnston & File, 1991; Masur, 1972; Zimmerberg & Farley, 1993). Additionally, estrous-phase-dependent effects have been noted. Females in proestrus exhibit more activity in open field tests and make more open-arm entries in the elevated-plus maze relative to the other phases of estrous, which has been shown to be dependent on fluctuating estrogen-progesterone levels associated with proestrus (Blizard et al., 1975; Frye et al., 2000). Many studies have concluded that the open field and elevated plus maze tests lack external and construct validity as assays of emotion-related behaviors (that is, they don't correlate well with other tests or measures of emotionality), particularly for females, and behavior therein is much more influenced by baseline locomotor activity rather than anxiety (Archer, 1975; Birke & Archer, 1975; Fernandes, González, Wilson, & File, 1999; Gray & Lalljee, 1974; Masur, 1972).

Researchers have proposed that active and inhibitory (or passive) avoidance paradigms, which measure cued and contextual avoidance behavior of dangerous (e.g., shock-eliciting) locations, are more valid tests of anxiety in both males and females, and thusly permit direct comparison of sex differences (Archer, 1973; Gray & Lalljee, 1974). In active avoidance paradigms, animals must respond in a specific way to avoid shock (e.g., move to a different compartment or press a lever when a cue indicating shock is presented). Such studies suggest that females acquire the active avoidance response faster than males and extinguish slower, and that such effects are predominately driven by the organizational, rather than activational, effects

of neonatal testosterone (Beatty, 1979; van Haaren et al., 1990). In studies of inhibitory avoidance, which typically measure the latency to return to a location associated with prior shock, females have been shown to return faster than males, and that this differences is also largely a consequence of the organizational effects of testosterone (Beatty, Gregoire, & Parmiter, 2013; Drago, Bohus, Scapagnini, & De Wied, 1980; van Haaren et al., 1990; van Oyen, van de Poll, & de Bruin, 1980).

While these paradigms to study fear and anxiety have been useful and generated much of the knowledge we have on the mechanisms and influence of various treatments (e.g., drugs, stress, social interactions), all such studies are similarly limited in that they examine behavior in settings that restrict the natural repertoire of behaviors (largely to simplify analysis) and sample behavior over a short duration, which ignores the longer-scale spatiotemporal integration of information that animals rely on to behave in natural environments. The present study utilizes a longitudinal closed economy approach to model the approach-avoid conflict that is ostensibly shared by all species – acquiring resources, such as food and water, is costly not only in terms of energetics, but decisions to explore and forage must also be balanced by considering potential risks and threat of predation. As this design utilizes unpredictable shock localized to one area of the chamber, similar to inhibitory avoidance paradigms, it was hypothesized that females would exhibit less avoidance than males overall. Furthermore, results from this paradigm are compared to an alternative approach-avoidance paradigm that presents a mobile, robotic threat while rats attempt to retrieve food (Choi & Kim, 2010), as well as the traditional Pavlovian fear conditioning paradigm.

3.2 METHOD

3.2.1 *Animals*

Subjects were gonadally-intact male ($n = 8$) and female ($n = 16$) and ovariectomized female (OVX; $n = 8$) Long-Evans rats (Charles River Laboratories) initially aged 7-8 weeks. Ovariectomy surgeries were performed by Charles River Laboratories prior to shipment. Upon arrival, all animals were individually housed in eight closed economy chambers on a 12 h/12 h LD cycle. Estrous phase was determined daily via vaginal lavage in a subset of female rats (as described in Marcondes, Bianchi, & Tanno, 2002). In brief, rats were brought into a separate room individually, handled momentarily, and then held in place by hand and a pipette filled with 10 µl of 0.9% saline was gently and shallowly inserted into the vagina. The saline was washed in and drawn out, and immediately placed on slides for cytological examination under a light microscope. All animal experiments were conducted in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were reviewed and approved by the University of Washington Institutional Animal Care and Use Committee.

3.2.2 *Closed Economy Task*

The closed economy dimensions were 74.3 cm x 25.4 cm x 33 cm (length x width x height) and consisted of a 'nest' (20.3 x 25.4 cm) and a 'foraging' arena (54 x 25.4 cm). The nest floor was covered with sawdust, while the floor of the foraging arena was composed of 32 stainless steel rods (4.5 mm diameter) wired to a precision animal shocker (Coulbourn Instruments, Allentown, PA) for delivery of footshocks. A camera (Fire-I B/W Board camera; Unibrain Inc., San Ramon, CA) was mounted above each closed economy chamber and connected to a computer for tracking animal activity via ANY-maze software (Stoelting, Wood Dale, IL), which also measured

activation of the food levers and dispensers (Med Associates, Inc., Georgia, VT) and the shock generator connected to an ANY-maze Interface (AMi; Stoelting, Wood Dale, IL). Forty-five-mg grain-based pellets for rodents (#F0165, Bio-Serv, Flemington, NJ) were used for food. The total number of pellets dispensed was chosen as the primary *foraging* variable due to the necessity of the animal to be in the foraging area and actively pressing the lever in order to obtain and consume the food pellets. Importantly, all pellets obtained by each animal were consumed in the foraging area. The total distanced traveled (m) among both the foraging and nest areas, rather than within one specific area, was used as the primary *activity* variable for analyzing behavioral rhythms as this would include but not be confounded by avoidance behavior. White noise (70 dB) generated by the ANY-maze software was continuously played through computer speakers throughout the experiment to obscure external noises. The actual times corresponding to the onset of the dark phase (ZT12) occurred between 10 a.m. and 11 a.m. (varied by cohort) so any human work-related vibrations would be poorly associated with the experimental cycles.

After arrival, animals were given 12-14 d to acclimatize to the chambers, during which time they were shaped to press either of two levers 25 times initially to gain access to a continuous reinforcement schedule (FR25:CRF), which was reset if 1 min passed since the last lever press. The FR threshold was doubled every 2 days (except FR16 was increased to FR25) until stable lever pressing at the FR25 threshold was achieved. After 14 d baseline days ('Baseline'), all of the animals were exposed to 14 d of unsignaled, pseudo-random footshocks (0.8 mA; ~2 shocks/h; 'Shock'). If the animal was in the foraging area when ANY-maze triggered the footshock, the shock continued for up to 10 s or until the animal escaped to the nest. If the animal was in the nest at the time the program generated the footshock, it terminated instantaneously. Animals were removed from the closed economy chambers during the last hour of the light phase (ZT11-ZT12)

every 2-3 days so the chambers could be cleaned, the food and water replenished, and animals weighed.

3.2.3 *Robot Foraging Task*

Following the final day of the closed economy experiment, all rats were moved into standard home cages, housed individually, in a separate vivarium maintained on a 12h/12h LD cycle. The animals were food restricted (~10 g standard chow per day) and body weight reduced (over ~10 d) then maintained at 85% of their post-closed economy body weight in order to ensure they were food-motivated for the robot foraging task.

The robot foraging task took place in a custom-built 'semi-naturalistic' foraging apparatus consisting of a 'nest' area (29.2 cm length X 57.1 cm width x 59.7 cm height; equipped with a water bottle; 16.2 Lux luminance) with a remotely-controlled, vertically-opening gateway (10 cm X 10 cm) to an adjacent foraging area (201.9 cm length X 58.4 cm width X 60.9 cm height; 56.7 Lux). White noise (60 dB, A-scale) was played through computer speakers to obscure external sounds. The ANY-maze video tracking system (Stoelting co, Wood Dale, IL), with video feed from an ultra-digital wireless camera (LW2101, Lorex Technology, Inc., Ontario, Canada) affixed over the apparatus and connected to a Sony HD DVD record (RDR-HX900), was used to capture video image and automatically track the animal's movement (30 fps) from both nesting and foraging areas.

Rats were first habituated to the nest area of the robot foraging area by placing them in the nest area with ten 1-g grained-based pellets (Bio-Serv, Flemington, NJ) for 30 min each day over 4 days. Afterward, over 5 days, baseline foraging behavior was shaped by placing one pellet at increasing distances from the nest area (short (S): 25.4 cm; medium (M): 50.8 cm; long (L): 76.2 cm) contingent on successful retrieval at the previous trial. Successful retrieval consisted of exiting

the nest and taking the pellet back into the nest to eat. If a rat began to eat outside the nest, the pellet was taken away, the animal placed in the nest, and the trial restarted. Shaping was terminated once the rat could successfully retrieve the pellet in under 20 s at the L distance on two consecutive days.

On testing days, baseline behavior was measured at each of the three distances, starting with the L distance and moving closer to the nest. Subsequently, a LEGO® Mindstorms robot, shaped like a cat was placed at the far end of the foraging area. The robot was programmed to automatically surge forward 23 cm, swipe its "paw" down, and return to its original position when the rat crossed a distance-specific threshold (15 cm before the distance at which the pellet was placed), as determined by ANY-maze tracking software (Stoelting, Wood Dale, IL). Rats were tested three times at each distance, beginning with the L distance and moving closer to the nest, each day over 4 days. A maximum of 3 min was allotted for each trial. The total latency to retrieve the pellet and return to the nest (s), success rate (successful retrieval attempts/total retrieval attempts), and latency to exist the nest (s) were used as the primary dependent variables. Retrieval attempts were scored when the animal exited the nest and triggered the robot to surge.

3.2.4 *Fear Conditioning*

Twenty-four hours following the robot foraging task, all rats underwent typical tone-shock fear conditioning. Each rat was placed in a modular operant chamber equipped with a speaker module and located in a sound-attenuated chest (Coulbourn Instruments, Allentown, PA). The chamber was rectangular (27 cm width X 28 cm length X 30.5 cm height) with front and back walls made of clear Plexiglas® and two side walls made of metal plates. The grid floor was composed 17 stainless steel bars (5 mm diameter) spaced 15 mm apart center-to-center, and wired to a precision animal shocker (Coulbourn Instruments, Allentown, PA) for delivery of footshocks.

An ammonium solution was presented in the bed pan below the grid floor to provide a distinct odor. A 24-cell infrared activity monitor was used to assess freezing, which was defined as 3-s or more of inactivity. Baseline behavior was recorded for 1 min, which was followed by 3 trials of a tone CS (2.9 kHz, 85 dB, 20 s) which co-terminated with a footshock (1.0 mA, 1-s; 1 min ITI). The next day, rats were placed in a novel context (non-lighted; hexagonal chamber with a Plexiglas® floor; acetic acid odor) and the same 2.9kHz tone was presented continuously for 8-min following a 1-min baseline period, and then returned to their home cage. 4 h later, rats were brought back into the original fear conditioning chamber, and freezing to the context alone (no tone or shock; ammonium odor presented) was assessed over an 8-min period. The order of context- and tone-testing was counterbalanced among the rats to distribute any potential order effects equally across conditions. Female rats were fear conditioned during the proestrus phase of their estrous cycle, as determined by vaginal lavage cytology (as described in Marcondes, Bianchi, & Tanno, 2002). Males were handled similarly to control for potential effects the procedure may have on behavior. Subsequent retention tests were performed independent of estrous phase. Due to the large number of statistics and tests performed, only significant and the most relevant non-significant test statistics are reported.

3.2.5 *Statistical Analyses*

All data are presented as mean ± SEM. The data from the closed economy experiment was analyzed using mixed factorial ANOVAs on the daily means, except where noted, with the within-subject factors of time (day) and experimental condition (baseline, shock, or extinction) and between-subjects factor sex. To analyze estrous-cycle related fluctuation of locomotor activity in the closed economy, rats' apparatus-wide activity (distance traveled, m) was summed in 1-h time-bins, and decomposed into its component frequencies using Fast-Fourier Transforms (FFT). The

magnitudes of the frequencies in the 3 to 5 d range were used to test for statistical significant sex differences using independent, one-tailed *t*-tests. Additionally, continuous wavelet transform analysis on the 3-5 d range was employed (as in Blum et al., 2015; Leise, 2013) to quantify the temporal dynamics of estrous-cycle-related fluctuations using the Morse wavelet (Leise, 2013). The locomotor activity of intact and OVX female rats, in 1-min time-bins, across 42 consecutive days was used. For each transform, the average and standard deviation of the dominant infradian (3-5 d) period was calculated for each animal. This analysis was performed in MATLAB (R2015b) using modified versions of the publically-available *CWTspectrogram.m* (https://github.com/storchlab/CWT.git; Blum et al., 2015) and *AnalyticWT.m* (https://www3.amherst.edu/~tleise/CircadianWaveletAnalysis.html; Leise, 2013) scripts. Mixed-factorial ANOVAs were used to test for main effects and interactions between estrous phase and experimental conditions, followed-up by dependent, two-tailed t-tests, in the subset of females from which estrous phase samples were obtained. Mixed-factorial ANOVAs were also used to analyze the data from the robot foraging tests, with the within-subject factors of distance (L, M, S) and day and between-subjects factor of sex. Fear conditioning data was analyzed using repeated-measures ANOVAs separately for each test (within-subjects factor of time and between-subjects factor of sex) to examine acquisition or extinction rates, and independent, two-tailed *t*-tests were used to test for sex differences in retention based on the average time spent freezing as a percentage of the total time over the first 4 min of each test. In cases where the assumption of sphericity was violated (Mauchly's test), Greenhouse-Geisser corrected degrees of freedom were used. In cases where Levene's Test for Equality of Variance was significant, the degrees of freedom were corrected using the Welch-Satterthwaite method. Bonferonni-adjusted, two-tailed, paired-samples t-tests or independent t-tests were used for post hoc tests where appropriate.

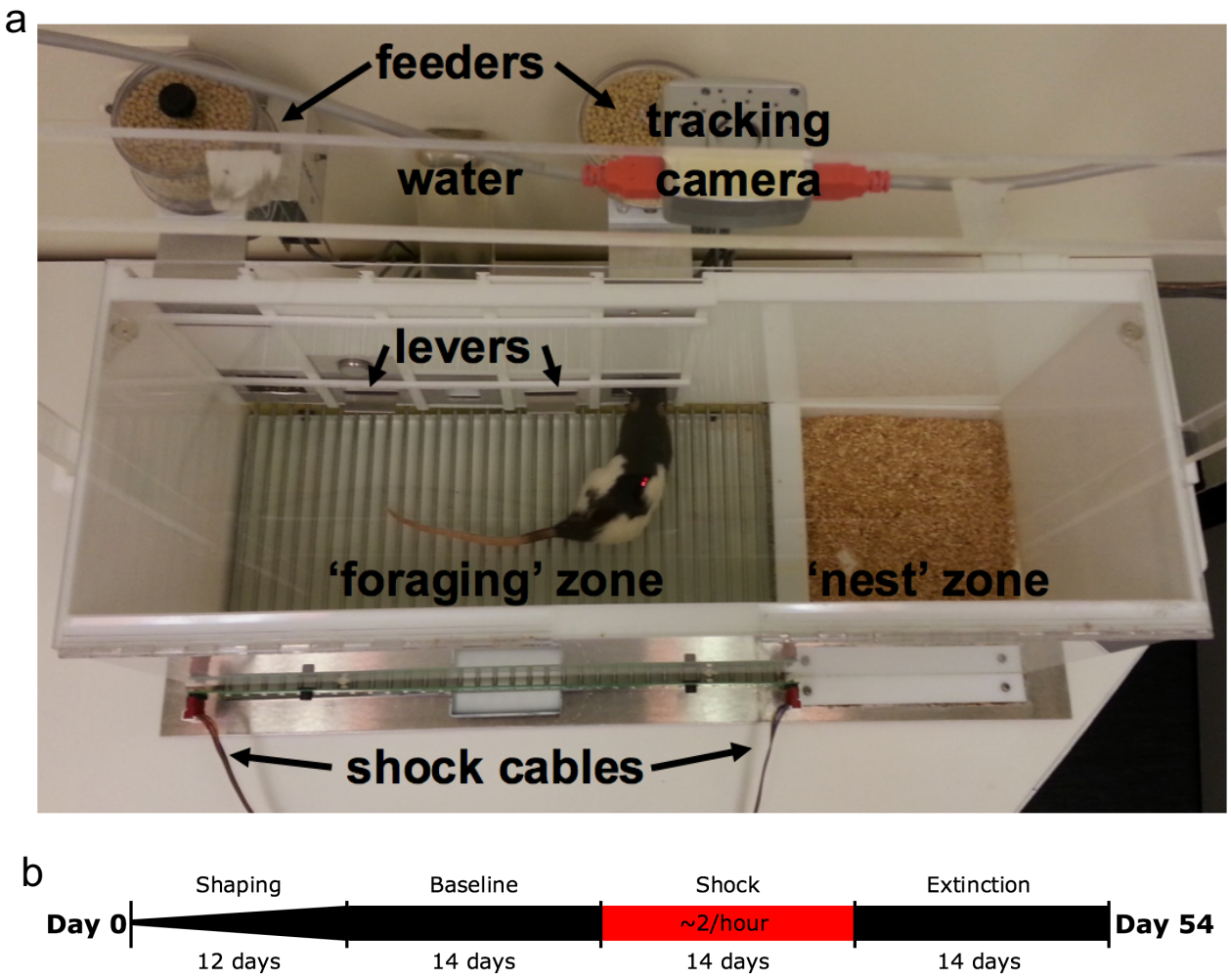

Figure 3.1. Experimental apparatus and design of the closed economy. (*a*) A photograph of a closed economy box. Measurements of lever presses, food dispensed, water bottle licks, shocks delivered and received, and locomotor tracking and spatial position via overhead-mounted tracking camera were coordinated by ANY-maze (Stoelting Co.) I/O interface and software. (*b*) Male (n = 8) and female (n = 8) rats were housed in the closed economy box upon arrival and were shaped on an FR25:CRF schedule over 12 days, followed by 14 days of baseline measurement, 14 days of shock, and 14 days of extinction (no shock) conditions.

3.3 RESULTS

Male (n = 8) and female (n = 8) rats living individually in closed economy chambers (Figure 3.1a) and maintained on a 12-h:12-h LD cycle were shaped to respond on either of two levers on an FR25:CRF reinforcement schedule to obtain food pellets, which was reset if 1 min had passed since the last lever press. Males and females did not differ in their rate of acquisition of the FR-threshold during shaping [repeated-measures ANOVA; p = 0.689]. Figure 3.2a shows the mean expected number of shocks each group would have received if shocks were delivered based on their naïve, baseline foraging behavior, the actual mean number of shocks each group received during the shock condition (triggered randomly and unsignaled, ~2 per h), and the expected mean number of shocks they would have received based on their foraging behavior during extinction (no shock). There were significant main effects of condition [$F_{2, 20}$ = 39.01, p < 0.001] and sex [$F_{1, 10}$ = 251.15, p = 0.011], and significant interactions between condition and sex [$F_{2, 20}$ = 5.37, p = 0.014], condition and time [$F_{26, 260}$ = 6.11, p < 0.001], and condition, time, and sex [$F_{26, 260}$ = 1.54, p =0.049] on shock frequency. At baseline levels of foraging, males would have been marginally more likely to receive shock (18.84 ± 5.13 shocks per day) than females (14.34 ± 2.98 shocks/day) [t_{14} = 2.15, p = 0.050], but during the actual shock condition, females and males received the same number of shocks, on average (females: 5.27 ± 1.96; males: 5.25 ± 1.06 shocks). A close examination of avoidance behavior on Day 1 of shock indicates that males and females acquired avoidance behavior, as measured by proportion of the total shocks they received over the course of the day, at the same rate [p = 0.340]. During extinction, males rapidly resumed baseline-levels of foraging, as indicated by the expected shock frequency (19.90 ± 6.74 shocks/day) [baseline vs. extinction: t_7 = 0.03, p = 0.975], whereas females remained below baseline-levels of foraging behavior (8.71 ± 3.85 shocks/day) [baseline vs. extinction: t_7 = 4.18, p

= 0.004]. These effects were primarily driven by the proportion of time spent in the foraging zone and not due to overall levels of activity as female rats were significantly more active (589.28 ± 189.97 m), as measured by distance traveled (Fig. 3.2b), than males (386.63 ± 89.97 m) [$F_{1, 14}$ = 10.14, p = 0.007], which was not significantly affected by the shock or extinction conditions [condition: $F_{2, 28}$ = 3.12, p = 0.060].

There were no statistically-significant sex differences in the mean daily number of water bottle licks (females: 7415.04 ± 1781.13; males: 8761.38 ± 2214.89 licks) throughout the experiment [$F_{1, 14}$ = 2.96, p = 0.107] (Fig. 3.2c), however water intake in general was significantly reduced during the shock condition [condition: $F_{2, 28}$ = 38.08, p < 0.001]. While males consumed more food overall (551.86 ± 79.87 pellets) compared to females (357.45 ± 66.11 pellets) [$F_{1,14}$ = 40.67, p < 0.001] (Fig. 3.2d), both sexes reduced their food intake during shock (females: 253.40 ± 86.97; males: 466.20 ± 93.75 pellets) and increased their food intake during extinction (females: 429.63 ± 78.39; males: 610.08 ± 49.71 pellets) by similar degrees relative to their baseline levels [condition: $F_{2, 28}$ = 34.79, p < 0.001; condition × sex: $F_{2, 28}$ = 0.34, p = 0.715; baseline vs. shock: t_{15} = 6.46, p < 0.001; baseline vs. extinction: t_{15} = 2.71, p = 0.016]. Males obtained meals (reaching the FR25 threshold) marginally more frequently than females during baseline (males: 10.38 ± 3.53; females: 7.55 ± 2.33 meals per day) [t_{14} = 1.79, p = 0.066], although they obtained meals at more similar rates during shock, which was significantly reduced among both groups [$F_{2, 28}$ = 7.56, p = 0.002], and during extinction (Fig. 3.2e). While both sexes reduced their overall meal frequency and total food intake during shock, males, but not females, increased how many pellets they obtained per meal (baseline: females: 57. 00 ± 18.26, males: 65.55 ± 31.74; shock: females: 46.16 ± 18.26, males: 78.34 ± 28.84; extinction: females: 67.75 ± 32.21, males: 83.04 ± 39.79 pellets/meal) [condition: $F_{2, 28}$ = 6.07, p = 0.006; condition × sex: $F_{2, 28}$ = 3.62, p = 0.040] (Fig.

3.2f). In terms of foraging efficiency (Fig. 3.2g), or the number of pellets obtained per lever press, which includes foraging behavior (lever presses) that fails to result in food (i.e., the FR25 threshold is not reached), there was a significant main effect of condition [$F_{1.41, 21.57} = 11.40$, $p = 0.001$], sex [$F_{1, 14} = 7.21$, $p = 0.018$], and a significant condition × sex interaction [$F_{1.41, 21.57} = 5.88$, $p = 0.007$]. Post-hoc tests revealed that male rats were significantly more efficient than females specifically during the shock condition [male vs. female, shock: $t_{14} = 3.28$, $p = 0.006$; baseline: $t_{14} = 1.13$, $p = 0.278$; extinction: $t_{14} = 1.89$, $p = 0.080$]. Whereas males' efficiency during shock (0.68 ± 0.09 pellets per lever press) did not differ from their baseline efficiency (0.67 ± 0.08 pellets/ press) [$t_7 = 1.01$, $p = 0.346$], they increased their efficiency during extinction (0.73 ± 0.08 pellets/ press) relative to baseline [$t_7 = 3.08$, $p = 0.018$]. On the other hand, females' efficiency was significantly impaired during shock (0.50 ± 0.13 pellets/press) compared to their baseline (0.63 ± 0.06 pellets/press) [$t_7 = 3.04$, $p = 0.019$], which was rescued during extinction (0.65 ± 0.07 pellets/press) [baseline vs. extinction: $t_7 = 1.46$, $p = 0.189$; shock vs. extinction: $t_7 = 3.22$, $p = 0.015$].

These data correspond to the finding that males were able to maintain their baseline-level body weight during the shock period (103.81 ± 3.64, % of baseline weight), but females were not and lost weight relative to their baseline (96.28 ± 4.87, % of baseline weight) [$t_{14} = 3.50$, $p = 0.035$] (Fig. 3.2h). Although males gained more weight (116.79 ± 3.01, % of baseline weight) during the extinction period compared to females (102.70 ± 6.48, % of baseline weight), faster weight gain in males may also be expected independent of avoidance due to lasting anxiety in females (Bell & Zucker, 1971), and it cannot be determined whether growth during extinction occurred at a normal or otherwise rate without a no-shock control group for both males and females.

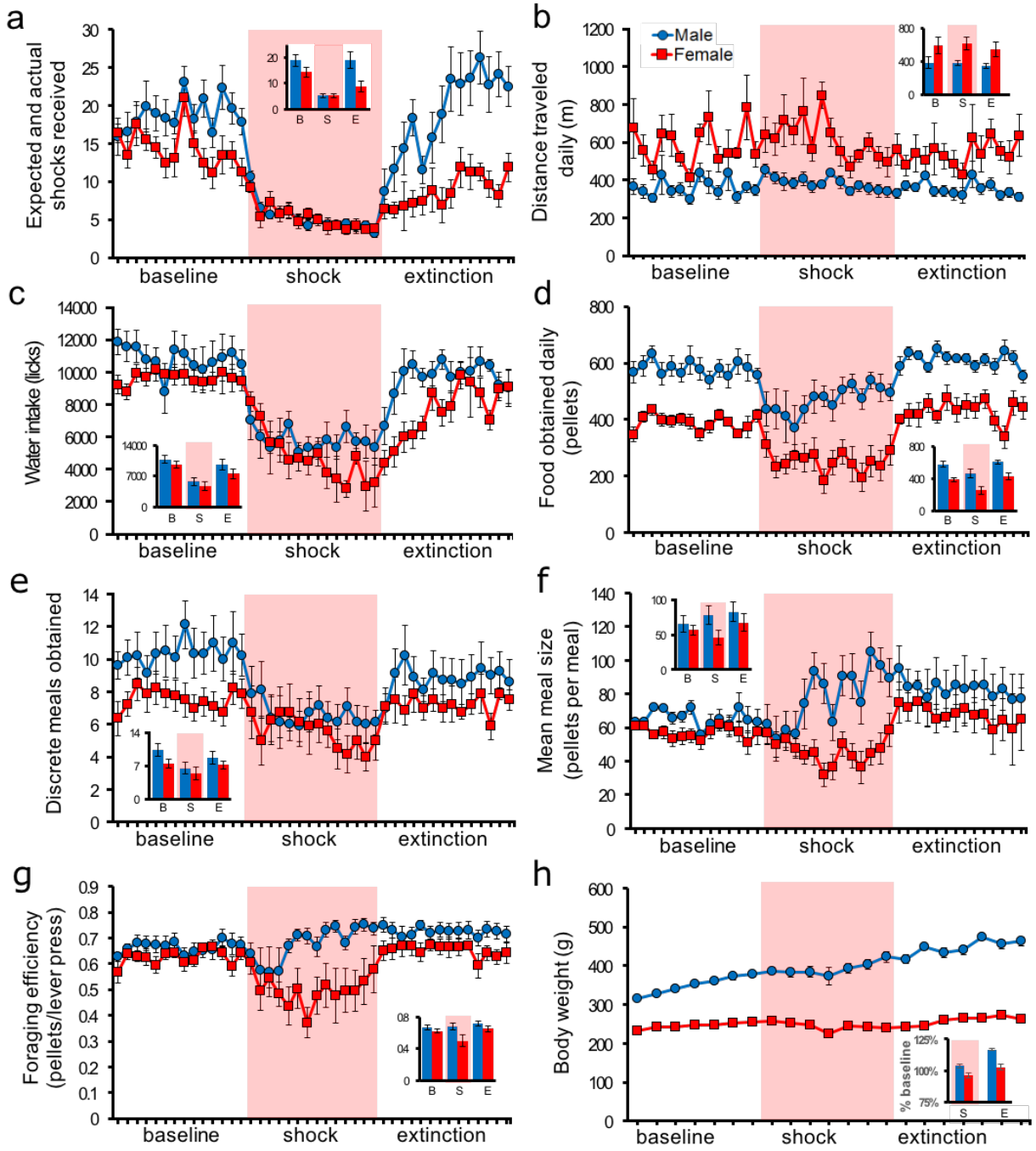

Figure 3.2. Sex differences in avoidance and foraging behaviors. Females (n = 8; *red/squares*) and males (n = 8; *blue/circles*). Mean daily (*a*) expected (during baseline, B, and extinction, E) and actual number of shocks received during the shock condition (S, red-shaded background), (*b*) activity (distance traveled, m), (*c*) water intake (number of licks), (*d*) food intake (pellets dispensed), (*e*) number of meals obtained (number of FR25 thresholds attained after reset), (*f*) meal size (pellets/meal), (*g*) foraging efficiency (pellets/lever press), and (*h*) body weight (g). Embedded figures (*a*-*g*) show the group means across each 14-d condition, (*h*) shows mean body weight during S and E as a percentage of B. All error bars represent SEM.

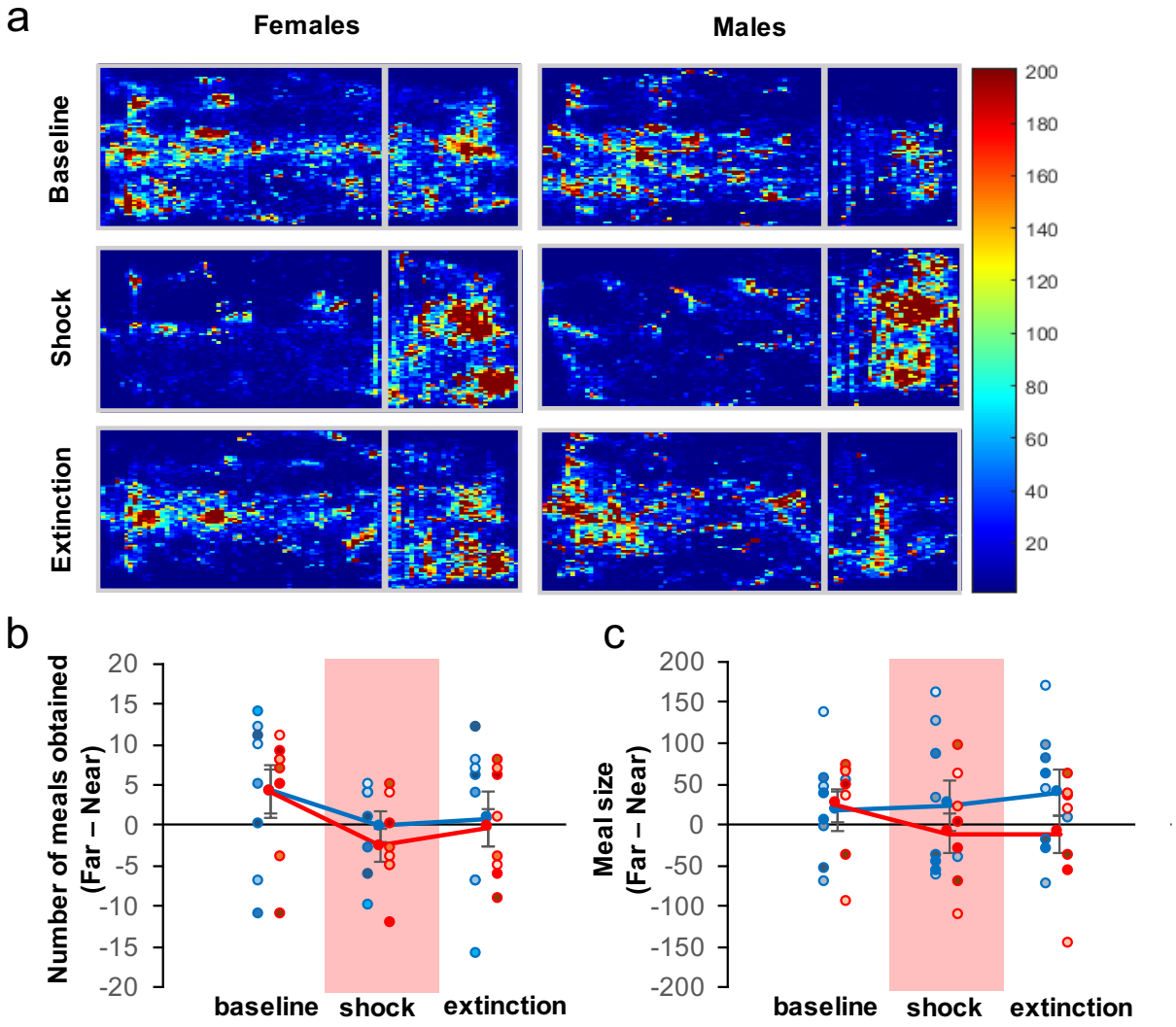

Figure 3.3. Effects of shock on foraging activity and lever preferences. (*a*) Heat maps of activity in the closed economy on the last day of baseline, the last day of shock, and last day of extinction. Lighter color indicates greater activity in a location relative to darker areas. All maps are on the same scale, and represent the aggregate behavior of all rats of each sex (left panel: females; right panel: males). (*b*) Number of meals obtained on the far lever minus number of meals obtained on the near lever. (*c*) Mean meal size of meals obtained on the far lever minus that of the near lever. All error bars represent SEM.

Changes in the spatial distribution of locomotor activity and in lever preferences across experimental conditions are shown in Figure 3.3. 2D histograms of locomotor activity (200 bins indicating frequency of areas occupied by rats, aggregated by sex) demonstrate that during the last day of baseline, when threat-naïve behavior is well-established, and on the last day of shock when avoidance behavior is well-established, males and females exhibit a similar spatial distribution of activity, and by the last day of extinction, males utilize the foraging space more than females (Fig. 3.3a). Although there were no significant differences between females and males in their established lever preferences (the difference in the number of meals or mean meal size between each lever on the last day of each condition) [$F_{1, 14} = 0.60$, $p = 0.452$], lever preferences did significantly shift toward the near lever across groups during shock (Fig. 3.3b) [baseline vs. shock: $t_{15} = 2.25$, $p = 0.040$]. However, there were no significant effects of sex [$F_{1, 14} = 0.740$, $p = 0.404$] or condition [$F_{2, 28} = 0.36$, $p = 0.700$] on the difference in meal sizes obtained on each lever (Fig. 3.3c). These data suggest that the difference in risk between foraging on the far lever and near lever is minimal.

As other studies have found that the locomotor activity of females fluctuates with their estrous cycle (Birke & Archer, 1975; Blizard et al., 1975; Frye et al., 2000), locomotor activity in the closed economy was examined for rhythmic fluctuations. A Fourier analysis of rats' locomotor behavior, measured by distance traveled (m), throughout the 14-d of both baseline and shock was performed, and the magnitudes of the component frequencies in the 1-7 d range were averaged by sex. As Figure 3.4 shows, both males and females exhibited normal circadian rhythms (1-d periods), but only females exhibited a peak in the 3-5 d range during both baseline [4.3 d: $t_{14} = 2.38$, $p = 0.026$] (Fig. 3.4a) and shock [4.3 d: $t_{14} = 2.63$, $p = 0.020$; 5.3 d: $t_{14} = 3.14$, $p = 0.007$] (Fig. 3.4b), which corresponds to the normal period range of the estrous cycle.

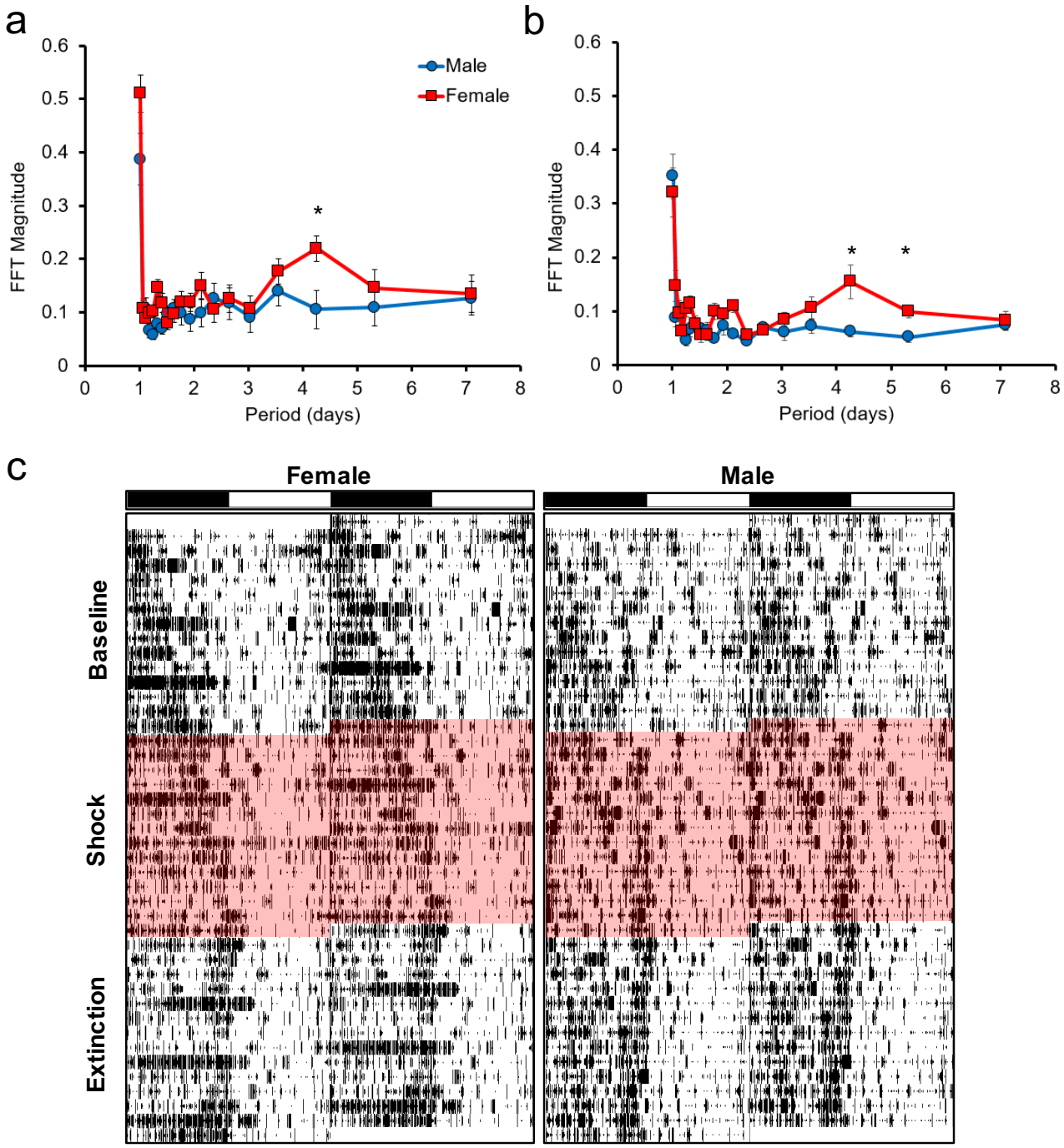

Figure 3.4. Fourier decomposition of circadian and infradian rhythms. Magnitude of each frequency (translated into periods) decomposed via Fast-Fourier transform of locomotor activity (distance traveled, m) during (*a*) baseline and (*b*) shock. All error bars represent SEM. Asterisks indicate $p < 0.05$. (*c*) Double-plotted actograms of locomotor activity (m) in 1-min time-bins from a representative female (left panel) and male (right panel) across experimental conditions (14 d baseline, 14 d shock, 14 d extinction). Red highlight indicates shock period. LD cycle is indicated by the black (dark phase; 12 h) and white (light phase; 12 h) bars above each plot.

Actograms (24-h raster plots) of a representative female and male locomotion across experimental conditions demonstrate the rhythmic fluctuation in activity levels in the 3-5 d range in females but not in males (Fig. 3.4c).

In order to examine the influence of estrous phase on risky foraging behavior, a separate group of intact-females ($n = 8$) and ovariectomized females (OVX; $n = 8$) were tested in the closed economy using the same methodology. As expected, intact females, like before, displayed greater rhythmic behavior in the 3-5 d range whereas ovariectomized females did not, as detected by Fourier transform [4.3 d: $t_{14} = 1.84$, $p = 0.044$] (Fig. 3.5a). One limitation of the Fourier transform is that it is not sensitive to changes in periodicity across time, so continuous wavelet transforms (CWT) on the locomotor activity (distance traveled, m) in 1-min time-bins were employed to achieve greater temporal resolution (Leise, 2013). Figure 3.5b shows a scatterplot of the mean and standard deviation of the period derived from the CWT, ranked by the maximum amplitude of the signal. Contrary to expectations, there were no differences in the periods, their standard deviations, or amplitudes between intact and OVX females to potentially characterize estrous-cycle-related fluctuations (all $p > 0.05$ based on independent two-tailed *t*-tests). Heat-maps of the CWT from representative intact (Fig. 3.5c) and OVX females (Fig. 3.5d) provide an example of the 3-5 d "scalloping" of circadian activity expected to occur with estrous-phase-dependent increases in activity in intact females (Leise, 2013), although this was not apparent in all intact animals.

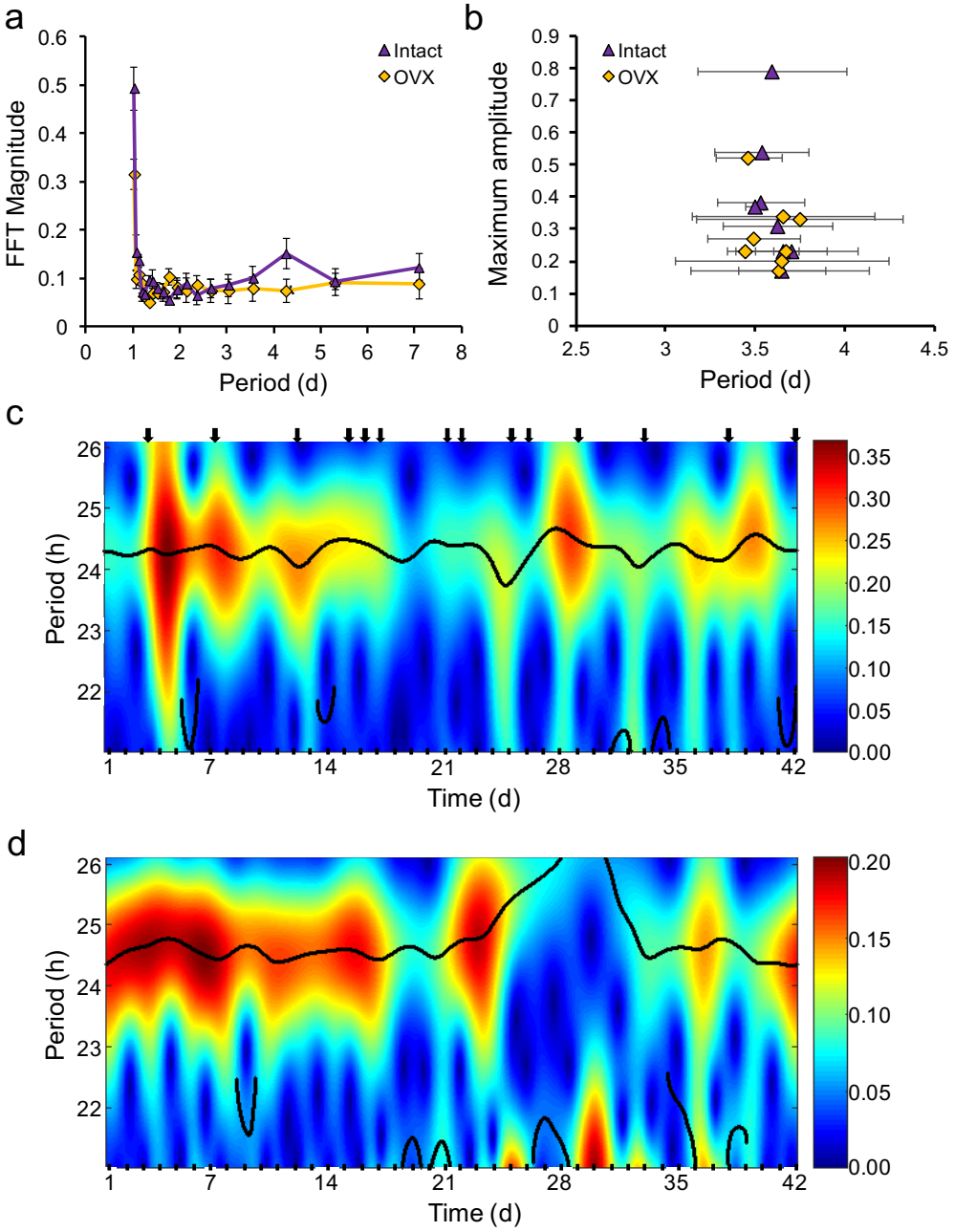

Figure 3.5. Wavelet analysis of infradian rhythms in intact and ovariectomized female rats. (*a*) Magnitude of Fast-Fourier transform component frequencies (translated into periods) of locomotor activity (distance traveled, m) during baseline. Error bars indicate SEM. (*b*) Scatterplot of mean locomotor period (in days) as determined by continuous wavelet transform for the 3-5 d frequency range, ranked by the maximum amplitude of the rhythm. Error bars indicate standard deviation. (*c-d*) Heat-maps of the coefficients derived from continuous wavelet transforms (CWT) of locomotor activity. The black line is the wavelet ridge indicating instantaneous period and the color indicates magnitude (note different scales). (*c*) CWT from a representative intact female rat. The black arrows indicate days when the rat was determined to be in the proestrus phase of their estrous cycle. (*d)* CWT from a representative OVX female rat.

In terms of avoidance behavior (Fig. 3.6a), intact and ovariectomized females avoided shock at similar rates during the shock period (intact: 4.48 ± 0.64; OVX: 3.97 ± 1.11 shocks) [condition: $F_{2, 28} = 32.80$, $p < 0.001$; group: $F_{1, 14} = 2.87$, $p = 0.113$] and extinguished their avoidance behavior at similar rates, as measured by the expected number of shocks to be received based on their foraging activity during the extinction condition [time: $F_{13, 169} = 3.34$, $p < 0.001$; time × group: $F_{13, 169} = 1.11$, $p = 0.355$]. There were no statistically significant differences between intact and OVX females in overall levels of activity [group: $F_{1, 14} = 3.68$, $p = 0.076$] (Fig. 3.6b).

While OVX and intact females had similar levels of water intake (Fig. 3.6c) [condition: $F_{2, 28} = 26.42$, $p < 0.001$; group: $F_{1, 14} = 1.36$, $p = 0.263$], OVX females consumed more food overall (468.07 ± 133.86 pellets) than intact females (353.52 ± 80.10 pellets), although both groups' feeding behavior (Fig. 3.6d) was similarly affected by the presence of shock [group: $F_{1, 14} = 23.94$, $p < 0.001$; condition: $F_{1.37, 19.21} = 23.59$, $p < 0.001$; condition × group: $F_{2, 28} = 1.39$, $p = 0.266$]. Rather than obtaining more discrete meals in general (Fig. 3.6e) [group: $F_{1, 14} = 2.60$, $p = 0.129$], OVX females consumed more pellets per meal (80.55 ± 18.91 pellets/meal) compared to intact females (62.72 ± 19.80 pellets/meal) [group: $F_{1, 14} = 13.17$, $p = 0.003$]. Furthermore, whereas both groups similarly reduced the overall number of meals they obtained during the shock condition [condition: $F_{2, 28} = 8.01$, $p = 0.002$; condition × group: $F_{2, 28} = 2.91$, $p = 0.071$; baseline vs. shock: $t_{15} = 3.07$, $p = 0.008$], there was a significant condition × group interaction on meal size [condition: $F_{2, 28} = 8.75$, $p = 0.001$; condition × group: $F_{2, 28} = 11.00$, $p < 0.001$]. Post-hoc tests showed that OVX females significantly increase meal sizes during the extinction period (108.86 ± 18.77 pellets/meal) compared to both baseline (85.80 ± 18.74 pellets/meal) [$t_7 = 4.21$, $p = 0.004$] and shock (60.55 ± 18.91 pellets/meal) [$t_7 = 4.34$, $p = 0.003$], but not during shock compared to baseline [$t_7 = 2.21$, $p = 0.063$], while intact females do not significantly change the size of their meals

[baseline vs. shock: $t_7 = 1.92$, $p = 0.097$; baseline vs. extinction: $t_7 = 1.18$, $p = 0.276$; shock vs. extinction: $t_7 = 0.54$, $p = 0.606$]. This interaction is also reflected in the rats' foraging efficiency data (Fig. 3.6g) [condition: $F_{1.22, 17.10} = 10.41$, $p = 0.003$; group: $F_{1, 14} = 11.40$, $p = 0.005$; condition × group: $F_{1.22, 17.10} = 4.53$, $p = 0.020$]. As with meal size, OVX females (0.70 ± 0.13, pellets/lever press) were more efficient overall than intact females (0.60 ± 0.08 pellets/press) and significantly increased in efficiency during extinction compared to baseline [$t_7 = 5.30$, $p = 0.001$] and shock [$t_7 = 3.47$, $p = 0.010$] conditions. Additionally, foraging efficiency was significantly decreased from baseline (0.73 ± 0.03 pellets/press) during the shock condition (0.58 ± 0.16 pellets/press) [$t_7 = 2.53$, $p = 0.039$] in OVX females. This is in contrast to intact females, which do not significantly change their foraging efficiency across conditions [baseline vs. shock: $t_7 = 0.49$, $p = 0.643$; baseline vs. extinction: $t_7 = 1.57$, $p = 0.161$; shock vs. extinction: $t_7 = 1.51$, $p = 0.174$]. Finally, while OVX females weigh more overall than intact females (OVX: 324.61 ± 23.00; intact: 239.69 ± 16.00 g) [$F_{1, 14} = 95.59$, $p < 0.001$], both groups exhibit similar changes in body weight across shock and extinction conditions (as a percentage of their body weight at the end of baseline) [condition: $F_{1, 14} = 21.60$, $p < 0.001$, group: $F_{1, 14} = 0.13$, $p = 0.721$].

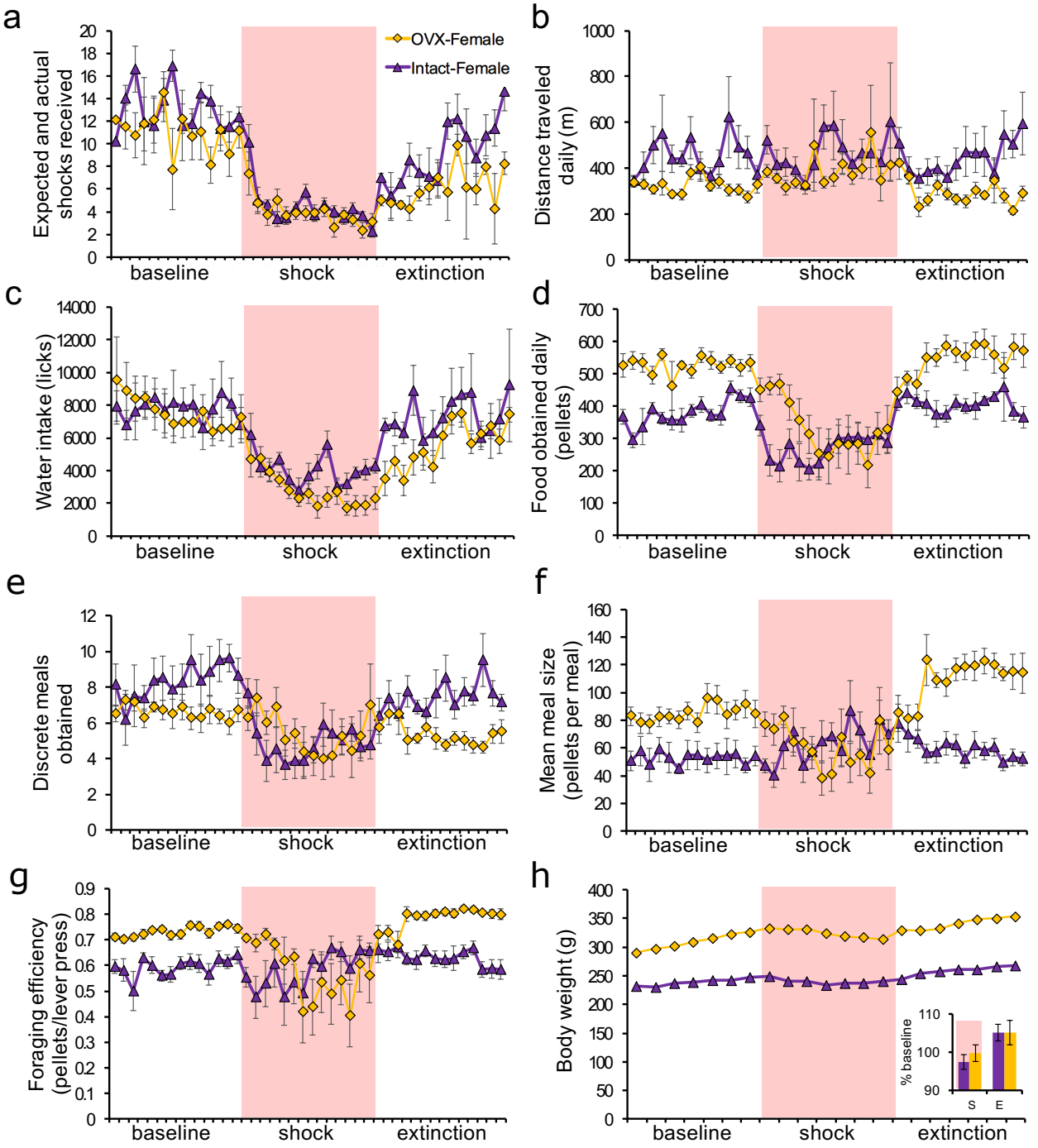

Figure 3.6. Influence of ovarian hormones on avoidance and foraging behaviors. Intact females (n = 8; *purple/triangles*) and OVX females (n = 8; *yellow/diamonds*). Mean daily (*a*) expected (during baseline, B, and extinction, E) and actual number of shocks received during the shock condition (S, red-shaded background), (*b*) activity (distance traveled, m), (*c*) water intake (number of licks), (*d*) food intake (pellets dispensed), (*e*) number of meals obtained (number of FR25 thresholds attained after reset), (*f*) meal size (pellets/meal), (*g*) foraging efficiency (pellets/lever press), and (*h*) body weight (g). Embedded figure in (*h*) shows mean body weight during S and E as a percentage of B. All error bars represent SEM.

To determine how estrous phase may influence foraging and defensive behaviors, estrous phase was determined daily in a subset of the intact females (n = 4) via vaginal lavage (see Methods). Behaviors were averaged across individual rats for each phase of estrous within each experimental condition. As suggested by the Fourier transforms, locomotor activity was significantly influence by phase of estrous, with increases in locomotion being associated primarily with the proestrus phase (Fig. 3.7a) [$F_{3, 33} = 15.55$, $p < 0.001$; P v D: $t_{35} = 3.86$, $p < 0.001$; P v E: $t_{35} = 3.51$, $p = 0.001$; P v M: $t_{35} = 3.23$, $p = 0.003$]. While there were no phase effects on the amount of food consumed (Fig. 3.7b) or the sizes of meals obtained (Fig. 3.7c) throughout the experiment, proestrus phase was associated with a significant reduction in foraging efficiency (Fig. 3.7d) [$F_{3, 33} = 3.35$, $p = 0.031$; P vs. D: $t_{37} = 2.43$, $p = 0.020$; P vs. E: $t_{37} = 3.12$, $p = 0.003$; P vs. M: $t_{37} = 3.02$, $p = 0.005$]. Additionally, proestrus was associated with more time spent in the foraging zone [$F_{3, 33} = 7.21$, $p = 0.001$; P v D: $t_{37} = 3.69$, $p = 0.001$; P v E: $t_{37} = 3.23$, $p = 0.003$; P v M: $t_{37} = 2.88$, $p = 0.007$], and subsequently, more shocks [$F_{3, 33} = 4.48$, $p = 0.010$; P v D: $t_{36} = 3.32$, $p = 0.002$; P v E: $t_{37} = 2.97$, $p = 0.005$; P v M: $t_{37} = 2.62$, $p = 0.013$]. Thus, estrous phase does appear to influence risky foraging decisions, with increased risk-taking associated with the proestrus phase, although this does not appear to be associated with an increase in gains (e.g., more food), but rather a decrease in foraging efficiency.

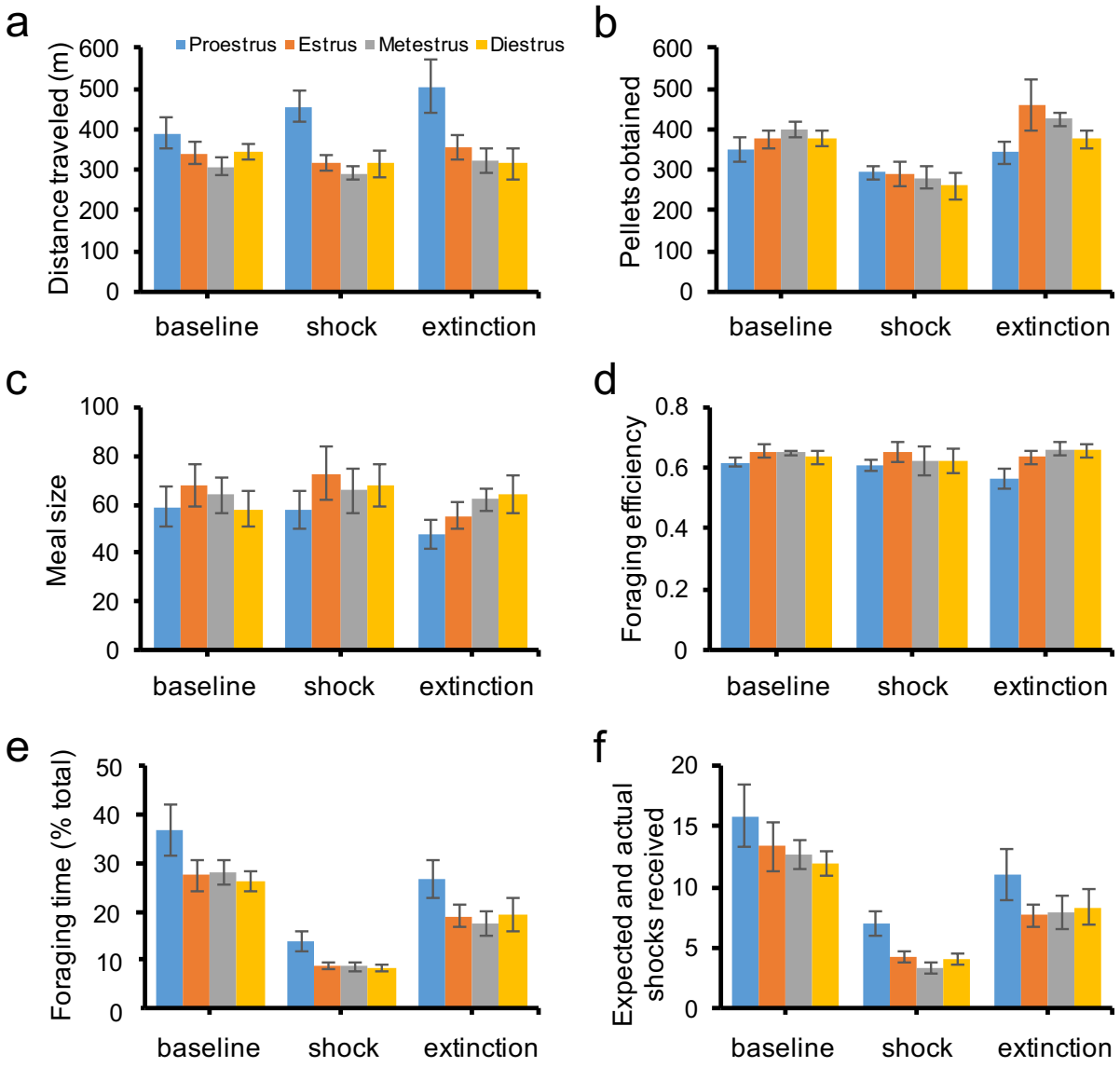

Figure 3.7. Influence of estrous phase on avoidance and foraging behaviors. (*a*) Distance traveled (m), (*b*) number of pellets obtained, (*c*) meal size (pellets/meal), (*d*) foraging efficiency (pellets/lever press), (*e*) time spent foraging (as a percentage of total time), and (*f*) expected (during baseline and extinction) and actual number of shocks (during shock) received during proestrus (*blue*), estrus (*orange*), metestrus (*grey*), and diestrus (*yellow*) phases averaged within each condition (baseline, shock, extinction).

After completing the closed economy experiment, intact males (n = 8) and females (n = 8) animals were transferred to standard rat cages in a common vivarium, maintained on a 12-h:12-h LD cycle, and food restriction began to gradually (~10 g of standard chow per day, over ~10 d) bring the rats down to 85% of their immediate post-closed economy body weight and motivate them toward obtaining food. Habituation to the nest area of the semi-naturalistic foraging field (Fig. 3.8) and shaping of foraging behavior occurred during this time (see Methods). One female rat did not engage in the task and instead consistently tried to jump out of the apparatus, and was therefore excluded from this analysis.

On baseline trials, across all test days, there were no significant differences between males and females in their total latency to retrieve the pellet and return to the nest (females: 12.62 ± 9.08; males: 17.21 ± 19.40 s; Fig. 3.9a, b) [$F_{1, 13}$ = 0.33, p = 5.77]. During the robot trials, female rats were able to retrieve pellets faster than males overall (females: 76.95 ± 31.60; males: 129.70 ± 49.88 s) [$F_{1, 13}$ = 6.44, p = 0.025]. Total retrieval latency decreased with repeated testing [$F_{3, 39}$ = 29.47, $p < 0.001$] and as the pellet distance from the nest decreased (L: 129.93 ± 39.09; M: 104.74 ± 52.28; S: 80.59 ± 56.39 s) [$F_{2, 26}$ = 28.70, $p < 0.001$], and there was a significant day × distance × sex interaction [$F_{6, 78}$ = 2.541, p = 0.027] corresponding to a faster decrease in retrieval latency on L trials over test days in females than males, according to a secondary analysis of distance-specific slopes (females: -56.94 ± 12.58; males: -23.43 ± 28.91 s/day) [L slope: $t_{9.82}$ = 2.97, p = 0.014]. Correspondingly, while there were no sex differences [$F_{1, 13}$ = 0.01, p = 0.946] or effects of test day [$F_{1, 13}$ = 0.26, p = 0.621] on baseline success rates (successful attempts divided by total attempts), females had higher success rates overall than males (females: 57.20 ± 17.33; males: 28.96 ± 27.41 %) [$F_{1, 13}$ = 6.30, p = 0.026] (Fig. 3.9c, d). As expected, success rates increased as a

function of test day [$F_{3, 39} = 28.54$, $p < 0.001$] and as pellet distance became closer to the nest (L: 26.56 ± 21.29; M: 41.99 ± 28.28; S: 57.87 ± 31.00 %) [$F_{1.37, 17.77} = 32.84$, $p < 0.001$].

There were no differences between sexes in terms of the latency to exit the nest area and begin foraging during baseline trials (Fig. 3.9e, f) [$F_{1, 13} = 0.93$, $p = 0.352$], nor were there any significant changes in exit latency during baseline trials across test days [$F_{3, 39} = 0.74$, $p = 0.537$]. However, during robot trials, there was a significant main effect of sex [$F_{1, 13} = 5.74$, $p = 0.032$], suggesting that females were quicker to begin foraging than males overall (females: 40.22 ± 23.95 s; males: 83.13 ± 45.14 s), as well as significant main effects of test day [$F_{3, 39} = 41.51$, $p < 0.001$], corresponding to a decrease in exit latency over time, and distance [$F_{2, 26} = 5.14$, p = 0.013]. Post-hoc analyses demonstrated that exit latency was greater for M trials (72.34 ± 49.60 s) relative to L trials (50.77 ± 29.92 s), with S trials (66.21 ± 50.57 s) falling in the middle [L vs. M: $t_{14} = 3.37$, $p = 0.005$; L vs. S: $t_{14} = 1.94$, $p = 0.072$; M vs. S: $t_{14} = 0.95$, $p = 0.360$].

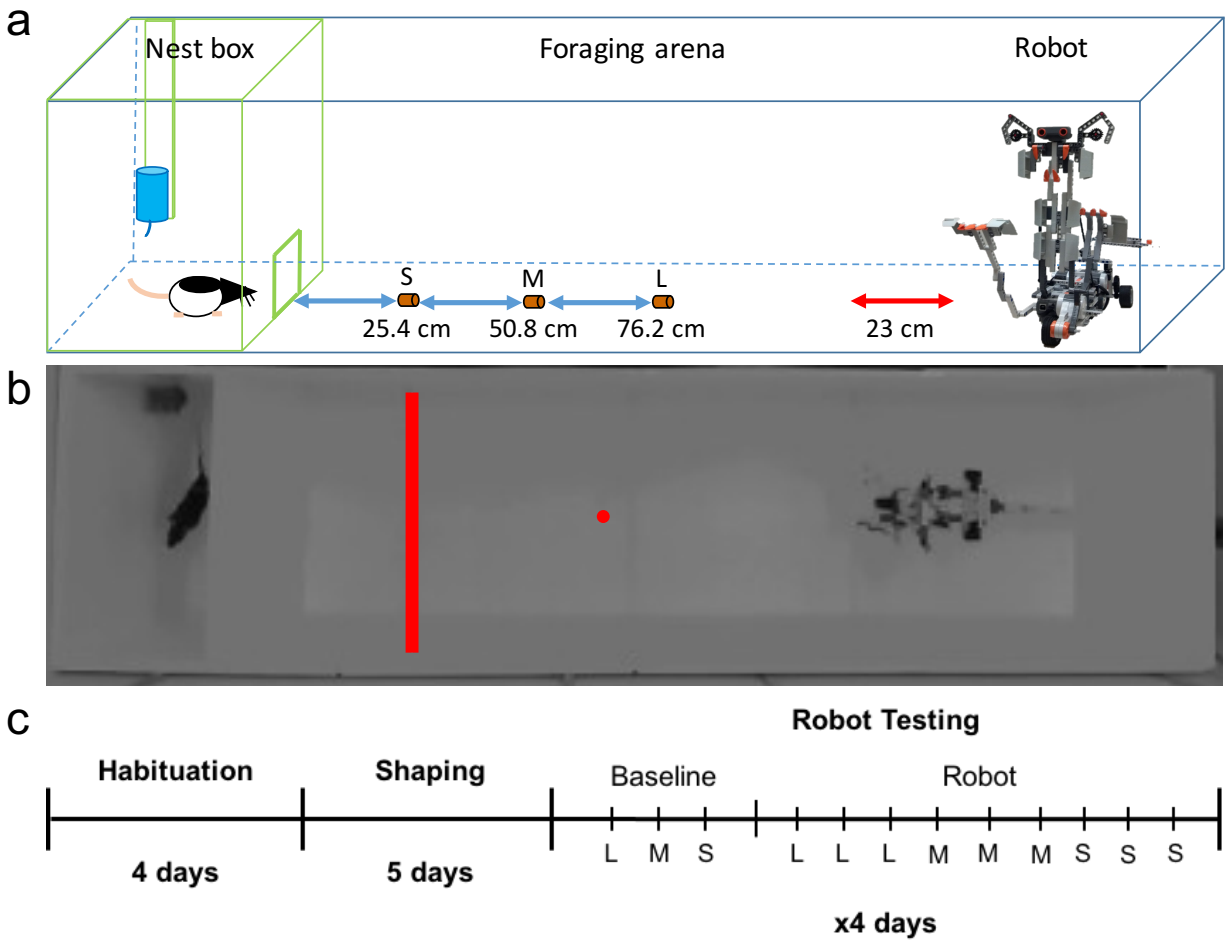

Figure 3.8. Experimental apparatus and design of the semi-naturalistic foraging task. (*a*) Diagram of the experimental apparatus, which consisted of a "nest" box equipped with a water bottle which opened to a "foraging" arena where pellets could be retrieved at three set distances (S, M, L) from the nest box. During robot trials, a robot shaped like a cat was placed at the opposite end of the nest box, and was programmed to surge forward 23 cm, swipe its "paw" down and back up, then reverse to its original position. (*b*) A photograph of the SNFF during a robot trial. The red dot indicates where food was placed at the L distance, and the red line indicates where the corresponding threshold to trigger the robot was located. (*c*) Timeline of the experiment. Habituation to the nest box occurred over 4 d, and shaping to retrieve the pellet and return to the nest box occurred over 5 d. Robot testing occurred over 4 consecutive days, and consisted of three baseline trials, one at each distance, and nine trials with the robot present, three at each distance.

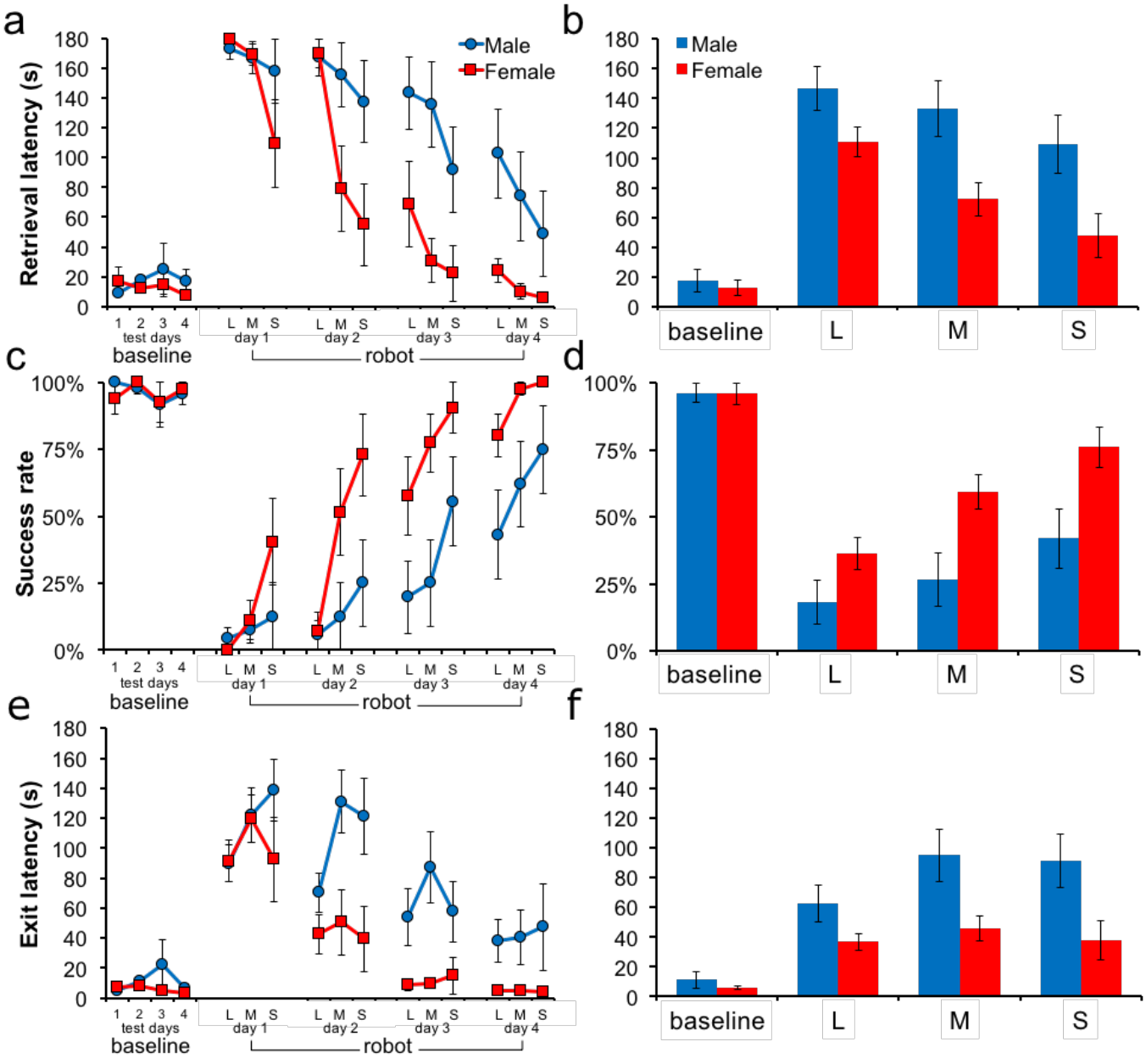

Figure 3.9. Sex differences in risky foraging behavior. (*a*) Total latency (s) to retrieve the pellet during each day of baseline (average across distances) and at each distance (L, M, S) during each day of robot testing, and during (*b*) baseline and each distance during robot testing averaged across days. (*c*) Success rate (successful attempts/total attempts to retrieve pellet) during each day of baseline and at each distance during each day of robot testing, and during (*d*) baseline and each distance during robot testing averaged across days. (*e*) Latency to exit nest box (s) during each day of baseline and at each distance during each day of robot testing, and during (*f*) baseline and each distance during robot testing averaged across test days. All error bars represent SEM.

Following the robot foraging test, the same rats underwent auditory tone-footshock fear conditioning (Fig. 3.10a). During training (Fig. 3.10b), there was a 1-min baseline period, followed by three 20-s presentations of the tone which co-terminated with a 1-s, 1.0 mA footshock, each followed by a 1-min inter-trial period. The levels of conditioned fear to both the tone and the conditioning context, as well as the rates of extinction of both, were assessed 24 h and 7 d following training. Training occurred during females' proestrus phase, whereas subsequent testing occurred independent of phase. There were no significant differences between females and males in their rate of acquisition [sex: $F_{1, 14} = 0.55$, $p = 0.472$, time: $F_{2, 28} = 44.52$, $p < 0.001$]. In the tone test 24 h and 7 d later (Fig. 3.10c), there were no statistically-significant differences between sexes in retention (average of first 4 min) of conditioned freezing or the extinction rate [24 h retention, $t_{14} = 0.75$, $p = 0.464$; 24 h extinction, time: $F_{3.29, 46.04} = 9.33$, $p < 0.001$, time × sex: $F_{3.29, 46.04} = 0.28$, $p = 0.855$; 7 d retention, $t_{14} = 0.24$, $p = 0.814$; 7 d extinction: $F_{1.62, 22.64} = 2.17$, $p = 0.145$, time × sex: $F_{1.62, 22.64} = 0.55$, $p = 0.798$]. Conditioned freezing to the conditioning context 24 h and 7 d later (Fig. 3.10d) was also similar between males and females [24 h retention, $t_{14} = 0.70$, $p = 0.499$; 24 h extinction, time: $F_{3.59, 50.29} = 3.26$, $p = 0.022$, time × sex: $F_{3.59, 60.29} = 0.56$, $p = 0.674$; 7 d retention, $t_{14} = 0.60$, $p = 0.560$; 7 d extinction: $F_{3.77, 52.75} = 1.98$, $p = 0.115$, time × sex: $F_{3.77, 52.75} = 1.04$, $p = 0.391$].

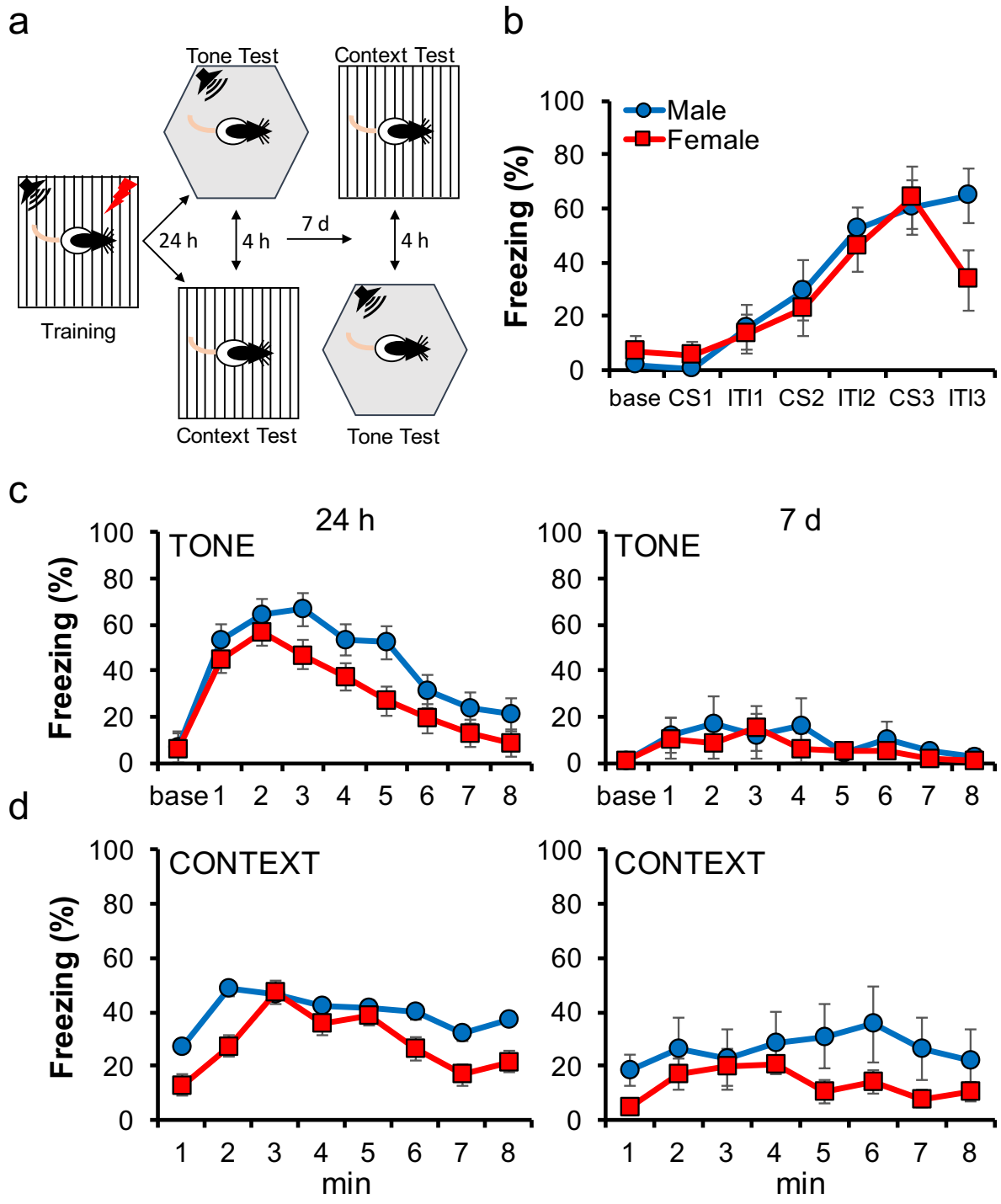

Figure 3.10. Sex differences in fear conditioning. (*a*) Design of fear conditioning tests. (*b*) Time spent freezing (percent of total time during block) during fear conditioning training. Base = baseline period, CS = conditioned stimulus presentation, ITI = inter-trial interval. (*c*) Time spent freezing (%) to the tone (tested in a distinct context) 24 h (left panel) and 7 d (right panel) after training. (*d*) Time spent freezing (%) to the original context 24 h (left) and 7 d (right) after training.

3.4 DISCUSSION

The closed economy experiment demonstrated that, while both males and females avoid unpredictable shock at the same rate, they appeared to do so be employing different foraging strategies. Whereas males increased their meal size and maintained their baseline foraging efficiency, thereby maintaining their baseline body weight, females instead sacrificed their metabolic needs in order to avoid shock, losing weight rather than risk encountering the threat. While the proestrus phase was associated with an increase in risky behavior, this led to more shocks received during the shock period and a decrease in foraging efficiency. Thus, the function of proestrus-related modulation of risky behavior does not appear to be directed toward foraging needs, and instead may be more related to reproductive behaviors, although this was not examined in the present study. The closed economy design is most similar to inhibitory avoidance paradigms in that shock was a diffuse, unpredictable threat associated with a specific place, and avoidance of shock was dependent on the inhibition of movement into the dangerous area. The finding that females extinguish slower than males in the closed economy is contrary to previous findings in inhibitory avoidance paradigms, which show that females are quicker to return to area associated with shock than males (Beatty et al., 2013; Heinsbroek, van Haaren, & van de Poll, 1988; Van Oyen, Van de Poll, & De Bruin, 1979). The duration of testing and repeated exposure to the shock contingencies in the closed economy experiment may explain this discrepancy, which would suggest that traditional inhibitory avoidance paradigms do not adequately address important temporal dynamics of fear and anxiety behavior.

Differences between intact and ovariectomized females were primarily limited to overall body weight and the amount of food consumed. OVX females obtained fewer discrete meals but obtained more pellets per meal than intact females, although this did not appear to be a strategy

employed to avoid shock as both groups received a similar number of shocks during the shock period and OVX females suffered a decrease in foraging efficiency during shock. Foraging efficiency did increase over baseline levels during the extinction condition for the OVX females, but this appeared to be more related to food motivation rather than a function of latent anxiety as extinction of shock avoidance occurred at similar rates between groups. Additionally, while the Fast-Fourier transforms detected a significant difference in infradian-range (3-5 d) periodicity between OVX and intact females, this was not reflected in the continuous wavelet analysis. It is possible that this more sensitive analysis capitalized on noise in the locomotor behavior as measured via the video tracking software, and more precise measurement of locomotor activity (for example, through the use of running wheels) is needed to reliably detect differences in infradian periodicity and its temporal dynamics. Potentially confounding factors such as the lack of a sham-ovariectomy control may be obscuring important differences. The fact that estrous phase was determined only once per day also limited the temporal resolution of the analysis. Nevertheless, these data suggest that the activational effects of ovarian hormones (as opposed to the organizational effects) play a relatively small role in determining naturalistic behavioral variability, and need not be actively controlled in experimental study if estrous-phase-specific phenomenon are not of interest, as others have suggested (Prendergast et al., 2014).

It may be tempting to conclude that females are more sensitive to threat than males based on the results of the closed economy experiment, however the results of the robot foraging test suggest caution in this interpretation. In the robot foraging test, female rats were able to retrieve the pellet at farther distances faster and in fewer attempts in the face of a potential threat – the cat-like robot – over time compared to male rats, indicating that they were less fearful of the robot than males. Like the closed economy experiment, approach and avoidance behaviors were placed

in conflict. However, unlike the closed economy experiment, the robot foraging test presented a more imminent, predictable threat, although the threat did not pose an actual danger. Thus, female rats may have learned that the threat was not an actual threat at a faster rate than males. This would suggest that females have greater threat *specificity,* rather than *sensitivity*, compared to males.

The results of the fear conditioning experiment correspond to previous research on sex differences in fear conditioning. When a discrete CS is used, males and freely-cycling females acquire conditioned freezing at similar rates, exhibit similar levels of fear in retention tests, and extinguish fear at similar rates (Milad et al., 2009) Females trained during their proestrus phase typically show reduced conditioned freezing during retention tests compared to males (Graham et al., 2009; Gupta et al., 2001; Maren et al., 1994), which was the case in this study, although the magnitude of that reduction was not statistically significant. Other studies have shown that females exhibit reduced retention, and faster extinction, of contextual fear memories compared to males (Maren et al., 1994). In the present study, females exhibit somewhat lower (albeit non-significant) levels of freezing during the context tests, and also froze less during the final inter-trial interval compared to the CS interval during training. Again, the ability to recognize the CS interval as dangerous and the ITI as safe may relate to greater threat specificity in females.

Previous research indicates that sexual dimorphisms exist in neural structures involved in fear, anxiety, and foraging decisions. Male rats have been shown to have larger medial amygdalae, which is involved in processing olfactory information and relaying it to diverse brain regions, such as the bed nucleus of the stria terminalis (BNST), ventral hippocampus, periaqueductal grey (PAG), ventral striatum, ventral tegmental area (VTA), dorsal raphe, and many hypothalamic regions, (Canteras, Simerly, & Swanson, 1995; Hines, Allen, & Gorski, 1992; Keshavarzi, Sullivan, Ianno, & Sah, 2014). The BNST, which is particularly important for anxiety and

defensive behaviors to uncertain threat-cues (Davis & Shi, 1999; Davis, Walker, Miles, & Grillon, 2009), has been shown to be sexually-dimorphic in rats and humans, being larger in males than in females (Allen & Gorski, 1990; Hines et al., 1992), Additionally, the medial preoptic nucleus of the hypothalamus has long been known to be a sexually dimorphic region (Gorski, Gordon, Shryne, & Southam, 1978), and it has been shown to have reciprocal connections with many of the same regions involved in fear and anxiety, such as the BNST, PAG, ventral hippocampus, and regions important for reward-motivated behavior such as the VTA, with efferent projections to striatal nuclei and the lateral habenula (Chiba & Murata, 1985; Simerly & Swanson, 1986). The ventrolateral part of the ventromedial hypothalamus has been shown to undergo estrous-phase-dependent plasticity, with marked increases in synapse density during the proestrus phase (Sá & Madeira, 2005), which carries reciprocal connections with the medial preoptic nucleus as well as strong projections to the PAG and superior colliculus and sparse projections to the nucleus accumbens, infralimbic and prelimbic prefrontal cortex, among others (Canteras, Simerly, & Swanson, 1994), and is likely involved in the proestrus-related modulation of behavior observed in the present research. Additionally, the hippocampus is likely to play an important role in the effects observed in the present research. It has been shown that the density of dendritic spines on pyramidal cells in the CA1 region of the hippocampus is greater in females during proestrus than in males (Dalla & Shors, 2009), although proestrus has also been associated with deficits in spatial learning and contextual fear conditioning (Markus & Zecevic, 2013). On the other hand, male rats exhibit greater LTP at performant path synapses in the dentate gyrus, which is correlated with greater conditioned contextual fear (Maren et al., 1994). Thus, there are many neural pathways by which sexually dimorphic fear, anxiety, and foraging behaviors can be coordinated, and while the

relative volumes or cell density of specific nuclei is not necessarily related to levels of fear and anxiety, they may contribute to functional differences in behavior.

It has been suggested before that fear and anxiety function differently in males and females (Dalla & Shors, 2009; Jolles et al., 2015), and the current study presents further evidence of that. The results of these studies indicate that males and females perform similarly when threat is highly predictable, but this is a situation that minimizes the functional difference between threat sensitivity and specificity. However, when threat is diffuse or uncertain, as in the case of the closed economy and robot foraging experiments, sexual dimorphism in fear-related behaviors become apparent, and females appear more capable of learning about threats – whether they present a real danger – and avoiding them if they are dangerous or ignoring them if they are not. Future studies should explicitly examine the threat sensitivity-specificity trade-off, and sex differences therein, by examining thresholds for defensive responses to uncertain/probabilistic threat cues.

Chapter 4. CONCLUSIONS

4.1 Summary of Results

The studies presented herein explored the functional aspects of fear and anxiety, how they can influence the temporal distribution of behavioral by serving as a non-photic entraining stimuli of circadian rhythms, how they differentially influence male and female rats' decisions to forage, and how female's reproductive cycle modulates such decisions.

In the first study, rats that received unpredictable shock during their normally-active phase of the day (i.e., in the dark) switched their foraging behavior toward the light phase of the day. The subsequent removal of photic input (i.e., the LD cycle) and shock led to a free-running rhythm in which the periodicity of the activity approximated the periodicity of activity during the nocturnal shock condition, indicating the cyclic threat had entrained rats' circadian rhythms. Furthermore, this was dependent on both the amygdala and suprachiasmatic nucleus, suggesting the two interact to modulate the circadian output of behavior. Determining the locus of these interactions and the cellular and molecular mechanisms underlying them present a promising avenue for future research. Circadian oscillations in clock proteins (i.e., *Per2, Bmal1,* and *dbp*) have been shown to occur in the amygdala (BLA and CEA), bed nucleus of the stria terminalis, as well as the dentate gyrus of the hippocampus, in addition to the well-known oscillations of the SCN (Harbour, Weigl, Robinson, & Amir, 2014; Lamont et al., 2005; Segall et al., 2006). Thus, the mechanistic basis for behavioral adaptation to cyclic threat may depend upon a complex and distributed network of oscillators in the brain.

The second study examined sex differences in the functional roles of fear and anxiety, demonstrating that males and females resolve approach-avoidance conflict using different strategies. In the closed economy experiment, wherein shock was unpredictable and a necessary

risk to obtain food and water, males decrease meal frequency, but increase meal size, and maintain a stable body weight (although with arrested growth). On the other hand, females decrease their meal frequency without the corresponding increase in meal size, and subsequently lose weight. It appears, then, that females are more willing to sacrifice their metabolic needs to avoid threat. However, when the potential threat in an approach-avoid conflict situation is not actually dangerous, as was the case in the robot foraging task, females learn to acquire food pellets faster, and have more success, than their male counterparts. When the threat is highly predictable, and isolated from appetitive motivation, as in the case of tone-shock fear conditioning, females and males performed similar. Together, these experiments show that fear and anxiety serve different functions for males and females, and that females may have a greater ability to learn about threats than males. Future research on sex differences in fear and anxiety may benefit from an explicit examination of sex differences in the trade-offs between threat *sensitivity* and *specificity.* A "signal detection" approach may be valuable, wherein the rates of responding accurately to probabilistic cues of threat (i.e., responding defensively when a threat is actually present) are compared to rates of responding inaccurately (i.e., responding defensively when a threat is not present).

Finally, the influence of the estrous cycle and the role of ovarian hormones in the closed economy experiment was examined. The estrous cycle has clear effects on the temporal distribution of locomotor activity, and it was found that spikes in activity and risky behavior were associated with the proestrus phase, although this did not correspond to increased gains toward metabolic needs. However, few differences were found between intact females and females that were ovariectomized in adulthood, suggesting that ovarian hormones do not have a large effect on the day-to-day behavior of female rats. However, ovarian hormones are critical during early

development, and such organizational effects and their functional consequences for fear, anxiety, and foraging behavior should be examined in ethobehavioral studies in the future.

While the longitudinal, closed-economy approach utilized throughout this dissertation allows for the simulation of approach-avoidance conflict in a dynamic environment, it is not without limitations. Foremost, the lengthy duration of experiments and the amount of data collected is very resource intensive. While within-subjects designs can help mitigate issues concerning smaller sample sizes, obtaining large sample sizes is costly in terms of time so this approach may not be as beneficial when investigating phenomenon with smaller effect sizes. Furthermore, the chambers used in these studies were only slightly larger than a standard home-cage, and thus restricted the degree to which spatial factors could influence behavior. Furthermore, animals were housed individually, whereas rats naturally live in social environments, so future research would likely benefit from simulating such conditions in ethobehavioral studies. However, the studies discussed in this dissertation demonstrate the utility of the closed economy approach, particularly for understanding the temporal dynamics of behavior.

4.2 Concluding Remarks

While significant progress has been made through the use of fear conditioning paradigms, the evolutionarily conserved roles of fear in guiding and shaping foraging behavior have been overlooked in contemporary fear research. Fear conditioning studies in humans and animals focus on specific responses for brief periods of sampling, whereas ethobehavioral fear studies allow the tracking of relatively integrated behavioral responses to fear-evoking experiences. Furthermore, innate fear and its mechanisms are far less researched than acquired fear, but are at least equally important to survival. Both innate and learning systems interact at a fundamental level and the extent to which the neural processes underlying them are separable remains unclear. Ultimately,

understanding their functional relationship will provide a more comprehensive understanding of the brain's fear system than trying to model them as distinct systems. State-of-the-art techniques for manipulating neurons and circuits provide neuroscientists with powerful means of understanding the brain, and coupling these with experimental methodology that captures naturalistic variability in behavior and the situations in which it is expressed will help researchers model the functional relationships between the organism and its environment. Future research should also examine individual differences within and across fear paradigms to better understand not only trait-level variability but also the correspondence (or lack thereof) between different measures of fear. Selective breeding for anxious (Landgraf et al., 2007) or depressive (Overstreet, Friedman, Mathé, & Yadid, 2005) traits are useful toward these ends, but understanding natural trait variability (e.g., Bush, Sotres-Bayon, & LeDoux, 2007; Galatzer-Levy, Bonanno, Bush, & LeDoux, 2013; Hall, 1936; Hartley, Fischl, & Phelps, 2011; Mobbs et al., 2010) will improve functional models of fear circuitry. Different species, and individuals within, are likely to have to have different functional characteristics that are dependent on the ecological and evolutionary history of each species. Thus, understanding the functional properties of fear and anxiety behavior in rodents and humans, within their respective eco-evolutionary contexts, and mapping the divergence and convergence phylogenetically, will help researchers understand how to scale findings from one species to another. These approaches will further help bridge the brain and behavioral sciences with ecological science to bring a unified understanding of the widespread biological functions of fear. Consequently, ethological studies of fear may inform translational and clinical approaches to treating anxiety-related disorders in humans, which are linked to alterations in behaviors tied to risk-assessment and decision-making – aspects that cannot be directly investigated in traditional behavioral assays.

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