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Pharmacological Dissociation of Anxiety Model in the Chick Separation Stress Paradigm

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PHARMACOLOGICAL DISSOCIATION OF ANXIETY MODEL IN THE CHICK SEPARATION STRESS PARADIGM

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To my parents, Mr. and Mrs. Thomas H. Wicks, and grandparents, Dr. and Mrs. Robert J. Eustice, who have stood by me with unending love and support throughout my college career and multiple "adventures." Also, to Dr. Kenneth Sufka, without whose mentorship none of this would have been possible.

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Abstract:

The chick separation stress paradigm has been validated as an anxiolytic screening assay. However, whether the paradigm better models Panic Disorder (PD) or Generalized Anxiety Disorder (GAD) is unknown. To pharmacologically dissociate the chick separation stress paradigm as a model of PD or GAD, subjects were administered drug probes that were either: 1) only effective in the treatment of PD (phenelzine 3.125-25.0 mg/kg), 2) effective in the treatment of both PD and GAD (alprazolam 0.065-0.5 mg/kg; clonidine 0.1-0.25 mg/kg; imipramine 1.0-15.0mg/kg), 3) only effective in the treatment of GAD (buspirone 2.5-10.0 mg/kg; trazodone 0.1-3.0 mg/kg), or 4) capable of exacerbating symptoms of PD in humans (yohimbine 0.1-3.0 mg/kg). At 7-days post-hatch, chicks received either vehicle or drug probe intramuscular 15 min prior to social separation under a Mirror (low-stress) or No-Mirror (high-stress) condition for a 180 sec observation period. Dependent measures were distress vocalizations to index separation stress and sleep onset latency to index sedation. Phenelzine, alprazolam, imipramine, and clonidine showed significant anxiolytic effects at doses without significant sedation in the model, while buspirone and trazodone did not show significant anxiolytic effects. Paradoxically, yohimbine produced modest anxiolytic effects. These results suggest the chick separation stress paradigm better models PD than GAD as an anxiolytic screen.

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LIST OF ABBREVIATIONS

CSSP	Chick Separation Stress Paradigm
DMSO	Dimethylsulfoxide
DVocs	Distress vocalizations
GAD	Generalized anxiety disorder
LSD	Least significant difference
PD	Panic disorder
SEM	Standard error of the mean
SOL	Sleep onset latency

INTRODUCTION

The US philosopher William Barrett once wrote, "Anxiety is not fear, being afraid of this or that definite object, but the uncanny feeling of being afraid of nothing at all. It is precisely Nothingness that makes itself present and felt as the object of our dread." Everyone will experience symptoms of anxiety at some point in their lives. These symptoms become a problem, however, once they become repetitive, intrusive and associated with inappropriate thoughts or actions (Pincus 1995). There are 40 million (18.1% of adults within a given year) adults who suffer from an anxiety disorder, which makes it the most prevalent mental disorder in the US (Kessler 2005). These disorders are largely treatable with current medications. However, a problem arises when trying to find novel drugs which reduce anxiety effectively without the side-effects and dependency issues of the current medications (Willner 1991). Essentially, this problem hinges on the much greater dilemma of dealing with animal models used in the screening of novel drugs.

Animals have long been utilized as research subjects in the hopes of modeling some aspect of human behavior. However, the term model is hard to define since it is extremely difficult to replicate a particular human behavior in another species. One all-encompassing definition of "animal model" does not exist. It is, therefore, more precise to categorize animal models into three specific classifications, each with its own definition (Willner 1991).

The first classification is *behavioral bioassays*. These assays serve to model a total physiological action. The entire animal serves as a measuring device to determine

the functional state of a physiological system, similar to how the same measurement might be made in an isolated tissue or test tube. In large part, behavioral bioassays are used to study mechanisms responsible for changes in brain function, typically those resulting from chronic drug administration or brain lesions (Willner 1991). Therefore, although behavioral bioassays are valuable tools in the study of anxiety, the research of this laboratory is primarily focused on the second two categories.

The second classification is animal models used as a *screening test,* which serves to model a drug action. In short, "The search for novel psychotropic agents is based upon the action of known drugs, which serve as reference points against which to compare the performance of new candidates" (Willner 1991). In order to determine a novel drug compound, two forms of screening procedures exist. The first is to identify compounds likely to have a specific type of clinical action. This allows for the identification of clinically effective drugs that vary widely in the chemical structure. The second strategy for screening tests is to identify specific biochemical actions as targets for drug development. One drawback to this procedure is that it can only be accomplished once the specific mode of action of existing compounds has been established, which in many cases is not a feasible option. Also, the second strategy has the major disadvantage of inhibiting the discovery of chemically novel modes of treatment (Willner 1991). According to Willner, "Irrespective of the manner in which they are constructed, screening tests are subject primarily to one very simple requirement: the test should predict accurately the desired activity, it should accept drugs that are effective and reject those that are ineffective."

Also relevant to this study is the third category of animal models, those used as a model of human behavior or *simulation.* Although this category includes models of all forms of animal behavior, this study will focus on simulations of abnormal behavior. The purpose of these models is to simulate a symptom, or group of symptoms, of a particular disorder. Methods of constructing the simulation vary greatly; they include brain damage, selective breeding, selection of extreme individuals, and the application of a variety of factors assumed to be implicated in the etiology of mental disorders such as stressors, social isolation, or aging. The object of these manipulations is to produce a behavioral state that can be used as a tool to study aspects of the disorder being modeled (Willner 1991).

Regardless of its classification, once a particular animal model is selected for a disorder, it is necessary to assess its validity. The validity is the degree of confidence that can be placed in the data generated from the use of the model. Three validity measures are required to fully assess a model. The predictive validity is whether the model is able to discriminate efficiently between those agents that are clinically effective and those that are not. Face validity refers to a strong phenomenological association between the model and the disorder it simulates. The model should resemble the disorder in etiology, symptomology, treatment, and physiological basis. Since face validity is not always achievable, a more important measure is construct validity, the final measure of validity. Construct validity basically refers to a model being able to measure a fundamental theoretical concept. All valid models are required to have both predictive and construct validity (Willner 1991).

Within the subject of anxiety, four main categories of animal models exist. The first category is that of conditioned avoidance responses, which includes the four-plate test, passive and active avoidance, and the Geller-Seifter conflict test. These tests mainly involve mild aversive stimuli to form conditioned responses. For example, many procedures "use the aversive effects of footshock to condition the inhibition of normally ongoing behavior; it is hypothesized that the inhibition of behavior in anticipation of punishment is mediated by the hypothetical construct 'fear' or 'anxiety', and that such inhibition should be reduced by anxiolytic treatments" (Willner 1991). A second category of animal model is the drug-induced discriminative states. In many ways, these models represent the closest approximation to a bioassay model that can be found in anxiolytic psychopharmacology. A third model is that of brain stimulation. This involves the stimulation of many different sites that can produce behavioral indications of fear in animals such as the amygdala, the locus coeruleus, or the median raphe nucleus. The final category of animal models, and the category of particular interest in this study, is the unconditioned response tests. Many of the unconditioned response tests involve exploratory locomotion in a novel environment as a simulation of an anxiety-like state. However, this category also includes observation of social interactions or aggression and how these actions relate to anxiety (Willner 1991).

One particular unconditioned response is isolation-induced vocalizations that are emitted by infants of various species following separation from their mother or conspecifics. Several studies have identified these responses in guinea-pigs, chicks, and in rats and mice, in which case ultrasonic vocalizations are recorded (Borsini, 2002). In particular, the psychopharmacology laboratory at the University of Mississippi has

worked with chicks and developed the Chick Separation Stress Paradigm (CSSP) as a model of anxiety. In particular, over the past decade, this lab has developed the CSSP as an anxiolytic screening model. This paradigm is based upon the fact that, when separated from their conspecifics, young domestic fowl exhibit a stress response that is characterized by distress vocalizations (DVocs: Gallup and Saurez, 1980; Panksepp et al. 1980; Sufka and Weed 1994). Through several studies, this laboratory has developed a method to use DVocs to index anxiety (Watson and Sufka, 1996; Feltenstein et al. 2004; Feltenstein et al. 2003; Feltenstein et al. 2002). The model possesses construct validity as an anxiety model in that separation stress reliably increases corticosterone levels (Feltenstein et al. 2002), a neuroendocrine marker of many stress responses. Additionally, the model possesses predictive validity through the successful detection of diverse classes of anxiolytics (i.e., meprobamate, pentobarbital, chlordiazepoxide, imipramine and clonidine; Feltenstein et al. 2004). Furthermore, the model is insensitive to a wide range of non-anxiolytic compounds (i.e. amphetamine, scopolamine, caffeine, chlorpromazine, and haloperidol; Feltenstein et al. 2004).

One problem with the CSSP does arise in the form of the atypical anxiolytic buspirone. Buspirone is a clinically efficacious anxiolytic in humans (Ninan and Muntasser, 2004). However, in the CSSP, as well as in several other models of anxiety, buspirone was shown to be ineffective as an anxiolytic (Feltenstein et al. 2004; Stephens and Andrews 1991). Although this seems a limitation of the paradigm, the buspirone effect may be empirical data that further defines how to classify the model.

Anxiety disorders comprise a large number of disorders where the primary feature is abnormal or inappropriate anxiety. Two primary anxiety disorders are generalized

anxiety disorder (GAD) and panic disorder (PD). The etiology of GAD is to have excessive anxiety and worry, for more days than not, that are out of proportion to the likelihood or impact of feared events. Symptoms include heart palpitations, muscle tension, exaggerated startle response, and insomnia. On the other hand, with PD the patient has recurrent unexpected panic attacks which are followed by a month or more of persistent concern about having additional attacks. Panic attacks are sudden attacks of intense fear or discomfort with heart palpitations, sensations of shortness of breath, and dizziness (Pincus 1995). There has been precedence to qualifying animal models based on disorder. In a recent international conference on the PD/GAD differentiation, "...most participants agreed that generalized anxiety and panic disorder are different on the basis of clinical manifestations, drug response and animal model. ...It is also common knowledge that existing animal models generate different types of fear/anxiety." (Andreatini et al. 2001). Of particular interest in the current study is to understand the differential efficacy of buspirone in each of these disorders. Studies have shown buspirone to be limited to GAD and ineffective for panic disorder and other anxiety disorders (Ninan and Muntasser, 2004; Bandelow 2002; Sheeham et al. 1990).

The ineffectiveness of buspirone in the CSSP is therefore a key aspect that points towards the paradigm better modeling PD. Additionally, the CSSP appears to have face validity in that it bears many similarities to situationally-bound or cued PD since the symptom onset is rapid, intense and brief with clear etiological origins. It is, therefore, hypothesized that the CSSP is a better screening assay for anti-panic medications than for anti-anxiety medications in general.

In order to dissociate the specific anxiety disorder modeled in the CSSP, the following strategy was derived. If the CSSP is a model of PD, then it should only be sensitive to pharmacological compounds efficacious for PD and insensitive to drugs only effective for other types of anxiety disorders (e.g., GAD). Therefore, seven drug probes were chosen as agents to help with this differentiation. Phenelzine was first chosen, since it is only effective in the treatment of PD (Bandelow et al. 2002; Davidson 2004). It was then decided to select three additional drugs that are effective in PD: alprazolam, clonidine, and imipramine (Bandelow et al. 2002; Hoehn-Saric et al. 1981). These three medications, while shown to be more highly effective in the treatment of PD, are also effective in the treatment of GAD. For drugs exclusively effective in the treatment of GAD, trazodone and buspirone were chosen, since both are effective GAD treatments that have no effect on PD (Bandelow et al. 2002; Charney et al. 1986). The final drug probe chosen was yohimbine which has been shown to exacerbate symptoms of PD in humans (Charney et al. 1992). It is expected, therefore, to increase DVocs if the CSSP models PD.

MATERIALS AND METHODS

Subjects

Cockerels (*Gallus gallus*; strain W36; Cal-Maine Foods, Mendenhall, MS, USA) were obtained 1-day post-hatch and were housed in stainless-steel cages (34 x 57 x 40 cm) at a population density of 12-14 chicks per cage. Food (Purina Start and Grow, St. Louis, MO, USA) and water were available ad libitum through 1-quart gravity-fed feeders and water containers. Room temperature was maintained at 29∀1EC, and overhead fluorescent illumination was maintained on a 12h light-dark cycle. Daily maintenance was conducted during the first quarter of the light cycle.

Apparatus

The six-unit test apparatus contained Plexiglas viewing chambers (25 x 25 x 22 cm) situated in sound-attenuating enclosures. Each unit was illuminated by a 25-W light bulb and ventilated by an 8-cm-diameter rotary fan (Commonwealth Model FP-108AX S1). Miniature video cameras (SuperCircuit Model PC47MC) allowed for animal observation during tests. DVocs were recorded by microphones (Lafayette Instruments Model 3-675-001) mounted at the ceiling of the Plexiglas chamber and connected to digital sound-activating relays (Lafayette Instruments Model 63040A; settings: 75% sensitivity and 0.10 s delay) that triggered electromechanical counters (Lafayette Instruments Model 58004).

Procedure

Experiments were conducted at 7-days post-hatch. Groups formed a single factorial design with a hanging control that included two vehicle-control groups in which chicks were tested in isolation, with or without two mirrors (20 x 20 cm) positioned along the outside of the Plexiglas side walls, and four drug dose conditions tested under the No-Mirror condition.

The drug probes included one compound clinically effective for PD but not GAD (phenelzine: 3.125, 6.25, 12.5, 25.0 mg/kg), three compounds predominantly used for PD but also show clinical efficacy for GAD (alprazolam: 0.065, 0.125, 0.25, 0.5 mg/kg; imipramine: 1.0, 3.0, 10.0, 15.0 mg/kg; clonidine: 0.1, 0.15, 0.2, 0.25 mg/kg), two compound clinically effective for GAD but not PD (buspirone: 2.5, 5.0, 7.5, 10.0 mg/kg; trazodone: 0.1, 0.3, 1.0, 3.0 mg/kg) and one compound that exacerbates the symptoms of PD but without effect on GAD (yohimbine: 0.1, 0.3, 1.0, 3.0 mg/kg). DMSO served as the vehicle for trazodone and yohimbine. Alprazolam was dissolved in a solution that consisted of 20.0ml propylene glycol, 5.0ml ethyl alcohol, 0.75ml benzyl alcohol, 2.44g sodium benzoate, and 60.0mg benzoic acid in 24.0ml of distilled water. All other probes were dissolved in 0.9% physiological saline. Drug doses were selected based on published work on chicks from this laboratory or from the literature in rodent models of anxiety (Feltenstein et al. 2004; Feltenstein & Sufka 2005; Graeff et al. 1998; Mason et al. 1987; Watson et al. 1999).

Vehicle and drug injections were administered IM 15 min before tests. The stress manipulation involved placing a chick in the observation chamber either in a Mirror (low-stress) or No-Mirror (high-stress) condition for a 180 sec test period. Dependent

measures collected during the test session were 1) distress vocalizations (DVocs) and 2) sleep onset latency (SOL), defined as the latency to adopt a posture in which the chick's head is drooping and its eyes are closed. Animals were returned to their home cage following tests. These procedures were approved by the University of Mississippi IACUC (Protocol # 05-007) and were conducted in accordance with the principles of laboratory animal care as detailed in the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No.85-23, revised 1985).

Data were analyzed using *t*-tests and analysis of variance (ANOVA). Post hoc analyses were conducted using Fisher's LSD for DVocs and Mann-Whitney U for SOL. Percent effect scores were derived using the following formulas: Percent anxiolytic effect = [1- (mean DVocs for Drug Dose / mean DVocs for Vehicle/No-Mirror group)] x 100; Percent sedative effect = [1 - (Mean SOL / 180)] x 100.

RESULTS:

Phenelzine:

The effects of isolation and phenelzine on DVocs are summarized in Figure 1. Social isolation led to a clear increase in DVocs. Phenelzine, in turn, dose-dependently attenuated this stress effect. Consistent with these observations, an analysis of the data demonstrated that the DVocs for the iso-vehicle group were significantly higher than the mirror-vehicle group, $t(33) = 4.5$, $p < 0.0001$. An ANOVA of DVocs across the iso-phenelzine groups revealed a significant treatment effect, $F(4,76) = 3.35$, $p < 0.05$. Post-hoc analyses demonstrated that the mean DVocs for the 25mg/kg phenelzine group were significantly lower than the mean DVocs for vehicle-isolated birds, $p < 0.01$. No other relevant comparisons reached statistical significance.

Figure 1: The effects of phenelzine on isolation-induced distress vocalizations in a 3-min test session. Phenelzine was delivered IM 15 minutes prior to testing. Values represent mean ± Standard Error of the Mean (S.E.M.), sample sizes N= 16-18. The gray bar represents the social test condition and the black bars represent the isolated test condition.
*** Indicates a significant isolation stress effect. ** Indicates significant attenuation of the stress effect, all P values < 0.05.**

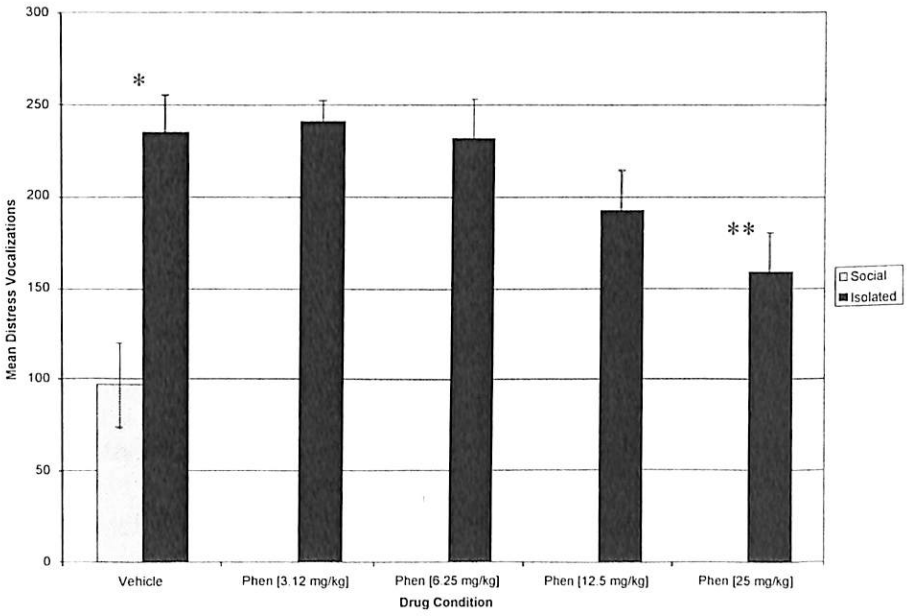
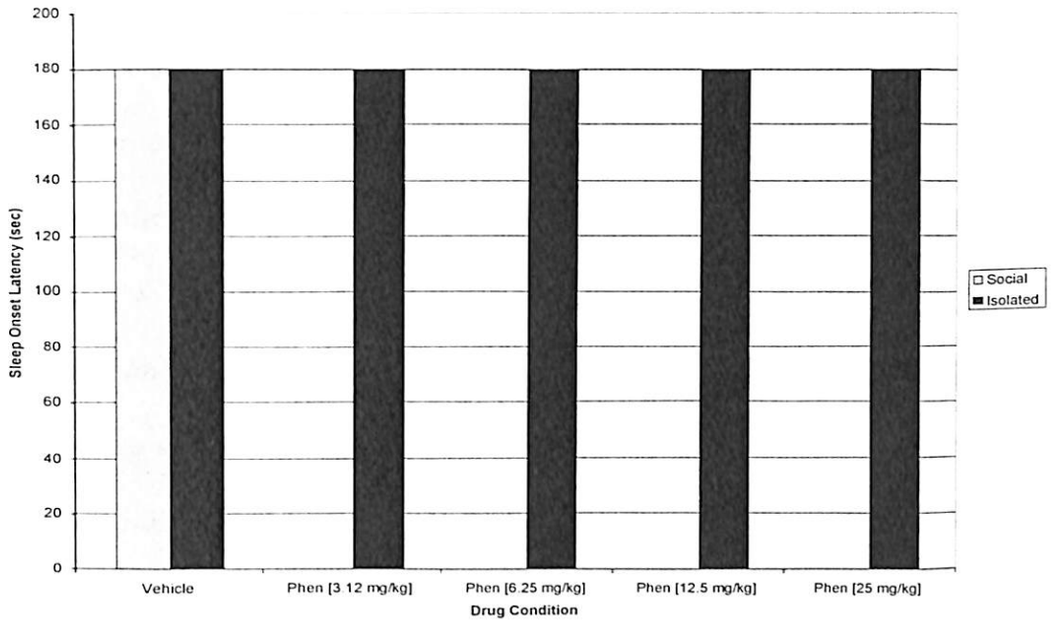


Figure 2: The effects of isolation and phenelzine on sleep onset latency in a 3-min test session. Phenelzine was administered IM 15 min prior to testing. Values represent mean ± S.E.M., sample sizes N = 16-18. The gray bar represents the social test condition and the black bars represent the isolated test condition.



The effects of isolation and phenelzine on SOL are summarized in Figure 2. Neither the isolation manipulation nor phenelzine probes affected SOL. An analysis of these data using a Kruskal-Wallis one-way ANOVA for non-parametric data failed to reveal a statistically significant treatment effect.

Alprazolam:

The effects of isolation and alprazolam on DVocs are summarized in Figure 3. Social isolation led to a clear increase in DVocs. Alprazolam, in turn, dose-dependently attenuated this stress effect. Consistent with these observations, an analysis of these data demonstrated that the DVocs for the iso-vehicle group were significantly higher than the mirror-vehicle group, $t(32) = 11.7$, $p < 0.0001$. An ANOVA of DVocs across the iso-alprazolam groups revealed a significant treatment effect, $F(4,81) = 51.0$, $p < 0.0001$.

Post-hoc analyses demonstrated that the mean DVocs for the 0.5mg/kg, 0.25mg/kg, 0.125mg/kg, and the 0.065mg/kg alprazolam groups were significantly lower than the mean DVocs for vehicle-isolated birds, $p < 0.0001$. No other relevant comparisons reached statistical significance.

The effects of isolation and alprazolam on SOL are summarized in Figure 4. The isolation manipulation failed to affect SOL. An analysis of these data using a Kruskal-Wallis one-way ANOVA revealed a significant sedative effect $H(5) = 77.20$, $p<0.0001$. Post-hoc analyses demonstrated that the mean SOL for the 0.5mg/kg, 0.25mg/kg, and the 0.125mg/kg alprazolam groups were significantly lower than the mean SOL for the vehicle-isolated birds $p<0.0001$. No other relevant comparisons reached statistical significance.

Figure 3: The effects of alprazolam on isolation-induced distress vocalizations in a 3-min test session. Alprazolam was delivered IM 15 minutes prior to testing. Values represent mean ± S.E.M., sample sizes 15-18. The gray bar represents the social testing condition and the black bars represent the isolated test condition.
*** Indicates a significant isolation stress effect. ** Indicates significant attenuation of the stress effect, all P values < 0.0001.**

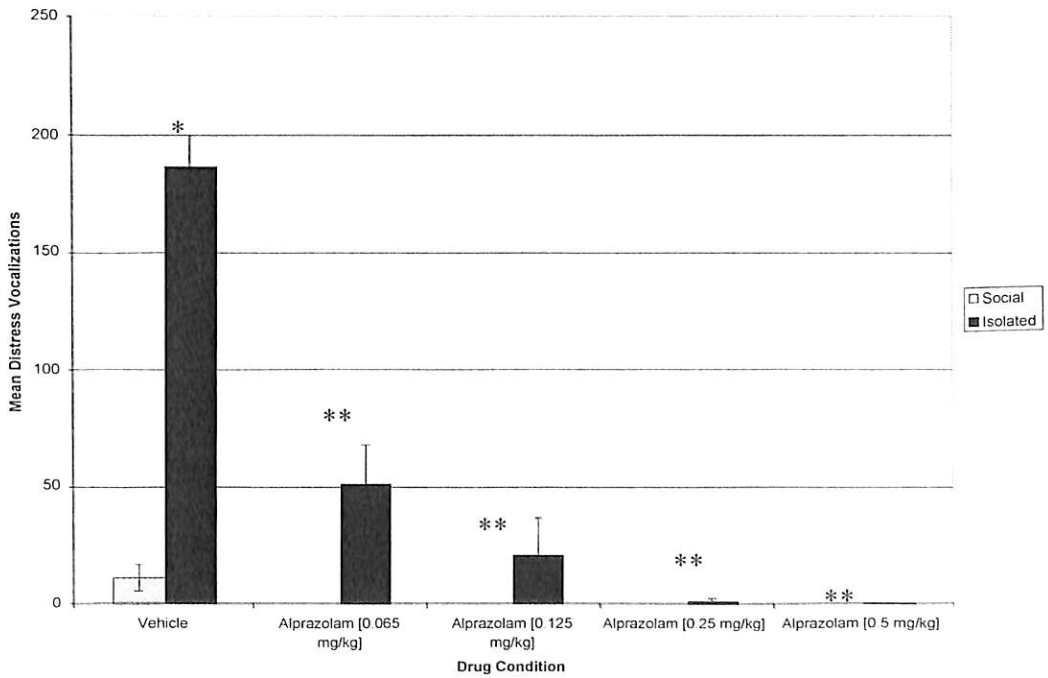
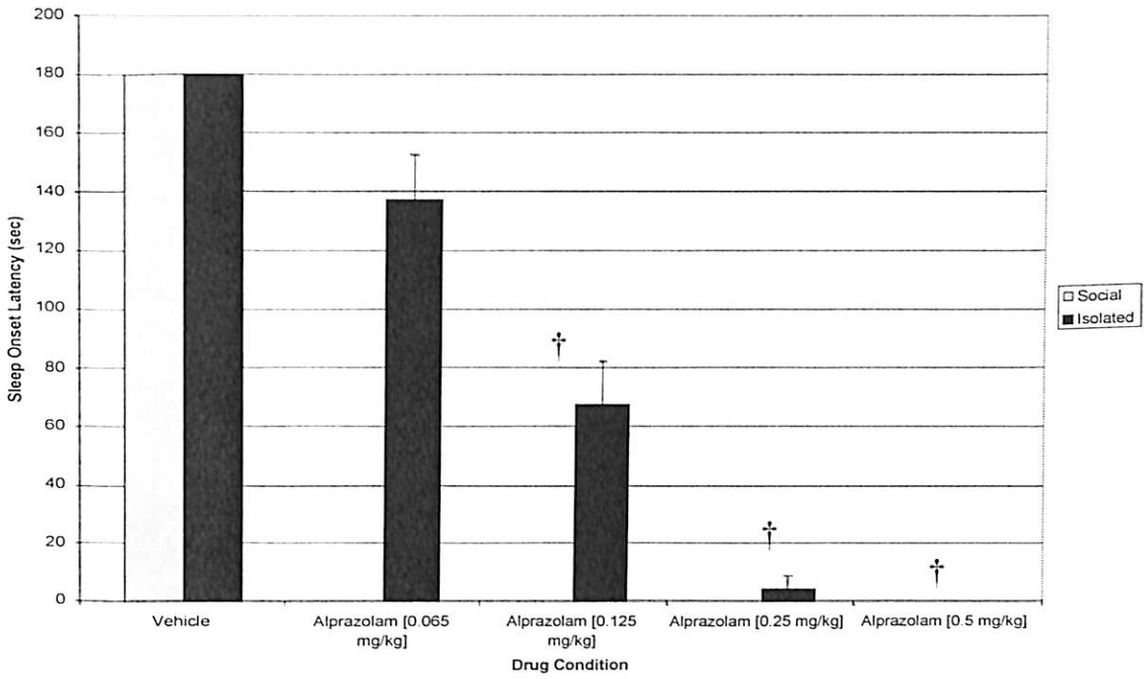


Figure 4: The effects of isolation and four doses of alprazolam on sleep onset latency in a 3-minute test session. Alprazolam was delivered IM 15 minutes prior to testing. Values represent mean ± S.E.M., sample sizes 15-18. The gray bar represents the social test condition and the black bars represent the isolated test condition.
† Indicates a significant decrease in sleep onset latency, all P values <0.0001.



Imipramine:

The effects of isolation and imipramine on DVocs are summarized in Figure 5. Social isolation led to a clear increase in DVocs. Imipramine, in turn, dose-dependently attenuated this stress effect. Consistent with these observations, an analysis of these data demonstrated that the DVocs for the iso-vehicle group were significantly higher than the mirror group, $t(30) = 5.78$, $p < 0.0001$. An ANOVA of DVocs across the iso-imipramine groups revealed a significant treatment effect, $F(4,85) = 28.3$, $p < 0.0001$. Post-hoc analyses demonstrated that the mean DVocs for the 10.0mg/kg and 15.0mg/kg imipramine groups was significantly lower than the mean DVocs for vehicle-isolated birds, $p < 0.001$. No other relevant comparisons reached statistical significance.

Figure 5: The effects of imipramine on isolation-induced distress vocalizations in a 3-min test session. Imipramine was delivered IM 15 minutes prior to testing. Values represent mean ± S.E.M., sample size N = 14-18. The gray bar represents the social testing condition and the black bars represent the isolated test condition.
*** Indicates a significant isolation stress effect. ** Indicates significant attenuation of the stress effect, all P values < 0.0001.**

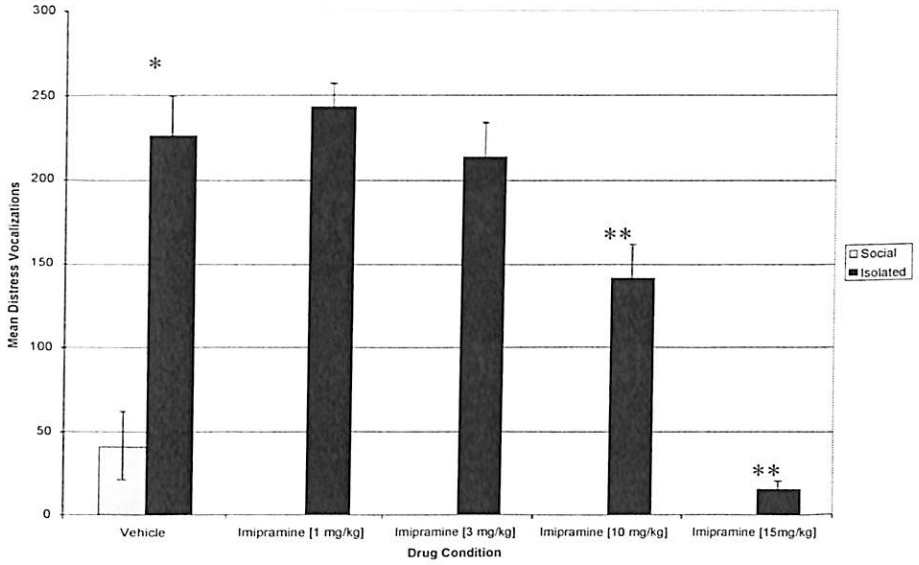
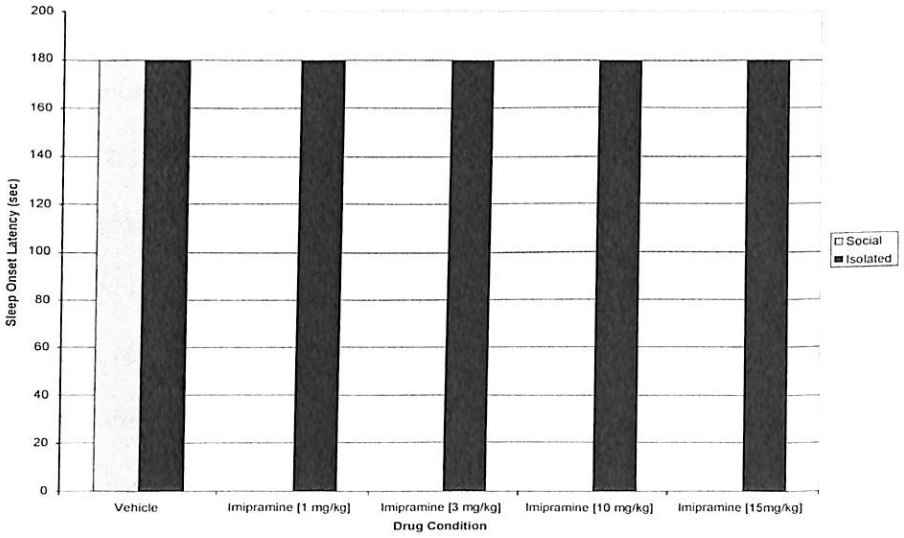


Figure 6: The effects of isolation and four doses of imipramine on sleep onset latency in a 3-minute test session. Imipramine was delivered IM 15 minutes prior to testing. Values represent mean ± S.E.M., sample size N = 14-18. The gray bar represents the social test condition and the black bars represent the isolated test condition.



The effects of isolation and imipramine on SOL are summarized in Figure 6. As evident, both isolation and imipramine failed to affect SOL. An analysis of these data using a Kruskal-Wallis one-way ANOVA failed to reveal a statistically significant treatment effect.

Clonidine:

The effects of isolation and clonidine on DVocs are summarized in Figure 7. Social isolation led to a clear increase in DVocs. Clonidine, in turn, dose-dependently attenuated this stress effect. Consistent with these observations, an analysis of these data demonstrated that the DVocs for the iso-vehicle group were significantly higher than the mirror group, $t(26) = 11.7$, $p < 0.0001$. An ANOVA of DVocs across the iso-clonidine groups revealed a significant treatment effect, $F(4,71) = 365.4$, $p < 0.0001$. Post-hoc analyses demonstrated that the mean DVocs for the 0.25mg/kg, 0.2mg/kg, 0.15mg/kg, and the 0.1mg/kg clonidine groups was significantly lower than the mean DVocs for vehicle-isolated, $p < 0.0001$. No other relevant comparisons reached statistical significance.

The effects of isolation and clonidine on SOL are summarized in Figure 8. The isolation manipulation failed to affect SOL. However, clonidine produced a dose-dependent decrease in SOL. An analysis of these data using a Kruskal-Wallis one-way ANOVA revealed a significant sedative effect $H(5) = 18.20$, $p<0.0001$. Post-hoc analyses demonstrated that the mean SOL for the 0.2mg/kg and 0.25mg/kg clonidine groups were significantly lower than the mean SOL for the vehicle-isolated birds $p<0.0001$. No other relevant comparisons reached statistical significance.

Figure 7: The effects of clonidine on isolation-induced distress vocalizations in a 3-min test session. Clonidine was delivered IM 15 minutes prior to testing. Values represent mean ± S.E.M., N = 11-17. The gray bar represents the social testing condition and the black bars represent the isolated test condition.
*** Indicates a significant isolation stress effect. ** Indicates significant attenuation of the stress effect, all P values < 0.0001.**

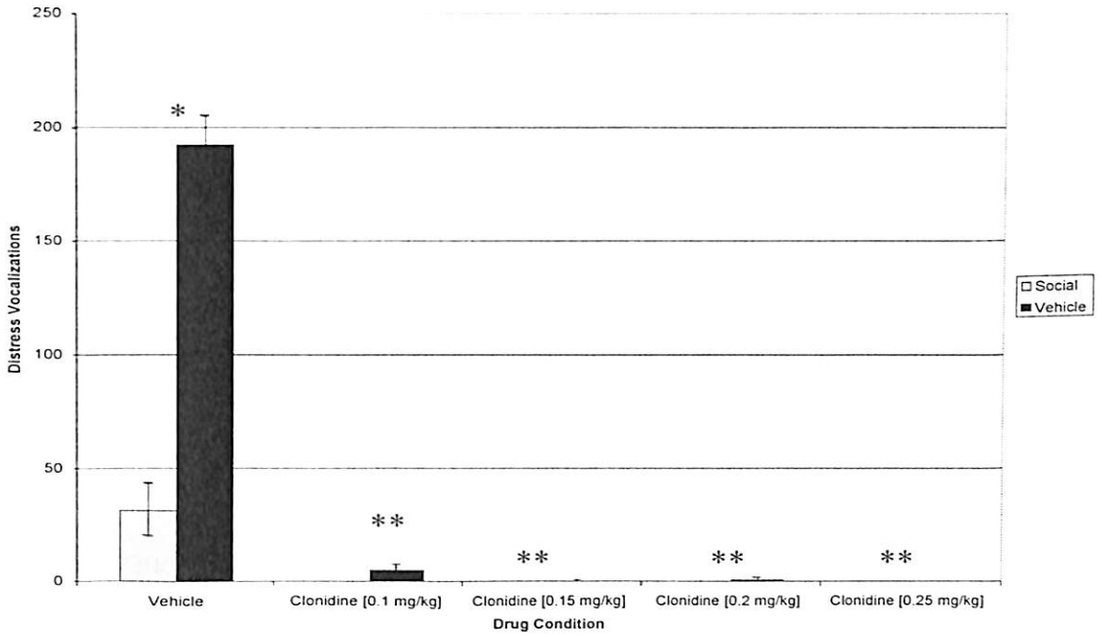
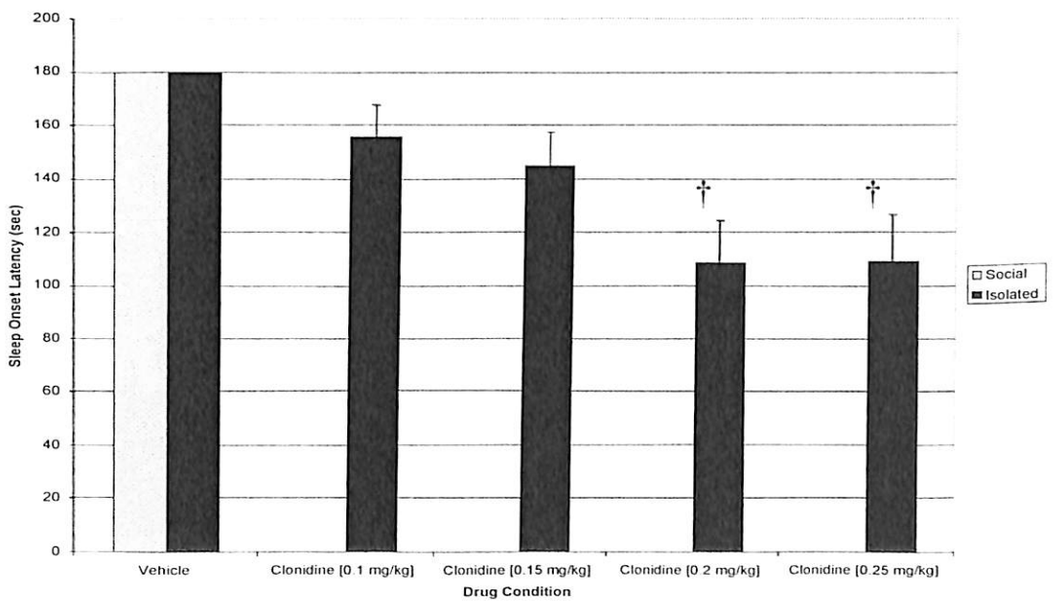


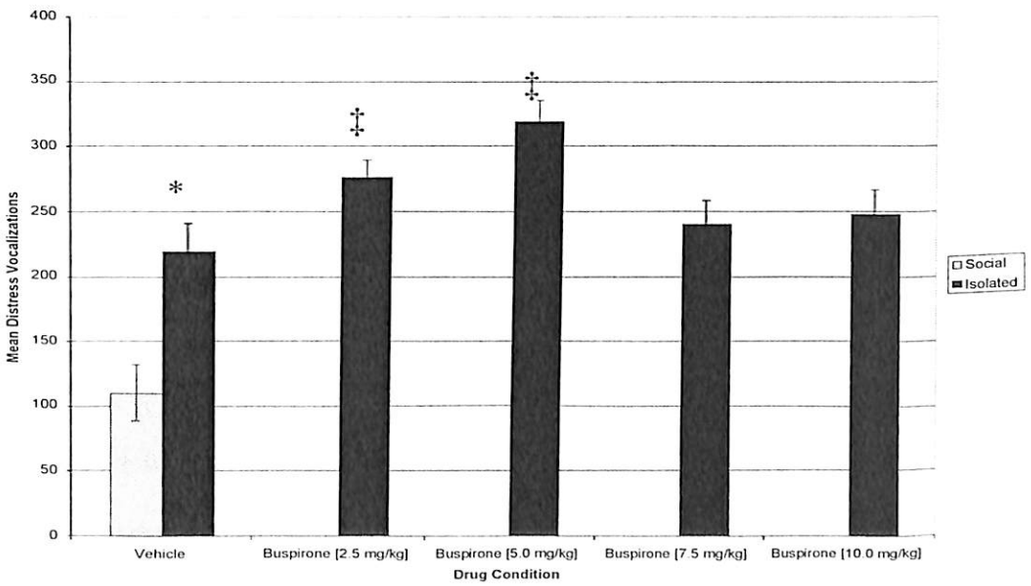
Figure 8: The effects of isolation and four doses of clonidine on sleep onset latency in a 3-minute test session. Clonidine was delivered IM 15 minutes prior to testing. Values represent mean ± S.E.M., N = 11-17. The gray bar represents the social test condition and the black bars represent the isolated test condition.
† Indicates a significant decrease in sleep onset latency, all P values <0.005.



Buspirone:

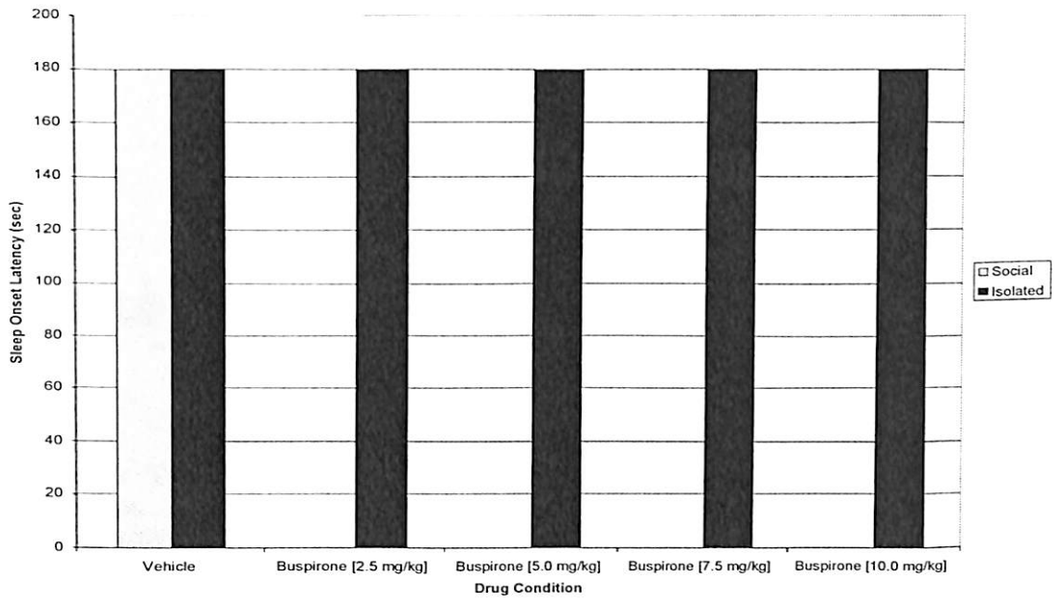
The effects of isolation and buspirone on DVocs are summarized in Figure 9. Social isolation led to a clear increase in DVocs. Buspirone, however, failed to attenuate this stress effect. In fact, buspirone produced an anxiogenic effect in the two higher doses. Consistent with these observations, an analysis of these data demonstrated that the DVocs for the iso-vehicle group were significantly higher than the mirror group, $t(32) = 3.62$, $p < 0.001$. An ANOVA of DVocs across the iso-buspirone groups revealed a significant treatment effect, $F(4,76) = 4.29$, $p < 0.005$. Post-hoc analyses demonstrated that the mean DVocs for the 2.5mg/kg and 5.0mg/kg buspirone groups was significantly higher than the mean DVocs for vehicle-isolated birds, $p < 0.05$. No other relevant comparisons reached statistical significance.

Figure 9: The effects of buspirone on isolation-induced distress vocalizations in a 3-min test session. Buspirone was delivered IM 15 minutes prior to testing. Values represent mean ± S.E.M., sample size N = 14-18. The gray bar represents the social testing condition and the black bars represent the isolated test condition.
*** Indicates a significant isolation stress effect. ‡ Indicates significant increase of this stress effect, all P values < 0.05.**



The effects of isolation and buspirone on SOL are summarized in Figure 10. Neither the isolation manipulation nor buspirone probes affected SOL. An analysis of these data using a Kruskal-Wallis one-way ANOVA failed to reveal a statistically significant treatment effect.

Figure 10: The effects of isolation and four doses of buspirone on sleep onset latency in a 3-minute test session. Buspirone was delivered IM 15 minutes prior to testing. Values represent mean ± S.E.M., N= 14-18. The gray bar represents the social test condition and the black bars represent the isolated test condition.



Trazodone:

The effects of isolation and trazodone on DVocs are summarized in Figure 11. Social isolation led to a clear increase in DVocs. Trazodone, however, failed to attenuate this stress effect. Consistent with these observations, an analysis of these data demonstrated that the DVocs for the iso-vehicle group were significantly higher than the mirror group, $t(34) = 13.3$, $p < 0.0001$. An ANOVA of DVocs across the iso-trazodone groups failed to reveal a significant effect, $F(4,83) = 1.80$, $p < 0.5$. No further analyses were conducted on these data.

Figure 11: The effects of trazodone on isolation-induced distress vocalizations in a 3-min test session. Trazodone was delivered IM 15 minutes prior to testing. Values represent mean ± S.E.M., sample size N = 16-18. The gray bar represents the social testing condition and the black bars represent the isolated test condition.
*** Indicates a significant isolation stress effect, all P values < 0.05.**

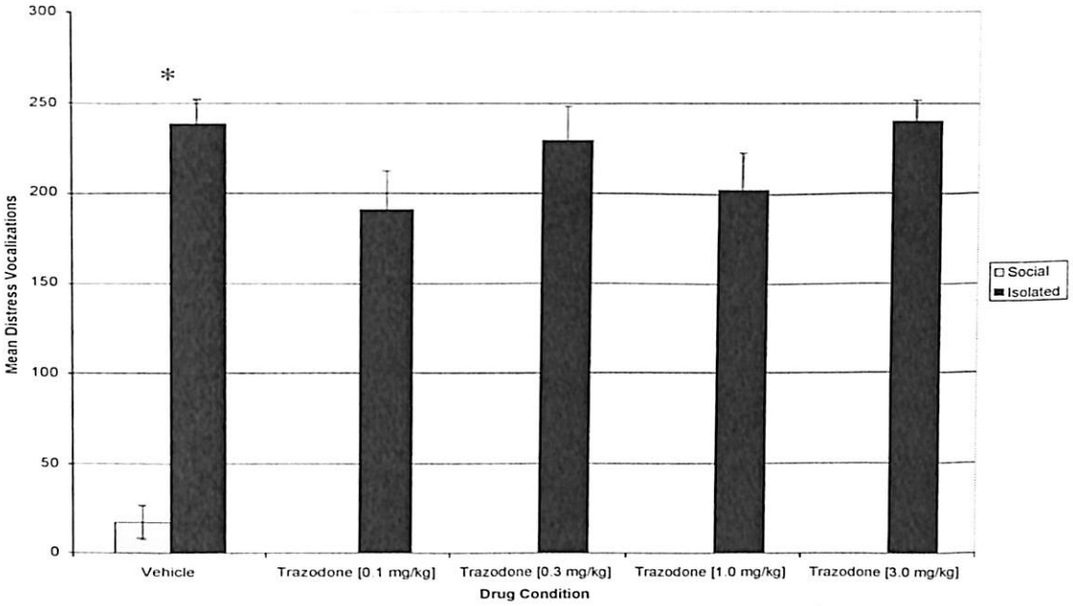
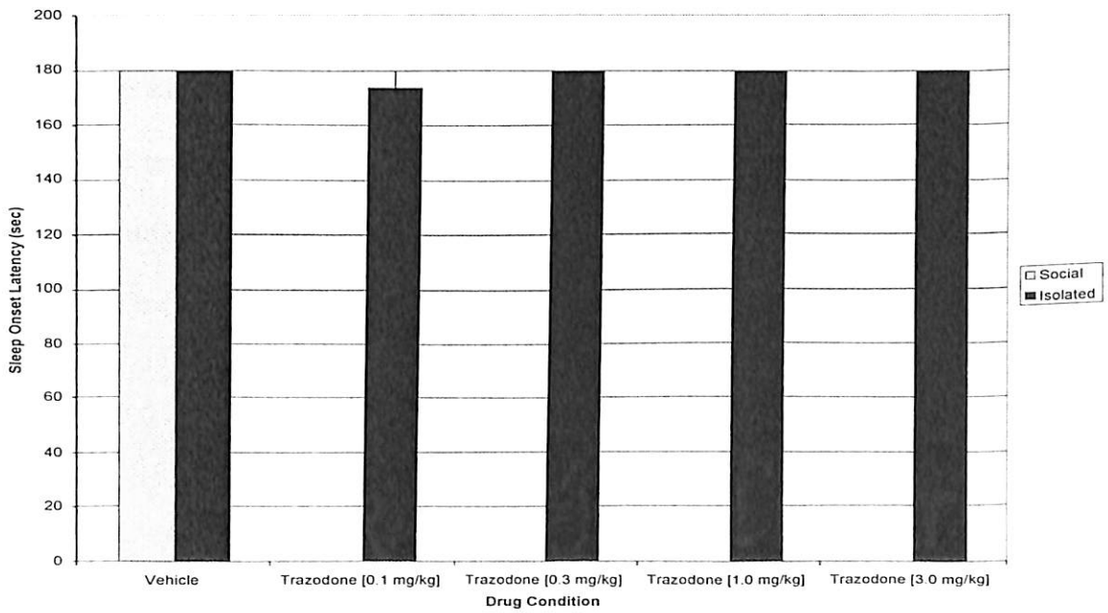


Figure 12: The effects of isolation and four doses of trazodone on sleep onset latency in a 3-minute test session. Trazodone was delivered IM 15 minutes prior to testing. Values represent mean ± S.E.M., sample size N = 16-18. The gray bar represents the social test condition and the black bars represent the isolated test condition.



The effects of isolation and trazodone on SOL are summarized in Figure 12. As evident, both isolation and trazodone failed to affect SOL. An analysis of these data using a Kruskal-Wallis one-way ANOVA failed to reveal a statistically significant treatment effect.

Yohimbine:

The effects of isolation and yohimbine on DVocs are summarized in Figure 13. Social isolation led to a clear increase in DVocs. Yohimbine, however, failed to enhance this stress effect. In fact, yohimbine attenuated the stress effect at three of the four doses. Consistent with these observations, an analysis of these data demonstrated that the DVocs for the iso-vehicle group were significantly higher than the mirror group, $t(33) = 7.21$, $p < 0.0001$. An ANOVA of DVocs across the iso-yohimbine groups revealed a significant effect, $F(4,81) = 3.27$, $p < 0.05$. Post-hoc analyses demonstrated that the mean DVocs for the 3.0mg/kg yohimbine group was significantly lower than the mean DVocs for vehicle-isolated birds, $p < 0.05$. No other relevant comparisons reached statistical significance.

The effects of isolation and yohimbine on SOL are summarized in Figure 14. Neither the isolation manipulation nor phenelzine probes affected SOL. An analysis of these data using a Kruskal-Wallis one-way ANOVA failed to reveal a statistically significant treatment effect.

Figure 13: The effects of yohimbine on isolation-induced distress vocalizations in a 3-min test session. Yohimbine was delivered IM 15 minutes prior to testing. Values represent mean ± S.E.M., sample size N = 16-18. The gray bar represents the social testing condition and the black bars represent the isolated test condition.
*** Indicates a significant isolation stress effect. ** Indicates significant attenuation of the stress effect, all P values < 0.05.**

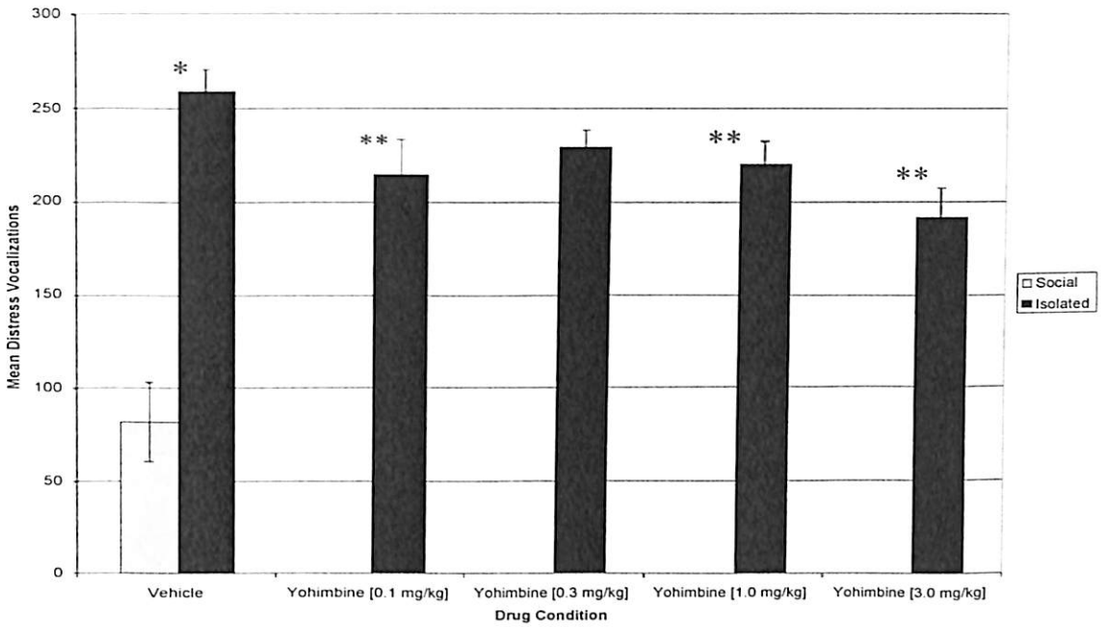
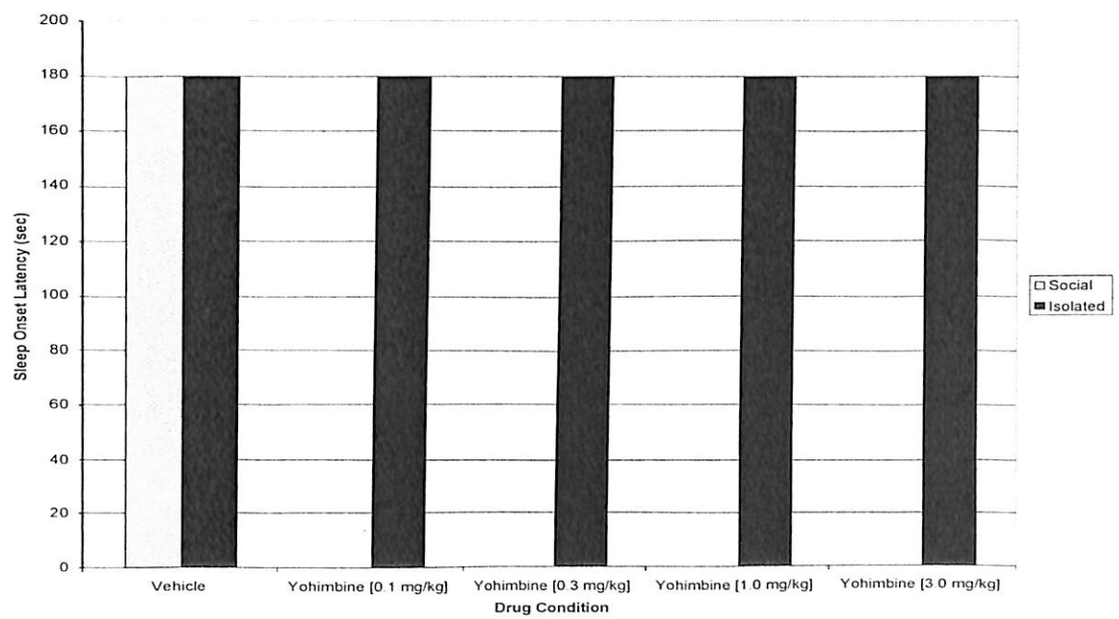


Figure 14: The effects of isolation and four doses of yohimbine on sleep onset latency in a 3-minute test session. Yohimbine was delivered IM 15 minutes prior to testing. Values represent mean ± S.E.M., sample size N = 16-18. The gray bar represents the social test condition and the black bars represent the isolated test condition.



To compare the relative effects of these drug probes on the two dependent measures, DVoc and SOL scores were converted to percent anxiolytic and sedative effect scores, respectively, and these data are summarized in Table 1. Although no inferential statistics were performed on these percent effect scores, in the cases where alprazolam and clonidine produced both significant anxiolytic and sedative effects, the anxiolytic effect was more pronounced at a given dose and/or occurred at a lower dose. This pattern of results argues that the anxiolytic effects of these compounds are not a confound of general sedation.

Table 1: Percent Anxiolytic and Sedative Effects of Drug Probes. Distress Vocalizations (DVoc), Sleep Onset Latency (SOL), * = significant isolation-stress effect, ** = significant anxiolytic effect, ‡ = significant anxiogenic effect, † = significant sedative effect, all $p^s < 0.05$.

	Dose 1	Dose 2	Dose 3	Dose 4
Phenelzine				
DVoc	-.2.34	1.62	18.43	32.47 **
SOL	0.00	0.00	0.00	0.00
Alprazolam				
DVoc	72.22 **	88.54 **	99.25 **	99.79 **
SOL	22.28 †	62.28 †	97.56 †	100.00 †
Imipramine				
DVoc	-11.52	13.35	35.15 **	88.73 **
SOL	0.00	0.00	0.00	0.00
Clonidine				
DVoc	88.96 **	99.27 **	97.55 **	100.0 **
SOL	11.22	19.61	37.39 †	38.83 †
Buspirone				
DVoc	-25.94 ‡	-45.61 ‡	-9.38	-13.33
SOL	0.00	0.00	0.00	0.00
Trazodone				
DVoc	20.07	3.56	15.21	-0.88
SOL	3.46	0.00	0.00	0.00
Yohimbine				
DVoc	17.01 **	11.41	14.89 **	26.04 **
SOL	0.00	0.00	0.00	0.00

DISCUSSION

Previous studies have validated the CSSP as a high-throughput, *in vivo* screening assay for anxiolytic compounds (Feltenstein et al. 2004; Feltenstein et al. 2003; Feltenstein et al. 2002). However, many forms of anxiety exist, each having its own unique characteristics. Through testing a series of pharmaceuticals used for the treatment of various forms of anxiety, this study set out to verify which form of anxiety is simulated by the paradigm. A series of experiments was designed to determine whether the paradigm was a screen for medications used in the treatment of panic disorder (PD) or generalized anxiety disorder (GAD). Seven known anxiolytic drugs with different specific treatment characteristics were chosen. One drug, phenelzine, is used exclusively in the treatment of PD. This would serve to validate the paradigm as a model of PD. Alprazolam, clonidine, and imipramine were then chosen to further validate the model, since they are effective in the treatment of both PD and GAD. Two drugs, trazodone and buspirone, were chosen as negative controls since they are both effective in GAD but have no effect in PD. Finally, yohimbine, which is shown to exacerbate symptoms of panic in humans, was chosen as a final validation of the paradigm as a screen for pharmaceutics used in the treatment of PD.

As hypothesized, if the CSSP is a model of PD, then isolation-induced DVocs should be attenuated both by the compound effective only in the treatment of PD and by the compounds effective in the treatment of PD and GAD. Likewise, the isolation-induced DVocs should *not* be attenuated by the compounds effective only in the treatment of GAD and should be enhanced by the compound known to exacerbate

symptoms of PD, but not GAD, in humans.

Seven-day old cockerels were administered intramuscularly either the vehicle or drug probe 15 minutes prior to the test period. During the testing period, the chicks were placed in a mirror (low-stress/social simulation) or no-mirror (high-stress/isolation) condition for 180 sec. During this period, the dependent measures of DVocs and SOL were recorded to index separation stress and sedation, respectively. The results obtained were then analyzed statistically to determine significance.

Consistent with previous reports (Feltenstein et al. 2002; Feltenstein et al. 2003; Feltenstein et al. 2004; Warnick et al. 2005), this study demonstrated that isolation (no-mirror condition) produced a robust increase in DVocs across all experiments. This response is a direct measure of the amount of stress experienced by the animal (Feltenstein, 2004). Furthermore, all drug probes effective in PD and the probes effective in both PD and GAD showed a significant attenuation of this increase without significant sedative effects.

Phenelzine was chosen as a probe representative of compounds that are exclusively effective in treatment of PD. As expected, phenelzine produced a dose dependent decrease in DVocs with a statistically significant decrease at the highest dose, a finding consistent with previous observations in this paradigm (Feltenstein 2005). No sedative effect was identified. This pattern of data argues that minimally the paradigm better simulates PD than GAD.

Similar positive results were seen in the three drug probes that have been shown to be effective in both PD and GAD: alprazolam, clonidine, and imipramine. All three probes showed statistically significant attenuation of DVocs, without sedation.

Significant effects on SOL were seen with both alprazolam and clonidine. However, in both cases the anxiolytic properties were more pronounced at a given dose and/or occurred at a lower dose, evidence that the anxiolytic effects of these compounds were not confounded by sedation. Furthermore, the relative efficacy and potency of the test compounds in the chick model were shown to be highly similar to the human clinical efficacy found in PD. In the chick paradigm, both clonidine and alprazolam were much more efficacious than imipramine, which in turn was more efficacious than phenelzine. This pattern exactly mirrors the one found in human clinical studies (Charney et al. 1986; Davidson and Connor, 2004).

In the case of trazodone and buspirone, the two drug probes shown to be clinically effective in GAD but not PD, no statistically significant attenuation of DVocs was revealed. These results are consistent with the hypothesis that the chick separation paradigm more closely models PD than GAD. It should be noted, however, that a significant anxiogenic effect was recorded for the two lower doses of buspirone. Although unexpected, the buspirone results are consistent with those in previous studies involving the CSSP (Feltenstein 2005).

While the failure of buspirone to attenuate DVocs supports the model as representative of PD, chronic administration of buspirone is often required for human clinical efficacy (Ninan and Muntasser 2004). However, four main reasons serve to explain why acute administration of buspirone was used in this study. First, other animal models have detected buspirone's anxiolytic effects after acute administration (e.g., Cole and Rodgers 1994). Second, the CSSP was able to detect the anxiolytic effects of other drugs that normally require chronic administration for therapeutic effects (i.e.,

imipramine and phenelzine). Third, it has been argued that successful *in vivo* pharmacological screens should be capable of screening drugs solely on acute administration (Willner 1991). And finally, human studies have shown that due to the method of administration, intramuscular injection, compounds that usually require chronic administration when given orally display acute effects when injected (Becker 1971).

Taken together, the effects of these drug probes reveal a pattern that is consistent with the hypothesis that the CSSP is a better simulation of PD than GAD. On the other hand, the final drug probe, yohimbine, gave rather indeterminate results. While yohimbine failed to exacerbate DVocs, it did reveal a statistically significant attenuation at the first dose and the two higher doses. These results are neither exemplary of a model of PD, in which case DVocs should have been attenuated, or GAD, in which case no statistically significant effect should have been recorded. These unexpected results are further thrown into question by the fact that yohimbine, an ∀2 antagonist, failed to exacerbate DVocs while clonidine, an ∀2 agonist, attenuated them. Although yohimbine has been shown to increase the symptoms of PD in humans (Charney et al. 1989), the modest anxiolytic effect of yohimbine detected in this study should not be surprising as this drug has been shown to attenuate stress behaviors in other animal models (e.g., punished drinking assay, Baldwin et al. 1989; Gower an Tricklebank 1988; conditioned ultrasonic DVocs, Molewijk et al. 1995; De Vry et al. 1993).

One might argue that the failure of yohimbine to exacerbate DVocs is a limitation to this paradigm as a valid simulation of PD. However, the lack of a stereotypical yohimbine effect should not be considered a fatal flaw when the model is being used as a

pharmacological screen. The primary importance of a screening assay is on its ability to successfully screen clinically active compounds without producing false negatives and, to a lesser degree, false positives (Willner 1991). False positives, as exemplified by yohimbine in this study, will be detected in subsequent screenings and never enter into clinical trials.

The results of this study serve to further validate the CSSP as a pharmacological screening assay for anxiolytics effective in the treatment of PD. As stated above, the CSSP has been used extensively in this lab and has been shown to be a valid model of anxiety (Feltenstein et al., 2004). Although some rodent based models of anxiety do exist, they tend to be time consuming, involve more stress for the animal, and are expensive. This test uses one testing period of three-minute duration whereas many rodent models require multiple trials and longer tests, placing more stress on the animal. Also, the chick separation stress model is highly cost-effective, which is illustrated by comparing purchase cost between rats and chicks; 1 rat = 110 chicks. Per Diem expenses are also less in the chick model. Furthermore, the CSSP is unique in that it meets the National Institutes of Health's 3R policy to Reduce, Refine, and Replace (Office of Laboratory Animal Welfare, 2002). The model reduces the number of purpose-bred research animals since the male chicks used in this experiment are a by-product of the commercial egg-laying industry (i.e., cockerels are discarded at hatch; Roach and Sufka, 2003). The screen has also refined the procedure since, as afore mentioned, it minimizes the stress-provoking stimuli to a single, short 3-minute test session. Finally, the screen replaces the standard rodent-based screening procedures for anxiolytics with a phylogenetically lower and less sentient species. These attributes strongly suggest the

adoption of the CSSP as an early preclinical screening assay for anxiolytics for the treatment of PD.

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APPENDIX

Table 2: Effects of Drug Probes on Isolation-induced Distress Vocalizations (DVocs) and Sleep Onset Latency (SOL)

Treatment	*N*	DVoc Mean (SEM)	SOL Mean (SEM)
Phenelzine			
Vehicle/mirror	18	97.0 (23.3)	180.0 (0.0)
Vehicle/no-mirror	17	235.9 (19.8) *	180.0 (0.0)

3.125mg/kg	16	241.4 (11.5)	180.0 (0.0)
6.25mg/kg	15	231.1 (21.6)	180.0 (0.0)
12.5mg/kg	16	192.4 (21.9)	180.0 (0.0)
25.0mg/kg	17	159.3 (21.0) **	180.0 (0.0)
Alprazolam			
Vehicle/mirror	16	11.1 (5.7)	180.0 (0.0)
Vehicle/no-mirror	18	186.8 (13.2) *	180.0 (0.0)
0.065mg/kg	17	51.9 (15.8) **	139.9 (14.3) †
0.125mg/kg	15	21.4 (15.7) **	67.9 (14.2) †
0.25mg/kg	18	1.4 (0.7) **	4.4 (4.4) †
0.5mg/kg	18	0.4 (0.2) **	0.0 (0.0) †
Imipramine			
Vehicle/mirror	13	28.2 (16.6)	180.0 (0.0)
Vehicle/no-mirror	18	226.5 (23.2) *	180.0 (0.0
1.0mg/kg	17	252.6 (10.9)	180.0 (0.0)
3.0mg/kg	16	218.9 (22.0)	180.0 (0.0)
10.0mg/kg	18	141.9 (20.1) **	180.0 (0.0)
15.0mg/kg	18	16.0 (4.8) **	180.0 (0.0)
Clonidine			
Vehicle/mirror	18	31.8 (11.7)	180 (0.0)
Vehicle/no-mirror	13	192.5 (19.4) *	180.0 (0.0)
0.1mg/kg	18	25.1 (14.0) **	159.8 (10.1)
0.15mg/kg	18	1.4 (1.2) **	144.7 (11.4)
0.2mg/kg	18	4.7 (3.7) **	112.7 (15.1) †
0.25mg/kg	18	0.0 (0.0) **	110.1 (16.3) †
Buspirone			
Vehicle/mirror	17	110.41 (21.1)	180.0 (0.0)
Vehicle/no-mirror	17	219.5 (21.5) *	180.0 (0.0)
2.5mg/kg	15	276.46 (13.0) ‡	180.0 (0.0)
5.0mg/kg	14	319.571 (16.1) ‡	180.0 (0.0)
7.5mg/kg	17	240.1 (18.4)	180.0 (0.0)
10.0mg/kg	18	248.7 (18.0)	180.0 (0.0)
Trazodone			
Vehicle/mirror	18	17.3 (9.6)	180.0 (0.0)
Vehicle/no-mirror	18	238.7 (13.6) *	180.0 (0.0)
0.1mg/kg	18	190.8 (21.6)	173.8 (6.2)
0.3mg/kg	16	230.2 (18.3)	180.0 (0.0)
1.0mg/kg	17	202.4 (20.7)	180.0 (0.0)
3.0mg/kg	16	240.8 (11.2)	180.0 (0.0)

Yohimbine			
Vehicle/mirror	18	82.3 (21.2)	180.0 (0.0)
Vehicle/no-mirror	17	259.2 (11.5) *	180.0 (0.0)
0.1mg/kg	16	215.1 (18.6) **	180.0 (0.0)
0.3mg/kg	18	229.6 (9.1)	180.0 (0.0)
1.0mg/kg	18	220.6 (11.7) **	180.0 (0.0)
3.0mg/kg	17	191.7 (15.5) **	180.0 (0.0)

* = significant isolation-stress effect, ** = significant anxiolytic effect, ‡ = significant anxiogenic effect, † = significant sedative effect, all $p^s < .05$.

Table 3: Comparative activity of drug probes in clinical cases of panic disorder (PD), generalized anxiety disorder (GAD), and in the chick separation stress paradigm.

Drug Probe	Effect in PD	Effect in GAD	Effect in the Chick Separation Stress Paradigm
Phenelzine	+	-	+
Alprazolam	+	+	+
Imipramine	+	+	+
Clonidine	+	+	+
Buspirone	-	+	-
Trazodone	-	+	-

+ = anxiolytic activity, - = absence of anxiolytic activity.