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Rapid and Reliable Sedation Induced by Diazepam and Antagonized by Flumazenil in Zebra Finches (*Taeniopygia guttata*)

Jonathan F. Prather, PhD

Abstract: Songbirds have emerged as attractive model systems in many areas of biological research. Notably, songbirds are used in studies of the neurobiological and neuroendocrine mechanisms that shape vocal communication, and zebra finches (*Taeniopygia guttata*) are the most commonly studied species. In these studies, some form of chemical restraint is often needed to facilitate procedures and to minimize the risk of injury during handling. To determine the minimum dose of the benzodiazepine diazepam that is adequate to achieve deep sedation across individual birds, a low dose (5 mg/kg) and a high dose (10 mg/kg) was administered intramuscularly to 20 zebra finches. Results showed that a 10 mg/kg dose of diazepam resulted in deep sedation, defined by dorsal recumbency, which was achieved in minutes and lasted for several hours. Sedation was induced without complication, because no birds displayed signs of distress during sedation or lethargy after recovery, and was adequate to permit minimally invasive surgical procedures. In addition, the duration of sedation was dose dependent, which provides additional information for researchers who seek to match the depth of sedation to their experimental requirements. Finally, complete recovery from the deeply sedated state was induced by a 0.3 mg/kg dose of the antagonist flumazenil, which enabled birds to more rapidly resume homeostatic behaviors to promote well-being and survival. Together, these results indicate that diazepam is a safe and reliable sedative for use in zebra finches and support specific recommendations to achieve rapid and reliable sedation and recovery.

Key words: sedative, benzodiazepine, diazepam, avian, songbird, zebra finch, *Taeniopygia guttata*

Introduction

Songbirds have been integral in pioneering or advancing many fields of biological study.[1,2] Among the many species that have been studied, zebra finches (*Taeniopygia guttata)* have emerged as the most commonly studied because several features distinguish them as especially attractive experimental subjects.[3,4] First, they readily perform their vocal and social behaviors in the laboratory, which enables researchers to record those behaviors very accurately. Second, the colonial nature of zebra finches enables many animals to be housed in the laboratory.[4] Finally, their success in captive breeding vastly facilitates studies of behavioral ontogeny.[5,6] Together, these advantages of zebra finches indicate the need for methods to facilitate their safe handling and reliable performance of experimental manipulations.

The contributions of investigations using zebra finches are most clearly evident in studies of the central and peripheral structures that underlie song learning, performance, and perception,[7] and the role of the neuroendocrine system in shaping sexually dimorphic neural structures and the associated behavior.[8–13] Such studies often require that birds be placed into a stereotaxic recording device or receive a subcutaneous implant of testosterone or other substance. Therefore, many experimental preparations in which zebra finches are studied require some means of chemical restraint to minimize stress and the likelihood of struggling, which could result in injury and reduced postoperative survival after otherwise

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minimally invasive procedures.[14] Inhalation anesthetics, for example, isoflurane, are presently the agents of choice for most investigations[15]; however, parenteral methods may be preferred in some cases. The reasons for this preference are typically practical ones, because agents delivered by injection are often less expensive than inhalation agents and the required equipment is much less expensive for injection methods. Furthermore, parenteral methods are potentially more broadly relevant, because they can be used equally well in the laboratory or in the field, whereas the equipment required for inhalation anesthesia makes those methods less impractical for field studies. Parenteral methods are also effective in inducing deep sedation, which may be preferable to general anesthesia in facilitating simple clinical or experimental manipulations, such as handling birds or placing subcutaneous implants.[12,16] In some cases, anesthesia can lead to respiratory, cardiac, or renal complications; however, the respiratory and cardiovascular effects of benzodiazepine sedatives, for example, diazepam, are minimal in healthy small animal patients.[17–19]

Previous studies used intramuscular administration of diazepam to sedate zebra finches during data collection.[20–24] Results of those studies revealed that diazepam is an effective sedative; however, each of those studies used a different dose (minimum of 4.4 mg/kg, maximum of 13.4 mg/kg, estimated based on the average weight of zebra finch of 15 g), which leaves open the fundamental question of what is the minimal dose to achieve deep sedation reliably across individuals. There is wide variation in the dose of diazepam that is adequate for clinical or experimental sedation,[18,19,25] and variation persists even when the effects are considered among only avian species.[26–28] Thus, the adequate dose for one species cannot simply be inferred from studies of other species.

In some clinical or laboratory situations, it may be beneficial to reduce the duration of diazepam-induced sedation, because accelerated recovery would allow the bird to begin regulating its own state more rapidly (eg, feeding, drinking, temperature regulation), which is important, given the high rate of avian metabolism.[29] Flumazenil reverses the effects of diazepam through competitive inhibition at the benzodiazepine-binding site on type-A gamma aminobutyric acid ($GABA_A$) receptors in the central nervous system.

The objectives of this study were to identify the adequate dose of diazepam in zebra finches that would achieve rapid and reliable deep sedation to facilitate handling in the clinic or the laboratory and to determine the degree to which sedation permits minimally invasive surgical manipulations such as those used in studies of the neurobiological and neuroendocrine control of song. In addition, the efficacy of flumazenil in facilitating rapid recovery and resumption of typical homeostatic behaviors was determined.

Materials and Methods

All of the procedures were in compliance with recommendations of the University of Wyoming Animal Care and Use Committee and state and federal regulations that govern the housing and use of songbirds.

Care and handling of experimental subjects

All experiments were performed by using adult (age $>$ 180 days posthatch) male and female zebra finches housed communally, with seed, water, and grit provided ad libitum (15:9 light : dark photoperiod). On the day of each experiment, the birds were transferred from their communal cage and housed individually before, during, and after drug administration. The animals were not fasted before drug administration.[26,30] After testing, the birds were closely monitored until eating, drinking, or vigorous activity was observed. Isolation was maintained until the next day to ensure that the birds were completely recovered when they were returned to their communal cages.

Drug administration

All experiments were begun within 2 hours after lights on to ensure that possible circadian effects were similar for each condition, and serial experiments on any individual bird were separated by 1 to 2 weeks to minimize the possibility of drug interaction.[31] Drugs were administered by injection with a small (30-gauge) needle into the pectoralis muscle of either side, with the dose determined by the bird's weight measured to the nearest 0.1 g. To ensure that the amount of drug that was delivered was close to the intended dose, very rare cases of bleeding at the injection site were excluded from further analysis, and the test was repeated at least 1 week later. Manual restraint was continued for 1 minute after injection, after which the degree of sedation was tested by attempting to place the bird onto its back in its individual cage.

Behavioral observation followed individual dosing of one or more of the following substances: 1) diazepam, which binds to $GABA_A$ receptors and causes broad decreases in neural activity by enhancing the inhibitory effects of endogenous

GABA[32]; 2) physiological saline solution (0.9%); 3) propylene glycol, which is used as a solvent in injectable diazepam; or 4) flumazenil (0.30 mg/kg), which antagonizes the sedative effects of diazepam. Diazepam was administered in either a low dose (5 mg/kg) or a high dose (10 mg/kg), and doses of control solutions (saline solution, propylene glycol) were delivered in volumes equal to that of the 10 mg/kg dose of diazepam.

Experimental design

This study comprised 3 closely related experiments. First, the birds were administered either a low dose (5 mg/kg) or a high dose (10 mg/kg) of diazepam, and the resulting sedation was quantified according to criteria described below. In alternating experiments separated by at least 1 week, 20 birds (10 males, 10 females) received each dose of diazepam. This experiment was intended to determine the minimal dose that was adequate to achieve a deeply sedated state, as defined by birds becoming dorsally recumbent in at least 95% of all individuals. Dorsal recumbency has been used as the standard of adequate sedation by researchers seeking to determine the appropriate dose for songbird care[31] and is very similar to stage III of anesthesia.[33–35]

In a second experiment, the birds were divided into 2 groups of 10 birds each. To determine whether diazepam sedation was adequate to enable procedures such as placement in a stereotactic device or minor surgery, half of the experimental subjects (10 birds) were deeply sedated (10 mg/kg dose) and placed into a stereotaxic recording device 15 minutes after injection. Placement in such a device required gentle spreading of the auricular feathers and insertion of ear bars into the holes of the external ears. The head, in addition, was secured by a bite bar placed in the bird's gaping beak and secured by using gentle pressure on the upper half of the bill. The depth of sedation was observed before handling to ensure that the birds were dorsally recumbent, and the reaction of the birds when placed into the device (eg, wing flapping, leg movement) and whether the bird returned to its sedated state within 2 minutes after placement into the device were noted. The other half of the subjects were also deeply sedated (10 mg/kg dose), and 15 minutes after injection, a small surgical incision (2 mm) was made in the skin overlying the abdomen. The reaction to the incision (eg, flinching, leg movement) and whether the bird returned to its sedated state within 2 minutes after placement into its cage were noted. After this procedure, the incision was sealed with surgical adhesive. This experiment was performed last in this study so that the sealed incision did not affect subsequent injections and was not irritated by subsequent handling. These techniques were meant to simulate procedures used in neurophysiological recordings of sedated zebra finches or minimally invasive surgical procedures such as drug delivery via a subcutaneous implant in studies of neuroendocrine influences on song behavior.

In the third experiment, the birds were sedated by using the 10 mg/kg diazepam dose and then were given an injection of the antagonist drug flumazenil (0.3 mg/kg[30]) 15 minutes after the diazepam injection. Relevant to each experiment, control solutions (saline solution or propylene glycol) were administered in tests interspersed with tests of diazepam, with all tests of any individual bird were separated by at least 1 week.

Behavioral observation and quantifying the depth of sedation

After injection of each substance in the first experiment, the birds were monitored in a well-lit, quiet room, with minimal disturbance from external factors. The status of each bird was recorded each minute for the first 10 minutes after drug injection, then every 5 minutes until 30 minutes after injection, then every 10 minutes until 60 minutes after injection, and finally, every 15 minutes thereafter until recovery. The bird's status was quantified by assessing the depth of sedation and degree of areflexia according to the following criteria, modified from those used in other studies of avian sedation.[31] For depth of sedation, minimal sedation (score = 1) was characterized by the ability to stand but with a stooped or broad-based stance, with closed eyes, or with fluffed or ruffled feathers. Birds in this state commonly leaned against the cage wall, expressing apraxic movements when attempting to walk or hop. Mild sedation (score = 2) was characterized by sternal recumbency and resistance to restraint when handled by the experimenter. Moderate sedation (score = 3) was also characterized by sternal recumbency, but the bird no longer resisted restraint. The birds, while under moderate sedation, could be placed onto their backs, but they retained the righting reflex such that dorsal recumbency was not possible. It was common that birds in this state would roll partially onto their sides. Finally, deep sedation

(score = 4) was characterized by sustained dorsal recumbency with no restraint. The onset of sedation was defined as the first observation in which the bird received a sedation score of ≥2, and the conclusion of sedation was defined as either the second of 2 consecutive observations in which the sedation score was <2 or the first time that the bird hopped, ate, or drank. Similarly, the onset of dorsal recumbency was defined as the first time that the bird would remain on its back after being placed in that position by the experimenter, and the conclusion of dorsal recumbency was defined as the first time that the bird righted itself from lying on its back.

In addition to quantifying depth of sedation, muscle tone was assessed by gently pulling one wing away from the body, with the goal of extending the wing fully and holding it in place. Muscle tone was quantified according to the following criteria.[31] Full muscle tone (score = 0) was evident as forceful retraction of the wing. Typically in this state, retraction occurred immediately after the experimenter began pulling the wing. In cases of lesser muscle tone (score = 1), the wing could be extended, often fully, but retraction was clearly evident. In this state, the bird could often pull its wing from the experimenter's hand. In the absence of muscle tone (score = 2), the experimenter could fully extend the wing, with little or no resistance or attempted retraction at any point. This measure of muscle tone provided an additional means of determining the depth of the bird's sedation, although the sedation score was the means through which onset and conclusion of sedation and dorsal recumbency were defined.

Statistical analysis

All statistical tests were performed by using Matlab (Mathworks, Inc, Natick, MA, USA), and the test used in each case is reported in association with the results of that comparison. Statistical significance was assessed by using a threshold of $P < .05$.

Results

Adequate dose to achieve dorsal recumbency

Diazepam was very effective as a sedative in adult zebra finches. In tests of 5 mg/kg and 10 mg/kg doses alternately applied in randomized sequence to the same set of 20 birds (10 males, mean weight [± standard error {SE}] = 15.9 ± 0.8 g; 10 females, mean [±SE] weight = 15.6 ± 0.9 g), both doses were effective in achieving at least some level of sedation. Importantly, the higher dose was significantly more effective in achieving a deeply sedated state, defined by dorsal recumbency. Dorsal recumbency was achieved in only 65% of birds that received the 5 mg/kg dose but in 95% of birds that received 10 mg/kg dose ($P = .04$, χ^2 test; n = 20 birds) and 100% of birds that were sedated with 10 mg/kg diazepam before administration of flumazenil (n = 20 birds, described below). When dorsal recumbency was achieved, the peak wing tone score was invariably achieved during that state. Birds typically reached dorsal recumbency while maintaining steady respiration (for the 5 mg/kg dose, 116 ± 4 breaths per minute; for the 10 mg/kg dose, 108 ± 4 breaths per minute) that was not significantly different between the 2 doses ($P = .11$, Wilcoxon signed rank test).

Sedation was induced more rapidly (Fig 1A), and both sedation and dorsal recumbency lasted longer (Fig 1B) after administration of the 10 mg/kg dose than of the 5 mg/kg dose of diazepam (Table 1). The higher dose induced a more sedated state, evident in the significantly greater peak sedation score (5 mg/kg, 3.60 ± 0.13; 10 mg/kg, 3.95 ± 0.05) and wing tone score (5 mg/kg, 1.65 ± 0.13; 10 mg/kg, 1.95 ± 0.05) for 10 mg/kg than for 5 mg/kg ($P = .03$ and .04, respectively, Wilcoxon signed rank test). The robust effects of the 10 mg/kg dose were common to both sexes, with indistinguishable effects on depth of sedation (peak sedation score: 3.9 ± 0.1 [males], 4.0 ± 0.0 [females], $P = .35$; wing tone score: 1.9 ± 0.1 [males], 2.0 ± 0.0 [females], $P = .35$, Mann-Whitney U test) and a tendency for the males to achieve the dorsal recumbency state slightly more rapidly than females (delay to onset of sedation: 1.6 ± 0.3 minutes [males], 2.7 ± 0.5 minutes [females], $P = .09$; duration of sedation: 308.3 ± 13.3 minutes [males], 258.4 ± 22.5 minutes [females], $P = .08$; delay to onset of dorsal recumbency: 2.7 ± 0.4 minutes [males], 6.0 ± 0.12 minutes [females], $P = .03$; duration of dorsal recumbency: 177.6 ± 23.5 minutes [males], 149.8 ± 24.5 minutes [females], $P = .42$, Mann-Whitney U test). No adverse effects were seen in either male or female zebra finches, because no birds regurgitated or experienced respiratory difficulty during any experiment. Therefore, the 10 mg/kg dose of diazepam appeared adequate to induce deep sedation characterized by dorsal recumbency for 150 to 175 minutes, which permitted approximately 3 hours of clinical or experimental opportunity, followed by complete recovery.

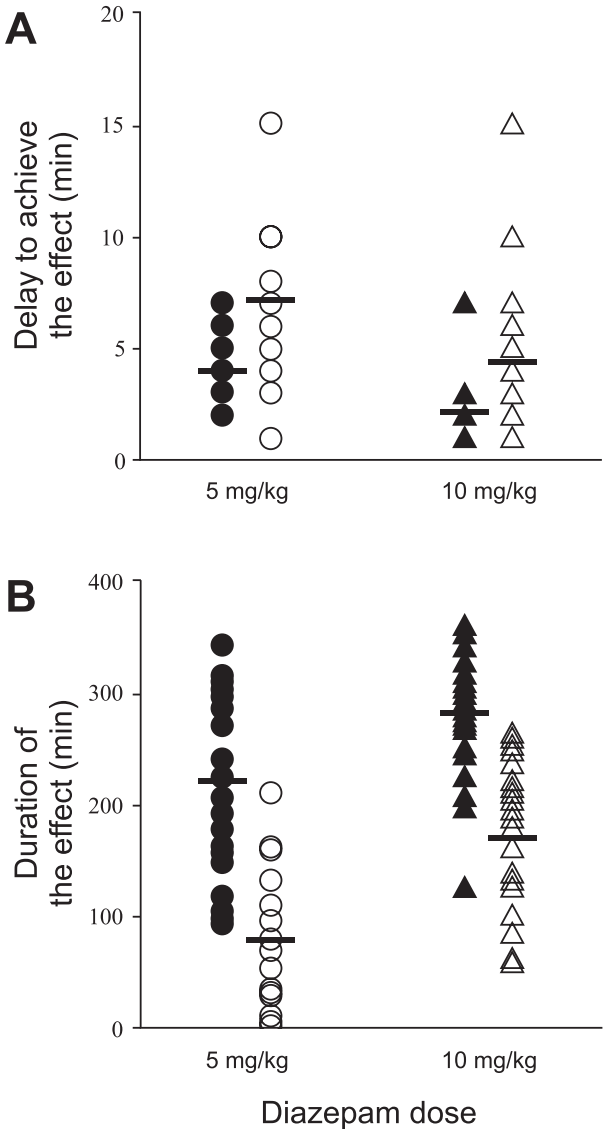

Figure 1. Diazepam-induced dose-dependent sedation in zebra finches. A 10 mg/kg dose of diazepam (triangles, right) was more effective than a 5 mg/kg dose (circles, left) in inducing sedation (filled symbols) and dorsal recumbency (open symbols) in adult zebra finches. The greater efficacy of the higher dose was evident as (A) a shorter latency to achieve the effect, and (B) a longer duration of the effect. Each point represents an individual bird (N = 20 in each column, with some points overlapping), and solid bars indicate the population mean. In no case did the control treatments of saline solution or propylene glycol induce any signs of sedation or dorsal recumbency (peak sedation score = 0 and wing tone score = 0 in all cases).

Diazepam sedation permitted minimal surgical manipulation

In the second experiment, the stability of diazepam sedation during manipulations such as those used in studies of neurobiological and neuroendocrine influences on song behavior was determined. All the birds were sedated with a 10 mg/kg dose of diazepam, and all the birds achieved the dorsal recumbency state before subsequent testing (n = 20). Fifteen minutes after injection of diazepam, the birds were administered 1 of 2 tests. In the first group (n = 10), the birds were placed into a stereotaxic device. In the second group (n = 10), a 2-mm incision was made in the skin overlying the abdomen. In experiments that tested the effects of stereotaxic restraint, all 10 the birds exhibited a mild reaction as they were removed from their cage and placed into the ear bars and beak restraints of the stereotaxic frame, with responses that included leg twitches, wing flaps, or twisting of the body. These reactions were of weak intensity and brief duration, with all 10 birds returning to a deeply sedated state within 2 minutes of being placed in the restraints. Sedation was assessed without attempting dorsal recumbency in the stereotaxic device, because the restraints did not permit that posture, but all of the birds returned to the dorsal recumbency state after removal from the device. Therefore, although the birds were transiently roused immediately after being placed into the stereotaxic device, the 10 mg/kg dose of diazepam was sufficient to facilitate procedures such as those commonly used in studies of the neurobiology of song behavior.

In the birds that underwent surgical incision, all 10 exhibited a mild reaction as the cut was made, with responses that included leg twitches, movement of the head, or subtle flinching movements of the body. These movements occurred only at the instant of the incision, and all 10 birds returned to a deeply sedated state within 2 minutes after the incision was made. Therefore, sedation by using 10 mg/kg of diazepam was sufficient to permit a minimally invasive procedure. Movements at the time of the incision indicated that a local anesthetic, such as lidocaine cream, should be used at the site where incisions are to be made.

Flumazenil induced rapid and persistent recovery from the sedated state

In the third experiment, the degree to which the duration of sedation was reduced by administering the antagonist flumazenil (0.3 mg/kg[30]) 15 minutes after injection of diazepam was investigated. All birds (n = 20) achieved the dorsal recumbency state before administration of flumazenil (Fig 2A), and the duration of sedation and dorsal recumbency was much shorter with administration of

Table 1. Effects of sedation with diazepam at 2 doses in zebra finches (N = 20). All values are mean ± SE. All comparisons were performed by using the Wilcoxon signed rank test.

Sedation parameter	Diazepam dose		*P* value
	5 mg/kg	10 mg/kg	
Delay to sedation onset (min)	3.95 ± 0.88	2.15 ± 0.48	<.01
Duration of sedation (min)	222.3 ± 49.71	283.35 ± 63.36	.02
Delay to DR onset (min)	7.15 ± 1.98	4.42 ± 1.01	.12
Duration of DR (min)	78.77 ± 21.85	171.06 ± 40.32	<.001

Abbreviation: DR indicates dorsal recumbency.

flumazenil than without antagonism (Fig 2B). Furthermore, males and females (n = 10 in each group) were similarly affected by flumazenil, because there were no sex differences in the reduction of delay to onset of sedation, duration of sedation, delay to onset of dorsal recumbency, or duration of dorsal recumbency ($P > .27$ in all cases, Mann-Whitney *U* test). Reports of the half-life of diazepam and flumazenil suggest that there could be a second phase of diazepam-induced sedation after the metabolism of flumazenil[25,36]; however, no relapse was observed in the zebra finches in this study.

Discussion

These results show that a 10 mg/kg dose of diazepam is adequate to induce rapid and reliable dorsal recumbency in zebra finches. Furthermore, sedation with diazepam (10 mg/kg) permits minimally invasive surgical manipulations such as those used in studies of the neurobiological and neuroendocrine control of song; however, supplemental application of local anesthetics is recommended to facilitate surgical success. Finally, the benzodiazepine antagonist flumazenil is effective in facilitating rapid recovery and resumption of typical homeostatic behaviors after diazepam sedation (10 mg/kg) in zebra finches.

Inducing deep sedation rapidly and reliably is useful clinically because it facilitates safe handling and treatment of the animals in diagnostic and minor therapeutic techniques. Specifically, sedation minimizes the likelihood of the bird struggling during treatment, which may result in injury and reduce survival after otherwise minimally invasive procedures.[14] Furthermore, sedation is useful for laboratory investigation of zebra finches. In some studies, deep sedation is desired,[21,22] whereas other experiments benefit from a less sedated state in which the animal can be transiently roused.[37] The depth of sedation reported here was dose dependent, such that 10 mg/kg resulted in dorsal recumbency in all but 1 individual, and 5 mg/kg resulted in a significantly less sedated state. This dose dependence provides additional resolution to enable researchers to match the level of sedation to their specific experimental needs.

An intriguing facet of these results is that male zebra finches are more susceptible than females to sedation by intramuscular administration of diazepam. Specifically, males took only half as long to achieve dorsal recumbency as did females. Furthermore, males tended to enter sedation more rapidly and stay in dorsal recumbency and in the sedated state longer than females, although those differences were not significant. These data may reflect differences in the number of $GABA_A$ receptors, the sensitivity of those channels to benzodiazepine activation, the subunit composition of at least some $GABA_A$ channels, or some combination of those differences in male versus female birds. This natural difference between male and female zebra finches provides an opportunity to investigate which of those features of $GABA_A$ receptors are most influential in establishing the efficacy of diazepam sedation. Future comparative studies of the effects of benzodiazepines and other sedatives will reveal the degree to which this greater response to diazepam in male zebra finches may be a species-specific phenomenon or a general difference in the response of male and female songbirds to neuromodulatory substances. An additional goal of future research will be to compare the efficacy of diazepam versus other benzodiazepines, such as midazolam, which is better absorbed and less irritating than diazepam when given IM, and other classes of sedatives, such as alpha-2 agonists, to further resolve optimal techniques of sedation to achieve clinical and laboratory goals for different species.

Future experiments will also resolve the degree to which intramuscular injection may be the best means of administering diazepam. Intranasal delivery would be desirable, because it would

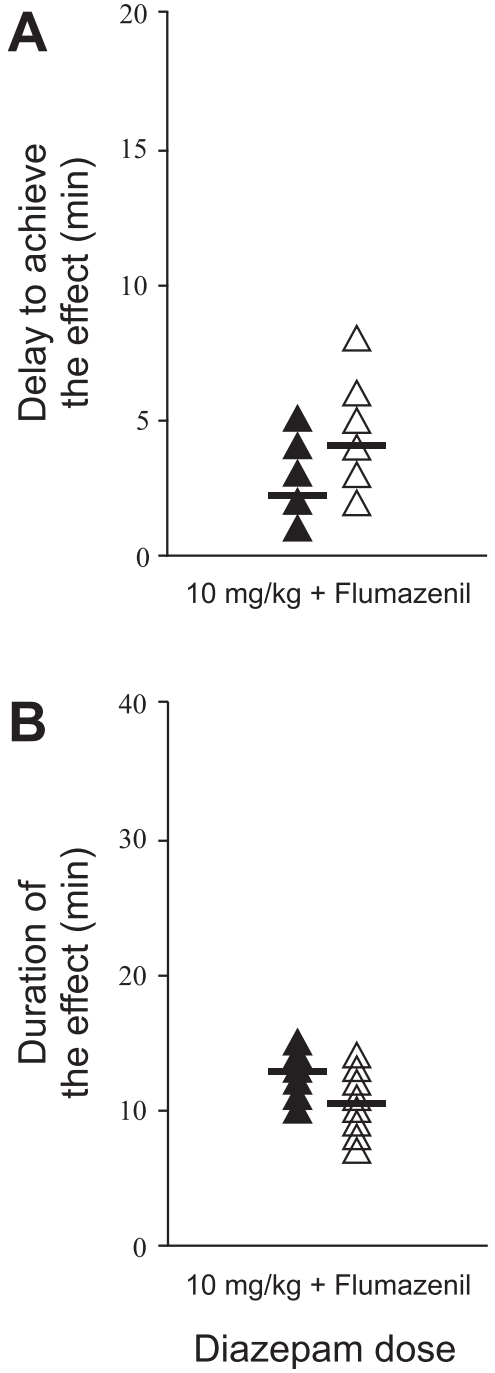

Figure 2. Diazepam sedation was rapidly reversed by the antagonist flumazenil in zebra finches. The birds that received a 10 mg/kg dose of diazepam achieved the dorsally recumbent state in all cases (N = 20, with some points overlapping). (A) All birds achieved sedation (filled symbols) and dorsal recumbency (open symbols) within 15 minutes. (B) Intramuscular injection of a 0.3 mg/kg dose of flumazenil 15 minutes after delivery of diazepam dramatically shortened the duration of dorsal recumbency and sedation (duration of sedation: 10 mg/kg without flumazenil, 283.4 ± 63.4 minutes; 10 mg/kg with flumazenil, 13.1 ± 2.9; $P < .001$; and duration of dorsal recumbency: 10 mg/kg without flumazenil, 171.1 ± 40.3 minutes; 10 mg/kg with flumazenil, 10.7 ± 2.4; $P < .001$; Wilcoxon signed rank test; solid bars indicate the population mean, note the different *y*-axis scales in this panel and Fig 1B).

avoid the discomfort associated with intramuscular or intravenous injection.[38] Previous data indicate that intranasal delivery of diazepam solution is effective in sedating canaries,[30] and another report indicates that intranasal delivery is also effective in zebra finches.[39] However, that study of zebra finches leaves open the question of what is the minimal adequate dose to achieve sedation via intranasal delivery, and it remains unknown whether intranasal methods may have the deleterious effects on lung tissue that have been reported in other species.[40] In addition, preliminary studies of intranasal administration suggest that intramuscular injection is the more reliable technique for sedating zebra finches. With intranasal delivery, many individuals shook their heads vigorously, even after restraint during administration (by following the method of Vesal and Zare[30]), expelling an unknown but substantial portion of the drug solution. This loss leads to an unknown dose and, therefore, an unpredictable duration of sedation. Future experiments will directly examine the degree to which differences in sedation reflect differences in the efficacy of individual drugs versus differences that arise from drug delivery via different means of administration.

These results led to a set of recommendations regarding the use of diazepam in zebra finch sedation. Specifically, a 10 mg/kg dose, together with local anesthetics in procedures that involve incision, is adequate to permit surgical and experimental methods commonly used in laboratory studies of the mechanisms underlying the neurobiological and neuroendocrine control of song. In addition, a 0.30 mg/kg dose of flumazenil is sufficient to reverse the effects of diazepam, inducing recovery from the sedated state within minutes. The ability to recover birds quickly after completion of a diagnostic or experimental procedure enables them to provide for their metabolic needs and thus promotes their well-being and survival.[29] More generally, diazepam is a safe method of inducing deep sedation without complication. None of the birds regurgitated, exhibited respiratory distress, or was lethargic during recovery or in the days that followed. Finally, diazepam was not only effective, but its

administration also required inexpensive materials, easily learned techniques, and only a brief period of restraining the bird. Together, these features recommend diazepam as a safe and reliable sedative for use in zebra finches.

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