Avian behavioral toxicity methods for use with the Japanese Quail Coturnix japonica as a model species

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Abstract: Establishing reliable indices of exposure to environmental contaminants can present a challenge when the effects are non-lethal and subtle. Although behavioral measures have use in toxicity tests, they are often overlooked for more traditional physiological responses. Many studies have shown that behavioral measures are not only often more sensitive, but also typically more persistent than the traditional physiological measures. The Japanese Quail (Coturnix japonica) is a useful model for avian toxicity tests due to the ease of husbandry and the short time required for individuals to reach reproductive maturity. Male copulatory behaviors are the most often used measures of behavioral alterations by chemical exposure for this species, although tests of sexual selection and locomotion have also been utilized successfully. Changes in individual behavior that result in decreased reproduction and fitness may cause downstream changes at the population level, and are therefore important to consider for inclusion in toxicity tests.

Key Words: Japanese Quail, Coturnix japonica, behavior, methods, toxicology, model species

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1. Introduction

Although behavioral toxicity tests traditionally have used rodent species, there is a growing body of publications that describe relevant studies using birds. Behavioral toxicology is a relatively recent subdiscipline of the field of toxicology. Synonymous with ethotoxicology, behavioral toxicology is the study of chemicals' effects on neural and behavioral functions. In the earliest publication referring to the behavioral effects of toxicants, Dioscorides wrote, “Lead makes the mind give way” (reviewed in Needleman, 1995). Rigorous study of the behavioral effects of toxicants did not occur...
until the mid 1960s, with the appearance of behavioral toxicology manuscripts in the peer-reviewed literature during the mid-1970s. Many of the harmful effects of chemicals on the nervous system that we now accept as common knowledge are relatively recently described; the neurological effects of embryonic exposure to alcohol was formally studied and described only as recently as 1968 (Brown et al. 1978). It is interesting to note that some of the earlier ethotoxicological studies performed as collaborative efforts between American and Soviet scientists were done with white Carneau pigeons *Columbia livia*. Inspiration for these then novel methods came from Sechenov and Pavlov’s works (reviewed in Weiss & Levine 1976). These initial experiments focused on establishing a sound approach and methodology to the budding science. Most of these early experiments used carbon disulfide, a common industrial chemical with well documented behavioral effects. As regulatory agencies became more aware of the behavioral effects of many other industrial contaminants, the need for toxicity tests to screen for potential neurotoxic effects became ever more apparent, further propelling the earlier methodology-establishing tests. Briefly, the Carneau pigeon studies determined the effects of carbon disulfide on conventional operant behavior via a chamber that rewarded birds with feed when appropriate response keys were selected (Weiss & Levine 1976). Although these initial tests with avian species were instrumental in establishing behavioral toxicology as a distinct discipline, traditional operant behavior tests are infrequently used in current ethotoxicity studies that tend to be aimed more at determining ecologically relevant endpoints.

While behavioral endpoints in toxicity tests are often recommended as part of a more comprehensive assessment, they are rarely included in the majority of reported avian tests. Many published studies have documented significant reasons for measures of behavior to be incorporated in the general suite of measurements. There is growing evidence that some chemicals cause behavioral effects at doses well below those demonstrated to cause anatomical changes (Gamberale & Iregren 1990). The brain and other parts of the nervous system are often more sensitive to chemicals than other somatic systems, with behavioral effects exhibited before many of the more traditionally measured endpoints, such as with reproduction and growth (Spyker 1975). Also, effects from exposures that occur during development may be delayed and manifested later during adulthood. Some studies have suggested that the nervous system may not be as adaptable to early life stage chemical exposures as compared to organ systems responsible for modulating more traditionally studied effects, i.e. reproduction, growth, and basic physiology (Quinn 2007a).

Though most of the current avian behavioral toxicity tests tend to focus primarily on the ecological implications of neurotoxic effects, this review will also be presented in an environmental context, which has different implications than the initial pigeon studies with carbon disulfide that was focused more on establishing standards for occupational safety. This review will also focus on behavioral tests using Japanese Quail, recognized as one of the most widely used and appropriate species for avian behavioral toxicity studies. Most of the measures described are currently used in toxicological studies. However, more innovative behavioral tests will be discussed to better understand the basic mechanisms and evolution of behaviors with hope that they will be included in future toxicology experiments.
2. Japanese Quail as a model species

The Japanese Quail and the Northern Bobwhite *Colinus virginianus* are two of the most commonly used species in laboratory-based avian toxicity tests. Both species are gallinaceous birds that are particularly useful in a laboratory setting because they breed readily in captivity, and their reproductive condition can also be easily altered through photostimulation/regression. Because both are precocial (i.e., well developed and fairly independent soon after hatching), the chicks require minimal care other than feed, water, and temperature control. In toxicity tests that assess reproductive aspects of exposure to environmental contaminants, Japanese Quail is the more appropriate model of the two species (Touart 2004). Japanese Quail mature as early as six weeks of age (compared to six months in Bobwhites) making them the preferred species when examining reproductive endpoints. Japanese Quail have also been bred specifically for egg production for centuries. When exposed to the appropriate lighting conditions Japanese Quail can reliably produce an egg daily for approximately two years after reaching maturity until reproductive senescence occurs.

Many studies that measure more basic aspects of reproductive physiology in Japanese Quail also test reproductive behavior. Recently, the U.S. Environmental Protection Agency has decided to pursue development of an avian two-generation test for potential endocrine active chemicals. This two-generation approach is being evaluated in order to evaluate the effects of endocrine disrupting chemicals (EDC) on all four key life stages where they might have the most impact on development and function: embryos, juveniles, subadults, and adults (Touart, 2004). The ability to reproduce easily in the lab and the short time required for egg incubation are two primary reasons for the selection of Japanese Quail as the test species over the Northern Bobwhite. One of the key measurements being assessed in this paradigm and used in the current one-generation test method is male copulatory behavior, one of the best characterized behaviors in this species (Ottinger & Brinkley 1978). The need to assess reproductive behavior is another reason why Bobwhites are rarely used in behavioral studies. Quinn et al. (unpublished data) have demonstrated the same methods commonly used for the Japanese Quail (described below) are not as feasible with male Northern Bobwhite. Male Bobwhites were observed to be unable to mate with females within the standard three minute time period used over three consecutive days; one male out of fifty achieved a successful copulation on an additional fourth day of testing. Male Bobwhites appeared to be too easily distracted and prone to stress to be able to focus on copulatory behavior when the female was placed in their enclosure.

The non-domesticated Northern Bobwhite’s predisposition to elevated levels of stress which can be induced in some strains while using gentle handling can make simple blood collection methods a lethal procedure in some individuals if the normal restraint required is not limited to a brief few minutes. Japanese Quail are much more amenable to being handling, making the development of an entire array of behavioral tests possible (described below).
3. Commonly used avian behavior tests using the Japanese Quail

3.1. Reproduction

Traditionally, the Northern Bobwhite and the Japanese Quail are two of the best established avian models for toxicity tests (ASTM 1990, USEPA 1985, OECD 1993). Although the current trend for toxicity tests appear to favor Japanese Quail over Northern Bobwhite for evaluating reproductive effects, some debate still exists as to which avian lab species is most appropriate. An abundance of control data from reproductive studies has been offered during the mid-1980s as a reason to prefer using the Northern Bobwhite (Piccirillo & Orlando 1985); however, numerous reproductive studies using Japanese Quail have been completed since. One of the few advantages that Northern Bobwhite proponents continue to use in their arguments is that toxicity studies that assist ecological risk assessment decisions in North America may benefit using data derived from a native species. This logic is complicated given the artificial controlled laboratory environment that is typically used for both species in these studies. It is known that Japanese Quail reach sexual maturity four and a half months sooner than Northern Bobwhite and are able to be used in common reproductive behavior tests when Bobwhites are not (Quinn et al. 2008). Additionally, when considering how non-primate data are used to derive toxicity benchmarks applicable to humans, the degree of phylogeny between these avian species is relatively close. This particular argument seems to have a demographic bias than based on apparent biological or evolutionary grounds.

An added advantage to using Japanese Quail in reproductive toxicity tests is that the hens are able to produce large numbers of eggs consistently. Although Bobwhite hens can also be photostimulated to produce eggs relatively consistently until the onset of reproductive senescence, the benefit of the Japanese Quail under laboratory stress is consistency; female Japanese Quail can produce approximately one egg per day reliably for nearly two years. Northern Bobwhites produce approximately one egg every two or three days for the same duration. Male Japanese Quail have an additional benefit over male Northern Bobwhite: the cloacal or foam gland. The cloacal gland is an external reproductive structure that develops and functions as testes mature to maintain reproductive functions. Growth of the gland begins during Japanese Quail’s short adolescent stage and is completed about 7–12 days later when the gland is able to produce thick, white foam. Although the role of this foam is unclear, current evidence suggests that it may aid in fertilization by preventing sperm aggregation and enhance sperm motility and metabolism (Singh et al. 2010). Because no similar external indicator of reproductive maturity or condition exists in the Bobwhite or most other avian species amenable to lab conditions, the cloacal foam gland is an extremely useful structure for monitoring gonadal development, reproductive fitness, and sexual maturation in the Japanese Quail (Touart 2004).

3.2. Male copulation

The use of the cloacal gland as an indicator of male reproductive maturity is a key indicator for male reproductive receptiveness, especially in studies that expose...
Quail to toxins prior to onset of maturity. Many male reproductive behavioral tests begin when at least ninety percent of males from the control treatment reach sexual maturity, as indicated by the gland’s foam production. Although copulation is instinctive, skill at the balancing the onset of reproductive maturity comes with experience. To account for this learning process, behavioral tests are conducted usually once per day with males over three consecutive days (Ottinger & Brinkley 1978). For each test, behavior is observed for three minutes as soon as a sexually receptive female is introduced into a male’s cage. Sometimes females may be aggressive, as indicated by pecking the male’s face or head, or unreceptive, recognized by a female either not allowing the male to mount or by blocking male access to her cloaca by sitting down or keeping their posterior backed against a wall or other immovable surface. These hens are to be replaced immediately. Behavioral measures include: latency to mount, latency to achieve successful copulations, number of mount attempts, and number of successful cloacal contacts. Secondary male behaviors may also be recorded, such as strutting and crowing that normally occurs immediately after a successful copulation. Where male Northern Bobwhite tend to be more easily distracted by any novel external stimuli even after females are introduced to them, normally developed Japanese Quail males primarily give the introduced females the majority of their attention from the onset of the test.

Observers should be ever mindful of aggression. It should be noted that although males that develop and mature while exposed to estrogen or estrogen-active chemicals may not appear to be different externally to human observers, they may behave atypically. Often, these males tend to shy away from the females they are introduced to, and this sometimes results in aggression from the females. Likewise, some females and males can be more aggressive than others. As a precaution against bullying, individuals of similar weights should be paired. Although bullying is usually expressed as aggressive pecks to the eyes or head, other more subtle acts may include blocking access to feed or water. While blocking of these resources is not an issue during a three minute test, researchers should be aware of this when long term pairings are initiated. These longer term pairings may be desired if fertilization and other more physiological measures are sought in the same studies that the behavioral tests are conducted. Eggs may be collected to test for fertilization at least a week after pairs or regular matings are made.

Tests that solely examine the effects of environmental toxins on the morphology and functioning of organ systems may not be sufficient to determine the complete toxicity of a compound. In fact, previous studies that have shown no physiological effects to the reproductive system following exposure to high levels of EDCs during sensitive time periods, have revealed abnormalities in mating behavior in the same animals (Quinn et al. 2007a, b). In this respect, the reproductive system of the Japanese Quail appears to be more resilient to one time embryonic exposures to chemicals than the nervous system. Intact and functional gonads do not contribute to an individual’s fitness if the performance of reproductive behavior is impaired; the reproductive machinery may be intact, but no contribution to fitness can be made if mating does not occur. Therefore, it is essential for behavioral measures to coincide with physiological ones during reproductive toxicity tests, not only because they
Japanese Quail behavioral methods are non-lethal methods for the measurement of alterations in brain development, but they also provide a method of linking physiological and functional changes with mate recognition processes (Scott & Sloman 2004).

3.3. Mate selection

A surprisingly large amount of research has been dedicated to investigating how social interactions influence mate selection in Japanese Quail. Although none of this research has yet begun to examine how chemical exposures could impact these relationships, they may show some promise for use in future toxicological studies. Summaries of these types of studies are provided in this report in the hope to inspire their adaptations and inclusions to the Japanese Quail toxicology test models. Some of the earliest work in this area established effects of female observations of familiar and non-familiar males mating on mate preference. The basic paradigm of these tests follow a similar structure of three phases (Galef & White 1998): (1) female Japanese Quail are presented to two non-familiar target males and preference for one is determined by relative time spent with each; (2) females either watch or do not watch the non-preferred males court and mate with a different female; and (3) females are presented with their original two males, and time spent with each is reassessed. The act of observing mating between a different female and a non-target male had no effects on the subject females' mate choices; however, time spent with the original non-preferred male increased over that of the preferred male when the female observed him courting and mating with a different female. Prior to these studies, female preference for males that other females find attractive was only observed in field studies involving avian leks (Höglund et al. 1990, Gibson et al. 1991). Mate copying may be an important fitness strategy in that it may help to reduce a female's mate selecting costs while increasing her chances of accurately assessing male quality (Gibson & Höglund 1992).

In most avian species (and across most taxa), female mate selection is more prevalent than that of males because females most often bear a majority of the burdens of reproduction. Shortcuts to this selection process are beneficial when they optimize females' ability to accurately select males that are more fit than others. Males are overwhelmingly less selective in their mate choices, and as a result, the behavior of male selection has remained widely overlooked. Without the burden of nesting or pregnancy, typical male fitness strategies are often enhanced by mating with as many females as possible in a mating season. This promiscuous behavior increases the male's chances of mating with a fit female as the number of females he mates with increases. With the constraints of egg production/incubation, pregnancy in mammals, and raising young, females may only mate once per mating season. Female fitness strategies are therefore optimized by accurate assessment of which one male in the population would contribute the best genes to her progeny.

White and Galef (1999) turned the tables on the above described female selection study design and began to assess social influences on mate selection in male Japanese Quail. Males were similarly presented with two females and the immediate preference for one over the other was ascertained. This preference decreased when
the male observed his preferred female not only mating with another male, but also when only courted with another male (when copulation was prevented by separation by a transparent barrier). Mating with a recently inseminated female would be a wasteful expenditure of sperm and energy, which apparently would be spent better in the pursuit and mating with a novel female. Similarly, male attraction to females has also been shown to decrease after observing brooding or other maternal cues (Ruscio & Adkins-Regan 2003).

Potentially more interesting than social influences on mating preferences for both genders is the acuity of their ability to recognize individuals. This recognition is further underscored by a variation of the above described methods that showed females, who had already established their preferences for specific males, target males mating with random females via active matrix, thin-film transistor (a type of video that provides a higher level of contrast and brightness than cathode ray tube monitors). Females were still able to recognize individual males two-dimensionally, and the observation of non-preferred males mating with other females increased their preference for those males similar to if they had observed the copulation occur live (Ophir & Galef 2003).

Mate selection has a profound effect on an individual’s fitness. Chemicals have been shown to alter physical secondary sexual characteristics in birds, such as feathers and soft part coloration, and behaviors that may serve to convey overall mate quality to potential suitors. As such, the assessment of how chemical exposures may impact the effects of more complex social interactions on mating would be a welcome addition to the established reproductive tests that use Japanese Quail as model species. In particular, quantitative assessment of female selection should complement the commonly used male copulation tests, allowing for a better overall assessment of potential reproductive effects of chemicals on both genders.

3.4. Motor behavior

Motor behavior in chicks can be easily measured in a laboratory setting in a runway-type arena, and is best assessed in chicks at week one and/or week two of age. In a method described by Quinn and Ottinger (2008), a runway 182 cm long with five parallel 12 cm wide lanes was used. A group of about ten randomly selected conspecifics is placed at one end of the runway, and individual chicks are then separated from the group to the opposite end of the runway and allowed to call and return to the main brood. The separated individuals can be given a maximum amount of time to reach conspecifics; three minutes should be adequate. Basic measures may include: the amount of time it takes individuals to reach conspecifics, the total distance travelled, and the number of lanes crossed within the time allotted.

This motor behavior test assesses an important survival skill: locating and returning to the brood when separated. Japanese Quail are precocial birds, with the ability to leave the nest and find their own food at day of hatch. It is necessary for chicks to remain with the brood after hatch for protection from predators and for thermoregulation; chicks are unable to effectively moderate their own body temperature prior to development of adult plumage. If a chick does become separated
from its brood, it typically calls to its conspecifics. Siblings then vocally respond to the chick's separation call, helping the separated individual to locate and return to the rest of the brood.

While this test was originally designed to test motor behavior, communication among conspecifics is also assessed. In Quinn and Ottinger (2008), the most interesting (and unexpected) result was the complete absence of vocalization from the treatment groups that received 50 μg of trenbolone acetate, an androgenic chemical, at day four of incubation. Although vocalization in this study was expressed as the percentage of individuals per treatment that performed stress vocalizations in the first trial and the total number of vocalizations made per individual in the second trial, future studies may record vocalizations and analyze them digitally for effects more subtle than presence vs. absence of sound.

Other avian species vocalize for a variety of additional critical behaviors to ensure survival and fitness: food begging, mate attraction, sexual selection, and territory defense. The significance of the myriad purposes of avian vocalization and the relative ease with which it can be measured support its inclusion in behavioral toxicity tests.

3.5. Righting response

The righting response is one of the more easily measured responses expressing fear and is used relatively frequently by the poultry industry. While ecological studies may be more interested in how fear contributes to survival, the poultry industry is concerned mainly with its effects on egg laying or growth (Jones 1996). The autonomic nervous system is the main part of the brain that is assessed in this test; however, this is not the only area that modulates stress. Because a test subject's perception of stress strongly influences individual responses, it is suggested that areas of the brain other than the hypothalamus may also play a significant role (Valence et al. 2008).

The righting response test is accomplished by placing individual birds on their backs and restraining them in this position for about 15 seconds. Once tonic immobility is achieved, the time taken for the bird to return to an upright, standing position is recorded. An additional measure that is often taken following the righting behavior is vocalization, as it serves as a warning to other individuals in social species (Saterlee et al. 1994). Although this is a fairly easy measure of a basic survival mechanism, tonic immobility is not always the primary fear response in all species. Tests using species other than Japanese Quail may more appropriately use other tests to measure panic, hysteria, and fleeing as appropriate. The righting response test may also not be best suited for all life stages of species other than Japanese Quail. For example, it is almost impossible to create tonic immobility in Northern Bobwhite chicks. A Bobwhite chick’s normal reaction to an initial stress is for the brood to disperse in random directions to confuse potential predators before remaining still. Fortunately for laboratory studies that do use Bobwhites, tonic immobility in adults is often achieved relatively easily by lightly restraining individuals on their backs for about five seconds.
4. Summary

One of the primary indications of behavioral toxicity tests is how exposure to chemicals alters baselines of the behaviors investigated. Natural behavioral baselines need to be known and understood in the ecological context from which they are derived so the biological significance of any changes can be appreciated. The argument has been made that some examined behaviors are used as biomarkers often without concern for the context in which the behavior was being observed (Parmigiani et al. 1998). Quantification of behavioral endpoints should be more than attaching numbers to arbitrarily selected measures (Weiss 1988). For example, if mean cloacal contacts in chemically-treated birds are one per three minutes of testing and are statistically significantly less than a control mean of four, is it biologically significant as well? How many copulations are typically required to result in a successful fertilization? The three minute test duration is used not only to pair adequate time in which to mate, but also to allow researchers to be able to reasonably test sixty or more pairs per day. Is this three minute interval an appropriate time in which to establish a baseline for mating behaviors? Although it may not be possible to answer all questions regarding the biological significance of statistically significant differences in behavior, it is important that they are given proper consideration. Because toxicological data are often used in support of risk assessment, it should ideally test aspects of individual survival, environmental fitness, or both. This differs from the more industrial/occupational-minded approach that additionally tests for potential chemical effects on behaviors that may alter job performance.

References


