A BEHAVIORAL PROCEDURE FOR MEASURING
CRITICAL FUSION FREQUENCY IN RATS

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A BEHAVIORAL PROCEDURE FOR MEASURING CRITICAL FUSION FREQUENCY IN RATS

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John Charles Heath

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THESIS ABSTRACT

A BEHAVIORAL PROCEDURE FOR MEASURING

CRITICAL FUSION FREQUENCY IN RATS

John Charles Heath

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The ingestion of methylmercury (MeHg) has been found to adversely affect primate and human visual fields and contrast sensitivity (Choi, Cho, & Lapham, 1981; Clarkson, 1989; Gilbert & Grant -Webster, 1995; Merigan, 1980; Rice, 1994; Rice & Gilbert, 1982). The frequency at which a flickering stimulus is perceived has been related to both phenomena and to possible damage to the parvocellular or magnocellular visual neural systems. The rats visual system, although different from the human visual system contains parvocellular regions related to their visual perception. A series of experiments were conducted to develop a method for testing the rats visual perception to a flickering stimulus. A behavioral discrimination procedure was developed using four Long Evans male rats. Three testing methods were used. No significant difference was found between or within subjects or between and within methods for the frequency at which a flickering stimulus is perceived as a steady stimulus.

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CHAPTER I

INTRODUCTION

Methylmercury (MeHg) is a known neurotoxicant that can be ingested directly or passed to the fetus from the mother via the placenta. Aversive effects on the functioning of the visual system, that have been observed in primates who were exposed during adulthood are differences in detecting a flickering light and constriction of the visual field, (Merigan, 1980). Changes in critical flicker fusion frequencies and contrast sensitivity were observed in monkeys subjected to developmental exposure (Rice & Gilbert, 1982, 1990). Conversely, docosahexaenoic acid (DHA), an omega 3 fatty acid, is important for the development of normal vision. Diets rich in DHA have been shown to have possible advantages over the development of visual acuity. Given the detrimental effects of MeHg and the beneficial effects of DHA, the possible interaction of the two deserves attention.

The initial objectives of this study were to observe the effects of MeHg exposure on vision in rats and to observe any interaction between DHA and MeHg. Subjects were the offspring of Long Evans rats that were exposed to 0.0 ppm, 0.5 ppm, or 5.0 ppm of mercury, as methylmercuric chloride, in their drinking water of which half received a diet high in DHA and half a diet deficient in DHA.

Forty-five subjects were present at the beginning of training, but by the end of the experiment, only 28 subjects survived. The reason for this poor survival rate was urolithiasis, which is the formation of kidney stones, caused by contamination in the diet. This problem was unrelated to the experiment (see appendix B). The presence of

urolithiasis made it difficult to assess whether the behavioral techniques used to try to establish a critical flicker fusion frequency in the subjects were responsible for the results that were recorded or whether they were prejudiced by the effects of urolithiasis. Furthermore, it became apparent as the data accumulated that the original approach was too ambitious and complex to achieve a psychophysical function from which the CFF could be calculated. Therefore, the emphasis of the experiment was changed. The factors of MeHg exposure and DHA diet were eliminated in favor of developing a reliable method for the measurement of CFF in rats that could be used in future developmental studies involving MeHg intoxication.

Methylmercury as a Neurotoxicant

The first well documented large-scale contamination by methylmercury (see appendix A) in a population was in Minamata Japan in 1956 (Harada, 1997), although it was not until 1957 that MeHg was suspected as the toxic agent. In 1959 it was reported that what had come to be known as Minamata Disease was most probably caused by acute MeHg poisoning. (Watanabe & Satoh, 1996). The signs and symptoms of Minamata disease include abnormal gait, dysarthria, ataxia, deafness and constriction of the visual field (Watanabe & Satoh, 1996).

Fetal Minamata disease was first detected in 1958. Fetal Minamata disease was diagnosed in young children whose mothers had been exposed to MeHg during gestation, at some stage in the Minamata exposure period, but who themselves had never been directly exposed to the compound after birth. The symptoms that these children portrayed

included mental retardation, cerebella ataxia, enhanced primitive reflexes and dysaphia. In addition, a high proportion of the children had seizures and pyramidal signs (types of pyramidal signs not reported by Watanabe and Satoh). Because of the severe condition of the children, sensory disturbance including visual function could not be examined (Watanabe & Satoh, 1996).

The mothers of children with Fetal Minamata disease appeared healthy when it was diagnosed, later a high percentage of these mothers developed signs or symptoms of Minamata disease and 57% experienced constriction of the visual field.

Direct exposure to MeHg is characterized by local lesions in the cerebellum with atrophy of the granule cells, preferential injury to the calcarine fissure, an area associated with visual field function,(Grusser & Landis, 1991) in the occipital lobe and other cortical regions associated with sensory function especially the precentral gyrus (Takeuchi, Morikawa, Matsumoto, & Shiraishi, 1962).

In-utero intoxication of MeHg from the mother to the fetus via the umbilical cord is especially insidious as the immature nervous system is especially sensitive to MeHg toxicity and the fetal brain may be affected even if the mother shows no sign of poisoning. High dose exposure may result in blindness, cerebral palsy, deafness and severe mental retardation while lower levels of MeHg could produce deficits in vision and hearing, delayed walking and speech development (Castoldi, 2001).

Visual Defects and Methylmercury

Although reports of visual defects by MeHg poisoning are frequent there has been little systematic assessment of vision performed in the evaluation of methylmercury poisoning (Faubert & Bellavance, 2003).

Acute and chronic exposure of MeHg appears to have different effects on the visual system. In addition, the effects of direct versus in-utero ingestion show marked differences in the brain.

Constriction of the Visual Field

As far, back as 1865 there were two cases of dimethylmercury poisoning in which either poor vision or blindness was referred to as a symptom. Constriction of the visual field was reported in 1940, in four cases of MeHg poisoning, by Hunter (Watanabe & Satoh, 1996).

Various studies of victims of the Minamata disaster, both from direct exposure (Hamada et al., 1993; Korogi, Takahashi, Okajima, & Eto, 1998; Kurland, Faro, & Sielder, 1960; Mukuno, Ishikawa, & Okamura, 1981; Uchino et al., 1995), and fetal exposure (Choi, 1986; Hamada et al., 1993; Takeuchi et al., 1962; Watanabe & Satoh, 1996) to MeHg report constriction of the visual field. Constriction of the visual field was also reported from victims of MeHg poisoning, in Iraq, in 1972, (Clarkson, 2002).Thus the reports of constriction of the visual field due to ingestion of MeHg are numerous. Furthermore, Merigan, in a series of experiments using macaque monkeys reported constriction of the visual field as a result of mercury poisoning (Merigan, 1980).

Studies in which constriction of the visual field, attributed to exposure to MeHg, were observed have reported no damage to the retinal area of the eye. This would suggest that the damage that occurs to vision may be further back in the visual neural pathway, possibly in the area of the calcarine fissure (Kurland et al., 1960).

In a study on the pathology of brain tissue of victims of Minamata disease, specific areas were identified as being damaged by MeHg. These included the cerebellum, the cerebral cortex and areas of the occipital lobe in the region of the calcarine fissure (Takeuchi et al., 1962). From photographs presented in this study severe damage can be seen in area 17, [also known as area V1] (Grusser & Landis, 1991), which is located at the mesial surface of the occipital cortex and at the posterior end of the outer surface of the occipital lobe (Grusser & Landis, 1991). Because of the complexity of the visual neural structure in this region, different lesions of area V1 may cause different types of visual field defects. The visual field defects caused by lesions to the occipital lobe can vary in almost all dimensions, ranging from a small areas of a single quadrant to practically total loss of visual field, except for the foveal area, as is seen by the illustrations produced by Wilbrand and Saenger. The loss of visual field is dependent on the size and location of the lesion (Grusser & Landis, 1991).

Contrast Sensitivity

Contrast is defined as the ratio of brightness of adjacent parts of a pattern (Leventhal, 1991), thus contrast sensitivity could be described as the ability to distinguish between the relative brightness of two adjacent parts of a pattern.

Subtle effects in contrast sensitivity were reported in a study conducted on six-year-old children in East and West Germany. A significant difference was found in children with higher urine Hg levels at spatial frequencies of 1.5 and 3 cycles/degree in the right eye and at 18 cycles/degree in the left eye. (Altmann et al., 1996). These results seem a little ambiguous when comparing the frequencies for each eye, although no explanation is given for this. In addition testing spatial contrast sensitivity, using the Arden test, has shown abnormal results for victims of Minamata disease (Mukuno et al., 1981). The Arden test is a test of contrast sensitivity that consists of five charts the first four of which measure one specific spatial frequency and the last that measures two spatial frequencies. The range of the charts are from 0.2cycles per degree to 6.4 cycles per degree. The contrast of each sheet increases logarithmically by 0.88 log units per centimeter in a vertical direction (Woo & Prentice, 1983). The Arden charts are considered a sensitive tool for patients with lesions in the cerebral cortex (Mukuno et al., 1981).

A visual contrast sensitivity test was used on second and fourth grade children, in Bohemia, in the Czech Republic, with some negative correlations between Hg hair levels and contrast sensitivity at spatial frequencies of 1.5 cyc/deg and 6 cyc/deg. However, the negative correlation was only found in the second grade children and not the fourth grade children. It was suggested, that the effects are not permanent, but rather due to developmental delay (Hudnell et al., 1996).

Flicker, Contrast Sensitivity, Visual Field and Luminance

A common factor that is associated with visual field and contrast sensitivity is the effect that both have on the ability to detect a flickering light.

Flicker fusion is the point of transition of a visual stimulus from an appearance of flicker to an appearance of steady light i.e., a flickering light is perceived as a steady light. The critical fusion frequency (CFF) is the frequency at which this transition occurs. (Brown, J. L. p251). The CFF is affected by certain parameters that must be kept constant. These include background adaptation with the light stimulus (contrast), and definition of the flicker stimulus by such factors as average luminance, and size and depth of modulation.

In the human eye, for small flickering targets (a diameter of 0.5 to 1.0 degrees of visual field), the CFF decreases from the center of the visual field to the periphery. However, with a larger flickering field the opposite is true, with a higher CFF at the perimeter and a lower CFF at the fovea (Grusser & Landis, 1991).

In animal studies, a relationship has been found between contrast sensitivity and flicker. Primates exposed to MeHg had deficits in their ability to detect flicker and the contrast required to detect fast flicker was higher than in controls (Merigan, 1980). Furthermore, Post-natally exposed monkeys were superior to controls at detecting flicker under low luminance conditions while monkeys exposed in-utero and post-natally showed impairment at middle to low flicker frequencies under high luminance conditions (Rice & Gilbert, 1990).

Thus, although most testing of human victims of MeHg intoxication with visual defects has been on contrast sensitivity and visual field function, the ability to detect flickering lights at different levels of contrast sensitivity and at different luminance levels appears to be affected by MeHg ingestion in primates.

It could therefore be suspected that because visual field function and contrast sensitivity both have an effect on the detection of a flickering stimulus the measurement of the CFF under different conditions may give an indication of alterations to the visual system caused by MeHg ingestion.

Flicker Fusion

Flicker fusion is a well-documented method of testing for visual field limitations and as a measure of the visual system, has been investigated for more than 250 years. An annotated bibliography of flicker fusion from 1740 to 1952 reported some 2000 titles, (Landis, 1953) . In a seminal paper on the laws and mechanisms of the critical frequency of fusion, Pieron gives a short history of the theories of CFF and a synopsis of the various theories pertaining to CFF.

In 1765, Sir d'Arcy investigated the phenomenon of the persistence of the sensation of a flash of light after the flash had ceased and the duration of that persistence. Plateau, in his 1829 thesis was the first to determine a flicker fusion frequency. Charpentier first measured the effect of luminance on the magnitude of the persistence effect, but it was Ferry in 1892 that determined that "the value of the period", the reciprocal of the critical frequency, that measures persistence of a luminous stimulus, varied inversely with the

logarithm of the stimulating intensity. This was verified by Porter independently a few years later, and thus became known as the Ferry Porter Law (Pieron, 1965).

It was then thought that persistence was the factor that determined CFF and that persistence time was determined by the longest dark interval that yielded complete fusion of the luminous sensation. However as research developed Hecht established that the CFF of rods was different to that of cones, thus determining that as well as persistence, the latency of the photochemical reaction to light and the absence of light was integral in the phenomenon of flicker fusion (Pieron, 1965). In 1938, Crozier presented evidence that the CFF rises as a sigmiodal curve as a function of the logarithm of illumination. His theory was that CFF levels were a probability curve controlled by an integration that corresponds to a progressive recruitment from a population of natural elements, (Pieron, 1965). Pieron tends to support this statement by surmising that CFF depends on five factors. These are heterogeneity of the retina (rods and cones), luminance, phase relations, the size of stimulated surface of the retina, the duration of observation and the repetition of the flicker cycle (Pieron, 1965).

Critical fusion frequency in human research has been used in many different areas as a measure of different functions of perceptual and physical qualities. In pharmacology, the CFF was significantly reduced for one to seven hours after the administration of promethazine, an antihistamine (Hindmarch, Shamsi, & S., 2001). A study on the effect of procyclidine, an anticholinergic used to control extrapyramidal side effects of antipsychotic drugs in schizophrenia, found that a dose of 15 mg reduced both CFF and heart rate (Sharma et al., 2002). As a measure of fatigue, it has been employed in research into

circadian rhythms and jet lag in which east west intercontinental flight was found to have a significant effect on CFF (Hauty, 1967). Looking at the effects of alcohol it was found that the CFF was not significantly affected (Liguori, D'Agostini, Dworkin, Edwards, & Robinson, 1999), however, when looking at the sleep patterns of nine female family caregivers it was reported that when fatigued the CFF was lower (Sato, Kanda, Anan, & Watanuki, 2002). Critical fusion frequency has been investigated as a diagnostic tool for the early detection of Alzheimer Disease. The descending component of CFF was found to be significantly different in patients who met DSM-IV criteria for Alzheimer Disease (Curran, Wilson, Musa, & Wattis, 2004). It has also been suggested that flicker sensitivity at medium and high temporal frequencies showed impairment of the magnocellular channels in negative symptom Schizophrenics (Slaghuis & Bishop, 2001). An investigation into the relationship of CFF to blood glucose level (BGL) in diabetics found that there was a correlation between the naturally fluctuating BGL and CFF, suggesting the feasibility of a non-invasive test of BGL for diabetics (Castano, Wang, & D., 2000).

Methods of Testing Flicker in Humans

A test that incorporates both flicker and contrast is the full field flicker test also known as the Erlangen Flicker Test (Nguyen et al., 2002). This method is used to measure temporal contrast sensitivity or the contrast sensitivity level at which a flicker can be detected against a steady luminance background.

The Erlangen flicker test has no need for fixation or dark adaption. The test consists of a white flickering light presented to the patient in a full field bowl. A

flickering stimuli is presented and the contrast between the stimuli and the background luminance is gradually increased until the patient reports seeing a flicker. It is then decreased until the flicker is undetectable (Horn, Jonas, Korth, Junemann, & Grundler, 1997). In using this test on patients to detect early diagnosis of chronic open angle glaucoma Horn et al., stated the full field flicker test can detect optic nerve damage in patients with increased intraocular pressure, glaucomatous optic disk abnormalities and normal visual fields. Temporal contrast sensitivity as determined with the full-field flicker test was significantly higher in the normal control group than in the preperimetric glaucoma group, which was significantly higher than in the perimetric glaucoma group (Horn et al., 1997). In a later study, the same test was used in patients, who had undergone penetrating keratoplasty i.e., cornea transplant that also had glaucoma. Patients with early glaucoma and no visual field loss had a temporal contrast sensitivity (TCS) of 1.36 ± 0.23, while patients with glaucomatous visual field defects had a TCS of 1.07 ± 0.33. The contrast threshold correlated significantly with functional glaucomatous damage such as visual field defects (Nguyen et al., 2002).

In a pilot study to try to determine a simple test for methylmercury induced visual defects Faubert and Bellavance used a similar protocol to the Erlangen flicker test. The aim of the study was to find a simple, portable visual test that could be used in indigenous populations, such as the Cree of Northern Quebec, and was reliable in showing an effect of MeHg on the visual system. The dependent measure used in this study was temporal modulation fields, which includes contrast sensitivity for small slow flickering targets and large fast flickering targets. This evaluates the contrast sensitivity of an individual,

throughout the visual field with different spatio-temporal combinations. A description of the test states that the stimulus was modulated in time about a mean luminance in different parts of the visual field. The test was carried out on a laptop computer. The conclusions of the study was the assessment would prove useful and reliable to assess for neurotoxic effects of MeHg (Faubert & Bellavance, 2003).

An interesting finding from this study was the effects of MeHg contamination on one particular individual designated M09. At a flicker rate of 2 Hz at a 20^0 visual angle the subjects log contrast sensitivity is reduced significantly compared with a control group, i.e.' it takes a greater contrast to detect a flickering stimulus, however at a flicker rate of 16 Hz his sensitivity curve is normal. This represents a constriction of the visual field at the lower frequency flicker but not at the higher flicker frequency because the log contrast sensitivity decreases with an increase in eccentricity at the lower frequency but not at the higher frequency. Unfortunately, individual Hg levels are not reported in this paper therefore we do not know the level of contamination of subject M09 or even whether the MeHg was the only contributing factor.

The importance of flicker in its sensitivity to different types of damage to the visual system, as well as its usefulness in helping with the diagnosis of other conditions would suggest it is an effective method for assessing visual defects. When we consider that visual field loss and change in contrast sensitivity are both symptoms of different types of MeHg intoxication, and that flicker is a proven tool in detecting both of these phenomena, it becomes important to assess the use of flicker in animal studies of MeHg related visual defects, in order to learn more about the damage caused by MeHg.

Animal Studies of the Visual Effects of Methylmercury

Animal studies involving the effects of methylmercury are numerous e.g,(Baraldi, Zanoli, Tascedda, Blom, & Brunello, 2002; Bemis & Seegal, 1999; Gunderson, Grant-Webster, Burbacher, & Mottet, 1988; Newland & Rasmussen, 2000; Newland & Reile, 1999; Newland, Yezhou, Logdberg, & Berlin, 1994; Rice, 1998) with the two main species being either species of monkey (Gunderson, Grant, Burbacher, Fagan, & Mottet, 1986; Rice, 1998) or rats (Baraldi et al., 2002; Bemis & Seegal, 1999; Newland & Rasmussen, 2000; Newland & Reile, 1999; Newland et al., 1994). However behavioral studies on the visual effects of MeHg have been almost exclusively in monkeys (Evans, Laties, & Weiss, 1975; Merigan, 1980; Rice & Gilbert, 1982, 1990).

The ability to detect flicker and visual field constriction deficits were both found in primates with chronic and high exposure levels to MeHg. In addition, the contrast required to detect fast flicker was higher in the primates exposed to the toxicant. (Merigan, 1980). When temporal visual functions were tested with monkeys that had been exposed post-natally, to MeHg it was found that the exposed animals could detect flicker at lower contrast sensitivity than controls under low luminance conditions. Under high luminance conditions, some of the subjects could also detect a low to middle flicker frequencies at lower contrast sensitivity levels than control subjects.

Some monkeys, exposed in-utero and post-natally, when tested under high luminance conditions showed impairment at low flicker frequencies i.e., it required a higher contrast for them to perceive the flicker. Like the post-natally exposed only group,

these subjects could detect a flicker at lower contrast sensitivity levels, to controls, under low luminance conditions (Rice & Gilbert, 1990).

Parallel Pathways

The neural passage, in humans and primates, from the retina to the visual cortex via the lateral geniculate nucleus is strongly dominated by two distinct pathways, the magnocellular and the parvocellular. Approximately 90% of the neurons that leave the retina follow these pathways. Although parvocellular and magnocellular pathways are different, they have some common characteristics, and ranges of sensitivity.

Magnocellular pathway cells are often reported to be responsive to higher flicker frequencies than parvocellular cells, however, this difference is only approximately 15% in peak temporal frequency and there is a substantial overlap between the two classes of cells. Lesions to the magnocellular pathway show that the parvocellular pathway detects higher flicker frequencies at a lower contrast sensitivity, while lesions to the parvocellular pathway demonstrate that lower flicker frequencies are detectable at lower contrast sensitivity levels (Merigan & Maunsell, 1993).

Faubert and Bellavance maintain that if MeHg produces selective damage to the parvocellular pathway then we should expect to see lower flicker frequencies detectable at lower contrast sensitivity levels. Conversely if damage was to the magnocellular pathway detection of higher flicker frequencies would require a greater contrast to detect large objects (Faubert & Bellavance, 2003).

Rice and Gilbert speculate that some of the results they obtained may have been caused by damage to the parvocellular pathway because MeHg is known to preferentially damage parvocellular neurons in visual areas of the brain. Their speculation is based on the theory that the parvocellular pathway is more responsive to slow flicker frequencies, while the magnocellular pathway has good high flicker frequency resolution (Rice & Gilbert, 1990).

From the information presented, it is apparent that three factors are important in determining visual damage caused by MeHg; temporal frequency, contrast sensitivity and luminance. The ability to detect temporal (flicker) frequency appears to depend on the contrast and luminance levels at which the stimuli are presented. In addition, the ability to detect temporal frequencies at high and low luminance levels may give some indication of damage occurring at different sections of the visual neural pathways.

The limited research that has been accomplished in the area of visual defects caused by MeHg intoxication has been either studies with human populations that are prone to MeHg ingestion because of diet, or experimental studies with primates. Both types of research have yielded valuable information, however, research using other species and especially longitudinal studies investigating the visual defects caused by MeHg ingestion have yet to be conducted. Studies involving other species may generate data that could be beneficial in this area of research.

Vision in Rats

Although the visual system of rats consists of a small subset of the visual system of humans (Rice, 1994) and may not normally be considered a good candidate for visual testing, their visual system does possess certain qualities that may be advantageous.

Because the rat visual system appears to be dominated by magnocellular connections and very little of that system utilizes parvocellular cells, it may seem that the rat is not a good candidate for testing visual impairment caused by MeHg. This is especially true given that both Rice and Gilbert, and Faubert and Bellavance consider that it is probable damage occurs in parvocellular cells (Faubert & Bellavance, 2003; Rice & Gilbert, 1990). However, there are reports of parvocellular activity within the visual system.

In the ventral lateral geniculate nucleus of the rat brain there are two layers. These can be characterized by magnocellular and parvocellular cells which are separated by a thin cell free section. The magnocellular laminar connects heavily with dorsal thalamic nuclei that are interconnected heavily with visually related functions. The parvocellular laminar also connects heavily with dorsal thalamic nuclei, however few of these nuclei impinge directly with visually related functions (Kolmac, Power, & Mitrofanis, 2000).

Swanson et.al., reported two parvocellular projections from the caudal region of the ventral lateral geniculate nucleus (Swanson, Cowan, & Jones, 1974). However, Coolen et.al., based on a study tracing neuronal pathways in the rat thalamus state that the lateral parvocellular subparafasicular nucleus appears to be important for processing visual stimuli

based on the inputs to this area from the medial and lateral geniculate nuclei and the visual cortex (Coolen, Veening, Wells, & Shipley, 2003).

Although the visual neural pathways of rats lack the distinct parvocellular pathways of the Human visual system, nevertheless it appears parvocellular cells play a significant role in processing visual stimuli. It is speculated that damage to these cells would affect the visual system of rats. Furthermore, a behavioral method of testing rats vision that incorporated temporal and luminance factors may well be able to detect deficits to the rat visual system that occurs from MeHg ingestion.

The choice of strain of rat is important because the visual ability of some strains is better than others. The use of the Long Evans strain of rat, especially the male, would be advantageous for two reasons. Long Evans rats were reported to have significantly better visual acuity than albino strains of rats (Prusky, Harker, Douglas, & Whishaw, 2002), also the male Long Evans has approximately 19% more neurons in its binocular and monocular regions, in area Oc1 of the primary visual cortex (Nunez, Sodhi, & Juraska, 2002). Whether the increase in the number of neurons attributes anything to improve the vision in the male Long Evans rat as apposed to the female is speculative, however, it may be an advantage.

Before the rats visual system could be investigated in this manner, it requires a method of testing temporal sensitivity i.e., flicker fusion that can reproduce a reliable CFF over time without factors pertaining to the method influencing the value of the CFF.

Behavioral methods that are non-aversive and employ positive reinforcement may be applicable for this purpose, and thus could be used for both developmental as well as longitudinal studies.

Rats and Flicker Fusion

A number of studies have been conducted on rats in connection with using flickering stimuli, (Goldzban & Clark, 1955; Ison, Bowen, & Del Cerro, 1998; Legg & Turkish, 1983; Mendelson & Wells, 2002; Wells, Bernstein, Scott, Bennett, & Mendelson, 2001; Williams, Pollitz, Smith, & Williams, 1985). The techniques used in the majority of studies have employed either aversive stimuli (Goldzban & Clark, 1955; Ison et al., 1998; Williams et al., 1985), or a surgical technique involving a craniotomy to insert electrodes into the visual cortex to test specific areas of the visual system, (Mendelson & Wells, 2002; Wells et al., 2001).

Reported CFF Levels in Rats

Luminance is the measure of light leaving a surface in a given direction.(Ditchburn, 1976) (p.380). In order to report a reliable value for the CFF of rats it is necessary to report the luminance level of the stimulus that is used for testing. Lower values of luminance are associated with lower CFF values (Grusser & Landis, 1991).

The study by Goldzband and Clark reported the CFF of 12 rats, with a mean value of 24 Hz. Individual results ranged from 31.4 Hz to 22.8 Hz however no luminance levels of the visual stimuli were reported (Goldzban & Clark, 1955). When recording single unit responses to flickering stimuli in cells in the rats visual cortex a mean value of 21.54±6.0 Hz was documented for the CFF. The luminance value reported in this experiment was 17 cd/m^2 (Wells et al., 2001). Another study, which used behavioral methods, reported a

CFF of 21 Hz using a full visual field. The log luminance value of –1.3 foot-lamberts, reported, converts to log luminance value of -.07 cd/m^2. (Williams et al., 1985).

Other studies have used flickering stimuli to study additional visual phenomena in rats without calculating or reporting the CFF. A study on the temporal processing of the visual system in rats used a flickering stimuli to examine visual persistence in young and aged rats, but did not measure the CFF (Ison et al., 1998). Similarly, low frequency flickering stimuli were used in a discrimination procedure using two choice runways, to study learning in rats prior to and after receiving lesions to the thalamus (Legg, 1988).

Experimental Methods

Different methods for obtaining the CFF in rats have been utilized. A technique using positive reinforcement has been developed in which a discrimination choice was presented to rats by means of a runway at the end of which were two transparent swing doors with a computer monitor behind each door. Food was used to reinforce the discrimination of a flicker stimulus on one screen from a uniform field on the other screen (Legg & Turkish, 1983). This technique produced good discrimination, and tests at different visual angles were possible by moving the computer screens further away from the transparent doors. The limitation, however, was that the eyes could not be tested separately (without surgically closing one eye) and the position of the angle of vision was controlled by the viewing distance measured from the response door to the face of the display. The angle of vision therefore was only accurate when the eyes were in the position from which the angle was measured. If the eyes of the subjects were in any other position,

the angle of the visual field becomes a relative angle. Furthermore, it could be argued that by changing the distance of the stimulus to simulate a change in angle of vision, what is being tested is flicker discrimination at different distances from the subject and not a change in visual angle. Any effects on motor function caused by MeHg may also have an effect on the animals ability to respond, by impeding its ability to open the doors, or increasing the time it takes to open the door. This inability to respond correctly because of motor dysfunction could be interpreted as visual dysfunction.

Use of Aversive Stimuli

Studies measuring shock induced positioning and fighting in rats suggest that the amount of positioning and fighting changes with the amount of early handling of the rat (Erskine, Stern, & Levine, 1975; Thor, Ghiselli, & Ward, 1974). Positioning is defined as rearing and facing another animal in a stereotype fighting posture. No fights occurred in the absence of shock however, early handled animals and late handles animals fought significantly more than animals that were not handled in the presence of shock. Positioning activity was also higher for males than females (Erskine et al., 1975). The occurrence of fighting was observed to increase as the number of sessions increased. This was true of the handled males and females and the non-handled males. After 10 sessions there was also a significant difference between the males and the females, with the males showing a greater amount of fighting behavior (Thor et al., 1974). If fighting and positioning are construed as aggressive behavior then it is suggested that the handling of subjects who repeatedly

experience aversive stimuli, in a longitudinal study, may become a significant problem. This may be especially true of male subjects.

The advantages of using non- aversive stimuli are two fold. First, from an ethical prospective, if non-aversive stimuli can be used successfully then the use of aversive stimuli, and any subsequent distress to the subjects, becomes an unnecessary factor and should be avoided. Secondly, the handling of a comparatively large number of subjects that have been continually subjected to aversive stimuli over a long period can create a situation where personnel are at risk of being injured by the subjects. This may be especially true of personnel who have little experience handling such subjects.

Methods of Determining Psychophysical Functions

In order to determine the threshold of a sensory stimulus it is necessary to present stimuli of different values, both above and below the sensory threshold, to the subject and allow the subject to determine whether they perceive the stimuli or not. Three classical methods exist which have a common objective, which is to determine the physical value of the intensity of the presented stimulus to which the subject responds that they perceive the stimulus a fixed percentage of the time (usually 50%). The three methods are the method of adjustment, the method of constant stimuli and the method of limits or serial exploration (Green, 1966).

The method of adjustment requires that the stimuli be presented either well above or well below the threshold and the subject adjusts the stimuli until it is barely perceived. The trials are presented many times, and require the active participation of the subject.

The threshold is considered the mean of the values at which the subject discloses that they perceive the stimulus (Gescheider, 1997). Because the subject takes an active role in changing the value of the intensity of the stimulus the method of adjustment is not well suited for animal subjects.

In the method of constant stimuli, the stimulus is repeatedly presented in a random order at a series of different values. The lower value of the intensity is below the threshold, while the upper value is above the threshold. The subject responds either a yes or no when the stimuli are presented and the threshold is determined as a function of the stimulus intensity. Variation of biological measurements tends to be normally distributed. When the proportions of responses to the value of the stimulus intensity are plotted against the stimulus intensity e.g., flicker frequency. The result is usually a normal distribution curve. The ogive curve is a cumulative form of this distribution and describes how the proportion of cases below a point on the normal distribution increases as the magnitude of the measurement increases (Gescheider, 1997). Thus, the probability of a yes response, for each stimulus intensity, when plotted against the stimulus intensity will be described by an ogive curve if the distribution is normal.

If the distribution is normal then the ogive curve has the property that when the probabilities are converted to z scores and are plotted against the intensity, the resultant curve can be described by a straight line. As the mean of a standardized distribution is zero then the value of the intensity of the stimulus that crosses the line when the z score is zero will equal the z score of a 0.5 probability of a yes response. Thus this value can be considered the threshold of the intensity of the stimulus (Gescheider, 1997).

In terms of flicker, the value of the flicker frequency when the Z score is zero will represent the frequency at which the response to whether the subject perceives the frequency as a flickering light has a probability of 0.5. Conversely, the probability of the subject not perceiving the stimulus as a flickering light i.e., the stimulus is perceived as a steady light, is also 0.5. This frequency can then be considered the critical fusion frequency or the lowest frequency at which a flickering stimulus is perceived as a steady stimulus.

When the method of limits or serial exploration is used, stimuli of differing intensity are presented to the subject in either an ascending or a descending order with the initial value being either above or below the threshold, depending on the direction of presentation. Several versions of this method are available including absolute thresholds, in which the stimulus is presented in either a descending or ascending series of intensities. When the subject reports either the presence or absence of the stimulus (depending on direction) the trial is terminated. Several trials are presented to the subject in both directions and the threshold is considered the mean of the intensities at which the trials were terminated (Gescheider, 1997).

Another version of the method of limits is the staircase or titration method. When this method is utilized, the stimulus is presented as in the absolute threshold method except that the direction of the presentation can be reversed during a session.

Considering an ascending session, the initial presentation of the stimulus is below the intensity level of the threshold. When the subject responds yes to the presentation of the stimulus the intensity is increased and it is re-presented. When the intensity level of

the stimulus reaches a value that the subject responds no, then the direction of presentation is reversed. The intensity then decreases until the subject responds yes and the direction is again reversed. If the intensity of the stimulus is crossing the threshold then the direction of the presentation will reverse several times. The threshold of the presenting stimulus is considered the mean of the values at which the reversals in direction take place. A descending series would be similar to the ascending series except that the initial value of the intensity of the stimulus would be above the threshold.

When this method is applied using a flickering visual stimulus, the intensity level that is changed is the frequency of the flicker. On an ascending series, when the frequency reaches a value that the stimulus is perceived as steady then the frequency will be reversed and will become slower until the subject responds that they have seen the flickering light. At this frequency, the direction is again reversed. The sequence would be the same for the descending series except that the directions would be reversed. The mean of the values at which the reversing of direction takes place would be considered the critical fusion frequency.

Responding to a Visual Stimulus

The use of a visual signal as a discriminative stimulus for lever pressing in rats has been used in a variety studies (Di Ciano & Everitt, 2003; J. Evenden, 1999; J. L. Evenden, 1999; Hampson, Jarrard, & Deadwyler, 1999; Rezvani, Bushnell, Burkholder, Glasgow, & Levin, 2001; Sanchez et al., 1997; van Haaren & van Hest, 1989; Wyble,

Hyman, Rossi, & Hasselmo, 2004). The condition under which the visual stimulus has been used has varied in different experiments.

A visual cue consisted of a light over a lever being illuminating whiled the house light was being extinguished as an S^D for the administration of cocaine (Di Ciano & Everitt, 2003). Similarly, Hampson et.al., used a light over a nose poke device as an S^D. When the rats initiated a nose poke the light was illuminated. When it extinguished, after a random period, the rats were required to press a lever in a delayed matching to sample procedure (Hampson et al., 1999) In an experiment studying the running behavior and bar pressing behavior of rats the illumination of the houselight was used as an S^D. Upon illumination of the houselight, the rats would initiate a trial by pressing the center of three levers. A green or blue light was then illuminated each associated with one of the two remaining levers. The rats were required to press the lever associated with the illuminated colored light in order to receive a reinforcer. Thus, colored lights were used in a discrimination procedure for lever pressing behavior in rats (Wyble et al., 2004). Rats were required to discriminate between two types of stimuli from a single source, each of which was associated with a different lever. The only difference between the stimuli was the intensity of the light for a 300ms period. The level of intensity increase varied from .027 lux to 1.2 lux above the background illumination (Rezvani et al., 2001). In an experiment with a similar procedure, different groups of rats were required to discriminate between lights that were either different if spatial position (one above the other), or a single light that was illuminated by different intensities. In each case, the different stimuli were each associated with different levers. The rats were required to

press the correct lever in order to receive a reinforcer (Sanchez et al., 1997). An interesting variation for the use of visual clues used the illumination of one of three lights to signal the probability that one of two levers would lead to the presentation of a reinforcer, if pressed. At the beginning of the trial the probability of the correct lever being signaled was approximately 0.3. As the trial progressed, the probability increased that the correct lever was being signaled by the presentation of a sequence of the three lights.

Several studies have used discrimination between a flickering light and a steady light as the prerequisite for pressing a lever associated with the stimulus. One study looked at the difference in responding between two different modalities of stimuli. One set of stimuli was a steady or flickering light while the other mode used a continuous or intermittent tone. Subjects that were exposed to the visual stimuli were less accurate than those exposed to the auditory stimuli, however, accuracy increased when a programmed delay between presentation and responding was short. Also, males made more correct responses than females (van Hest, van Haaren, & van de Poll, 1990). Two studies used the same procedure in which rats were trained to discriminate between flickering lights of two different frequencies of 5 Hz and 1 Hz. In both studies 80% accuracy was achieved with control subjects although in the later study the number of errors on the slow stimulus was greater (Bussey, Muir, Everitt, & Robbins, 1996; Winters, Robbins, & Everitt, 2004).

The use of visual stimuli has been shown to be a reliable S^D in a number of different ways. In addition, the use of flickering visual stimuli has also been used in discrimination procedures where lever pressing is the required response and positive

reinforcement has been used. However, no procedure has been found that uses lever pressing and positive reinforcement in a discrimination procedure to test the CFF of rats. In order to develop a method of determining the CFF of rats a series of experiments was conducted. Positive reinforcement was utilized making the method non-aversive and applicable for developmental and longitudinal studies and lever pressing was used as the required response.

CHAPTER II

EXPERIMENT 1

Introduction

In experiment one, subjects with varying levels of exposure to MeHg were trained to initiate a trial with a nose poke to a sipper tube, thereby illuminating a steady visual stimulus. The subjects were then required to break contact with the sipper tube when the steady stimulus changed to a flickering stimulus. This was a difficult task; stimulus control was obtained in only five subjects, for which an estimated CFF was calculated. An additional problem was that a large proportion of the subjects died of urolithiasis, which made the determination of why the method failed difficult to do. However, the experiment demonstrated that rats could be brought under stimulus control of a flickering light, using a behavioral method and using positive reinforcement.

Method

Subjects

The subjects were offspring of Long Evans rats that had been exposed to 0.0, 0.5, or 5.0 ppm of mercury, as methylmercuric chloride, in their drinking water. Half received a diet high in DHA and half a diet deficient in DHA. They were given regular drinking water beginning at weaning. Subjects in the experiment received MeHg only during gestation. Animals were housed in a standard shoebox type plastic box with a wire top and solid bottom. Ash wood chips were used for bedding. There were two animals per

cage separated by a plastic barrier. Each animal was partnered with an animal in the same diet group. Males were maintained at a weight of 320 gm +/- 10 gm and females were maintained at a weight of 260 gm +/- 10 gm. The colony room, which was temperature and humidity controlled, was on a 12 hour light-dark cycle (lights on at 8.00am).

Forty-five subjects were present at the beginning of training, however at the end of the experiment only 28 subjects. The rest died of urolithiasis, (see appendix B).

Apparatus

Sixteen standard Med Associates operant chambers, model # ENV 007 measuring 30.5 cm long x 24.1 cm wide x 29.2 cm high were equipped with metal bars forming a grid floor. A magazine pellet dispenser was located in the center of one end of the chamber to deliver a 45 mg sucrose pellet. Two levers were placed either side of the pellet dispenser, both of which were non-operational and were there for an unrelated experiment. At the opposite end to the pellet dispenser was a recessed area, painted matt black, measuring 50.8mm wide x 50.8mm high x 25.4mm deep. This was placed in the center of the wall with the front of the recess flush with the wall of the chamber. The bottom of the recess was 12.7mm from the top of the grid.

Projecting approximately 6.35mm, at a downward angle of roughly 45^0 from the center of the back wall of the recess was a standard sipper-tube. The front of the tube was placed approximately 6.35mm from the bottom of the recess. No liquid was accessible via the sipper-tube. The grid and the sipper-tube were electrically connected via a Med Associates Contact Lickometer Controller such that when a subject was standing on the

grid and touching the sipper-tube an electrical circuit was completed. The current required to complete the circuit was 0.3 microamps.

A LED was recessed into each of the two walls of the recessed area that were parallel to the back of the chamber. The LED's were 25.5 mm from the bottom of the recess and 12.7mm from the front of the recess such that when they were illuminated they were visible on the inside of the recess. Two additional LED's were placed on the same wall as the pellet dispenser, one directly above the center of each lever and at a height of 133 mm from the grid. All four LEDs had a nominal peak wavelength of 585nm, (within the yellow to orange range of the spectrum) with a nominal luminous intensity of 7.3 mcd. The light emitted was relatively broad spectrum but had a yellowish tint. Two, tone generators, one with a frequency of 4700 Hz and the other with a frequency of 2900 Hz, were placed in the top corners of the wall of the operant chamber that contained the pellet dispenser.

An incandescent house light was placed in the center of the chamber wall that contained the recess, immediately below the top of the chamber. All equipment in the chamber was computer controlled via a Dell personal computer operating on Windows 2000 operating system. All programming was accomplished via MED-PD IV version 4, release 1, programming software, supplied by Med Associates Inc.

Initial Training

A 0.2 second tone of 4700 Hz was paired with the delivery of one, 45 mg sucrose pellet from the pellet dispenser such that it attained the properties of a discriminative

stimulus (S^D) for the reinforcer. An autoshaping procedure was then implemented to train the rats to touch the sipper-tube.

After successful autoshaping a training procedure was initiated that required progressively longer continuous touches to the sipper-tube. The subject had to begin a touch of the sipper-tube while standing with its paws on the metal bars forming the grid. After a 0.5 second continuous touch of the sipper-tube had elapsed a randomly selected LED in the recess area was illuminated and, after a second period during which the illumination was held steady, the LED was flickered at a frequency of 3 Hz. If the subject released the sipper-tube within 5 seconds of the commencement of the flicker, the 4700 Hz tone was sounded for 0.2 seconds and a reinforcer was delivered.

The initial duration that the subject had to be in continuous contact with the sipper-tube was 0.5 seconds, and this requirement increased according to performance. If the subject completed the trial successfully then the length of time that elapsed before the flicker commenced was increased by 10%; thus, if the flicker had previously started after 0.5 seconds and the subject had released during the appropriate flicker period, then the new touch requirement changed to 0.55 seconds. The touch requirement increased progressively up to 10 seconds. When the touch requirement was greater than 0.5 seconds the right or left LED would illuminate after 0.5 seconds and remain steady until the new hold requirement was met or the rat broke contact with the sipper tube. When a subject reached the required steady LED hold time of 10 seconds the period that the illuminated LED was held steady was randomly varied between 4 and 10 seconds, in 0.5 second intervals.

Changes to Procedure

During the course of the experiment, changes were implemented to improve stimulus control, compensate for unwanted behavioral patterns that developed, and changes in the chamber environment that were required for other experiments being conducted in the same chambers. The changes are summarized in Table 1.

Table 1 Changes in procedure

Change Number	Change
1	Introduce flexan sheet on grid.
2	Maximum touch requirement reduced to 5 secs
3	Introduce 4 LED's flickering simultaneously
4	Introduce Correct rejection and Sham trials
5	Introduce blackouts
6	Delete Sham trials

Change 1.

The introduction of a flexan sheet, placed on top of the grid was implemented to assist subjects from other experiments that were having ambulatory problems on the grid. The flexan sheet allowed the three rods, of the grid, closest to the cavity that housed the LEDs and the sipper-tube to remain uncovered. The effect on this experiment was that when a subject was in a position to touch the sipper-tube with its nose only the front feet would make contact with the grid, completing the circuit, indicating a successful touch of the sipper-tube.

Change 2.

The touch requirement before flickering i.e., the period when the LEDs were illuminated in a steady state plus the initial 0.5 seconds, was reduced from ten seconds to

5.5 seconds. The range of steady LED periods was changed from 4 to 10 seconds, to 2 to 5 seconds. These steady LED periods were separated into half-second intervals and were randomly selected for each trial. This change was introduced because a large percentage of the subjects were unable to meet the criteria of a continuous touch on the sipper-tube for 10 seconds.

Change 3.

Instead of one LED being illuminated inside the recess, all four LEDs in the chamber were illuminated simultaneously. This change was necessitated by the behavior of several of the subjects who, instead of touching the sipper-tube with their nose reached into the recess with one front leg and touched the sipper-tube while simultaneously touching the grid with the other front leg. This behavior meant the eyes were not in the correct position for the testing of each eye individually. By illuminating the whole chamber with the four LEDs total visual field could be tested.

Change 4.

Two extra trial types were introduced into the procedure, a correct rejection trial, and a sham trial. The correct rejection trial consisted of terminating the trial at the end of the steady LED period and delivering a reinforcer if the subject was still touching the sipper-tube. This was initiated to reinforce touching for the full steady LED period. The sham trial consisted of a normal trial, but the flicker frequency was 99 Hz. This frequency was considered well above the CFF of rats and therefore would appear to the subjects as a steady light. In addition, the period allowed for a release of the sipper-tube during which the LEDs were flickering was reduced to one second. This was

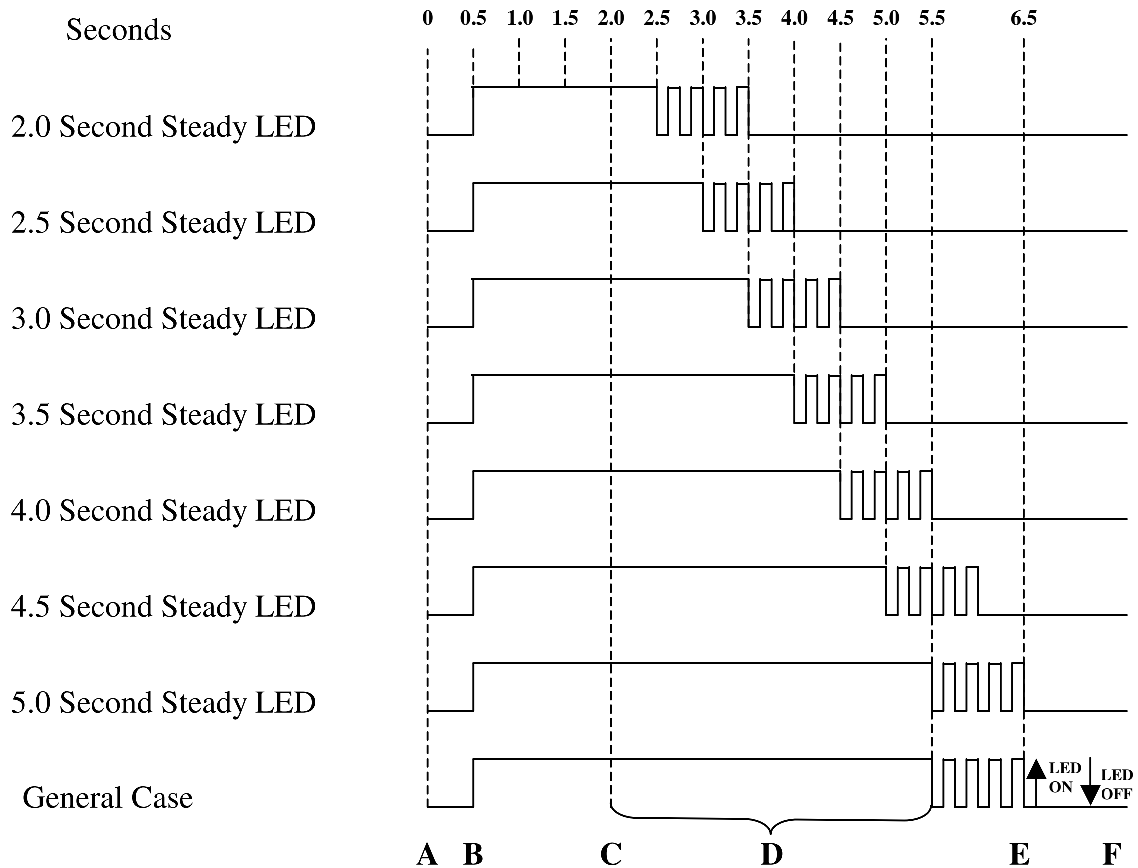
implemented to help determine when stimulus control over the flickering LEDs had been achieved.

Change 5.

Blackout periods of 10 seconds, which were accompanied by a 2900 Hz tone, were introduced for two conditions. The first condition was for an early release. This was defined as releasing from the sipper-tube before the end of the steady LED period. The second condition was for a late release from the sipper-tube. This was defined as any hold that lasted longer than the one-second-flicker period. The blackout was not imposed for any releases less than 2 seconds and touching the sipper tube during a blackout produced on consequences.

Change 6.

The sham trial was deleted from the procedure because it was too similar to the correct rejection procedure, in that the LED always appeared steady. The trial could only be completed successfully by the subject if they released from the sipper-tube during the flicker period, and because the flicker appeared as a steady light and the steady hold time was random, the subjects would not be able to discriminate when the flicker period commenced. Thus, a successful trial would only be achieved on a random basis.



A: **I**nitial touch to sipper-tube.
A-B: 0.5 second period during which there was no effect for touching the sipper-tube.
B: 0.5 seconds after initial touch to sipper-tube, steady LED is illuminated.
B-C: Release of the sipper-tube during this period resulted in LED being extinguished and trial reset.
C: Initiation of first blackout period**.**
C-D: Release of the sipper-tube during this period initiated a 10 second blackout and reset the trial.
D: Initiation of flicker period.
D-E: 1.0 second period when LED flickered at the required frequency.
E: Initiation of the second blackout period.
E-F: Release of the sipper-tube during this period initiated a 10 second blackout and reset the trial.

Figure 1 Final Procedure for a Single Trial

Final procedure

The final procedure, illustrated in Figure 1, was as follows. The houselight was illuminated indicating the beginning of the session. On the first touch of the sipper-tube, the house light was extinguished and remained out for the rest of the session. When a subject touched the sipper-tube one of three types of trial was randomly selected.

If a “flicker” trial was selected the following procedure was initiated. After 0.5 seconds constant contact of the sipper-tube had elapsed, four LED’s were illuminated in a steady manner for a randomly selected period of 1.5 to 5 seconds. At the end of this steady period, the LED’s flickered at the training frequency of 3 Hz. If the subject released the sipper-tube within 1 second of the onset of the flickering LEDs, a 4700 Hz tone was sounded for 0.2 seconds and a reinforcer was delivered on a VR 1.5 schedule. If the subject released the sipper-tube after 2 seconds of constant contact, but before the flicker started, or if the subject had not released from the sipper-tube by the end of the one-second-flicker period the LED’s were extinguished, the chamber went dark, and a 2900 Hz tone sounded for 10 seconds. Touching of the sipper-tube during this blackout period and for 1 second after the end of the blackout period had no effect except extending the blackout for the period of the touch. Releasing before the initial two seconds had elapsed turned off the LEDs and reset the trial.

If a “correct rejection” trial was selected, the same conditions occurred as in a flicker trial except that if the subject kept continuous contact with the sipper tube until the end of the steady period the LED’s were extinguished, the 4700 Hz toned sounded and a reinforcer was delivered. The same blackout conditions applied for an early release.

During a “test” trial a test frequency was selected, and the same conditions transpired as in the flicker trial, except that no blackout occurred for a late release and no reinforcers were delivered for a correct release. Each session consisted of a series of “test” trials at a particular frequency, starting at 3 Hz. Each frequency was tested for 10 trials. If, after the ten test trials, the subject obtained 50 percent or greater correct releases at the selected test frequency the test frequency was increased by 4 Hz. If the subject failed to release correctly for at least 50 percent of test trials the frequency was lowered by 2 Hz. At no time did the test frequency drop below 3 Hz.

Results

Mortality

At the beginning of the project, there were 45 subjects. When training commenced 16 subjects had died of urolithiasis, (see appendix B). These were replaced by 11 new subjects. When testing commenced, six more subjects had died, leaving 34 survivors. By the end of testing a further six subjects had died, leaving 28 survivors, which gave a total mortality rate of 45%.

The data from the 28 surviving subjects was analyzed together with data from three subjects that died but had generated enough data to warrant analysis with surviving subjects.

Response Rate

The mean number of responses per session emitted by the subjects for the last ten sessions ranged from 716.7 to 68. However, the percentage of responses per session that was less than 0.5 seconds in length was 85% and 84% respectively for the high and low values. Any touch on the sipper-tube that was less than 0.5 second in duration would not activate the LED's. For subjects that had a high percentage of responses under 0.5 seconds there responses did not initiate the illumination of the LEDs, which was the stimulus under which control had to come in order for the procedure to produce data that would generate a psychophysical function.

Of the 31 subjects, eight had a correct rejection rate greater than 70 % that ranged from 73% to 92%. Eleven subjects had a hit rate equal to or greater than 70 % ranging from 70% to 100%. Of the five subjects that had at least 70% for both categories the range for percentage of correct rejections and percentage of hits was 73% - 92% and 70% -84% respectively

A psychophysical function could be obtained for five subjects (2B, 7x1, 326x2, 336x2, 37x1) in which the percentage of correct rejections was above 70% and the percentage of hits was above 70%. The data from the five subjects were used to generate a function to estimate the CFF.

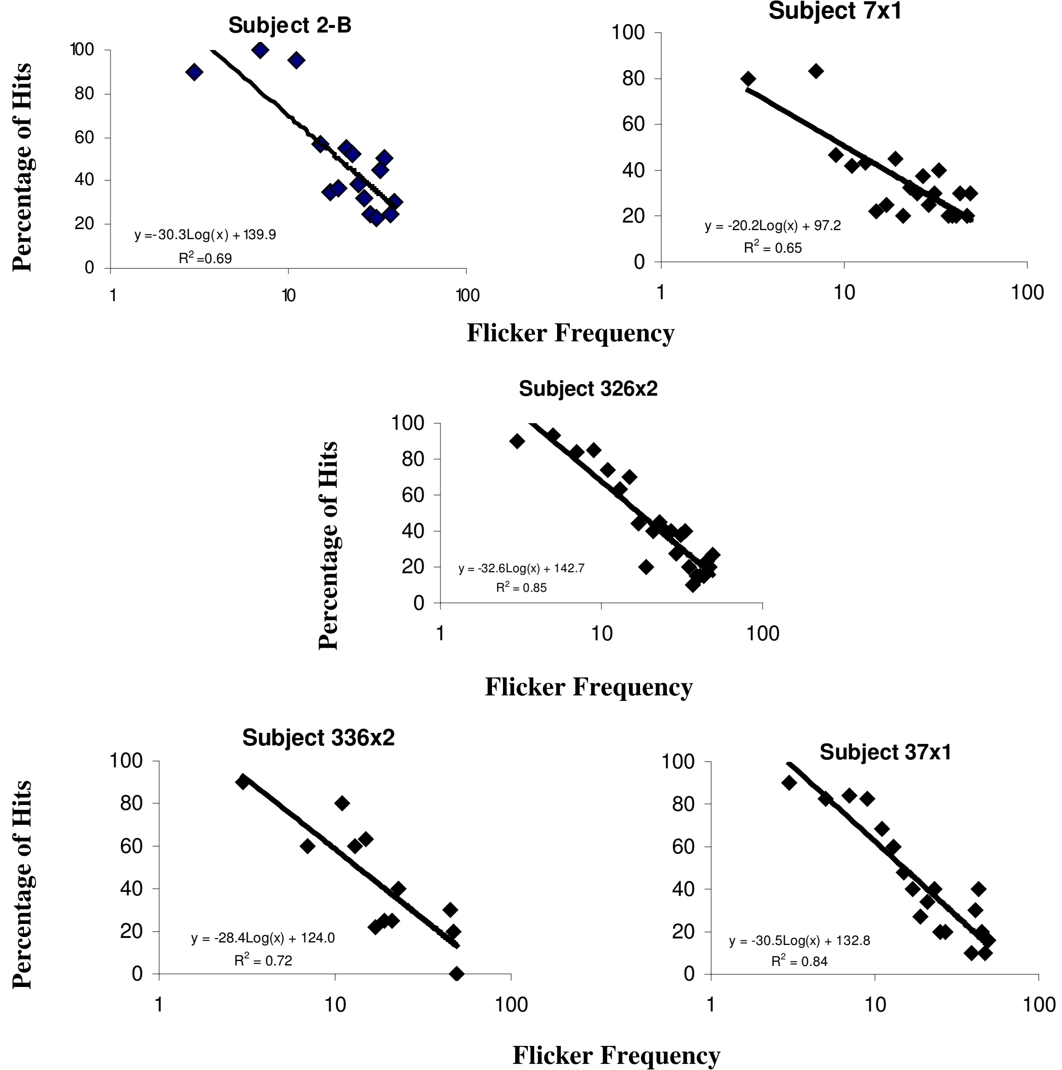


Figure 2 Percentage of hits
Percentage of hits for each flicker frequency tested for the five subjects that had correct rejection rates and hit rates greater than 70%. The independent variable is a log scale of the flicker frequency. The equation for each line represents a logarithmic function.

Figure 2 shows graphs generated from the percentage of hits for the five subjects that had hit rates and correct rejection rates equal or greater than 70%. The independent variable is a log scale of the flicker frequencies that were tested. The trendline shows the least squares fit through points using the equation $y = c \log x + b$, where $\log x$ is the logarithm of the flicker frequency and c is the constant that represents a subject's ability

to discriminate the flickering from the steady stimulus. The higher the absolute value of c the better the subject can discriminate. The variable b is the theoretical intercept on the y-axis when the log flicker frequency is zero i.e., the flicker frequency is one.

The frequency at which a 50% hit rate occurred was calculated for each of the five subjects, using the equation generated for each graph. The value x was calculated when $\left[\ y = \left(\frac{1}{2}a\right) + \min \%hits\ \right]$, where ($a$) was the difference between the maximum and minimum percentage of hits. The calculated 50% frequencies, which if the estimated CFF, for the five subjects are given in Table 2.

Table 2 Estimated CFF using the equations

Subject	Flicker Frequency
2-B	13.17
7x1	9.48
326x2	16.30
336x2	16.11
37x1	15.04

In order to produce the estimated CFF for the five subjects three criteria appear to be critical. The first two criteria are the percentage of hits and correct rejections. These should be equal or greater than 70% thus ensuring that the subject's behavior was under adequate stimulus control of the flickering LED's.

The third criterion is the response rate should be high enough to produce at least one reinforcer per minute. If this rate was not reached then enough behavior was not generated, and the subjects have did not come under stimulus control of the LED's.

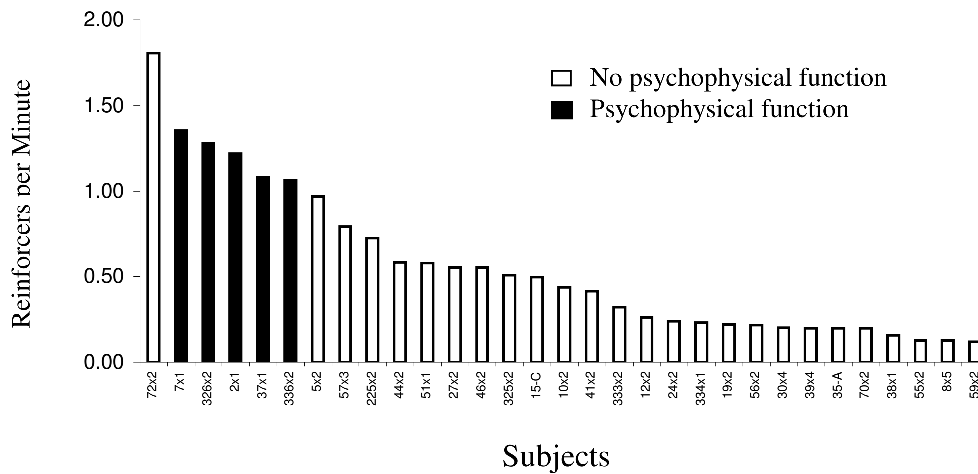


Figure 3   Reinforcers per minute for 31 subjects
Reinforcer rate is averaged over the last 10 sessions. Data is presented in order of magnitude. Black bars represent subjects for which a psychophysical function was obtained. White bars represent subjects for which no psychological function was obtained.

Figure 3 shows the reinforcer rate for the 31 subjects averaged over the last 10 sessions. Data are presented in order of magnitude of the reinforcer rate. The five subjects for which a psychophysical function was obtained are shown in the black columns. Only Subject 72x2 had a higher reinforcer rate than the five subjects for which psychophysical functions were generated, however, the hit rate for this subject was only 62%. All other subjects had a reinforcer rate of less than one reinforcer per minute, and a hit rate, correct rejection rate or both less than 70%.

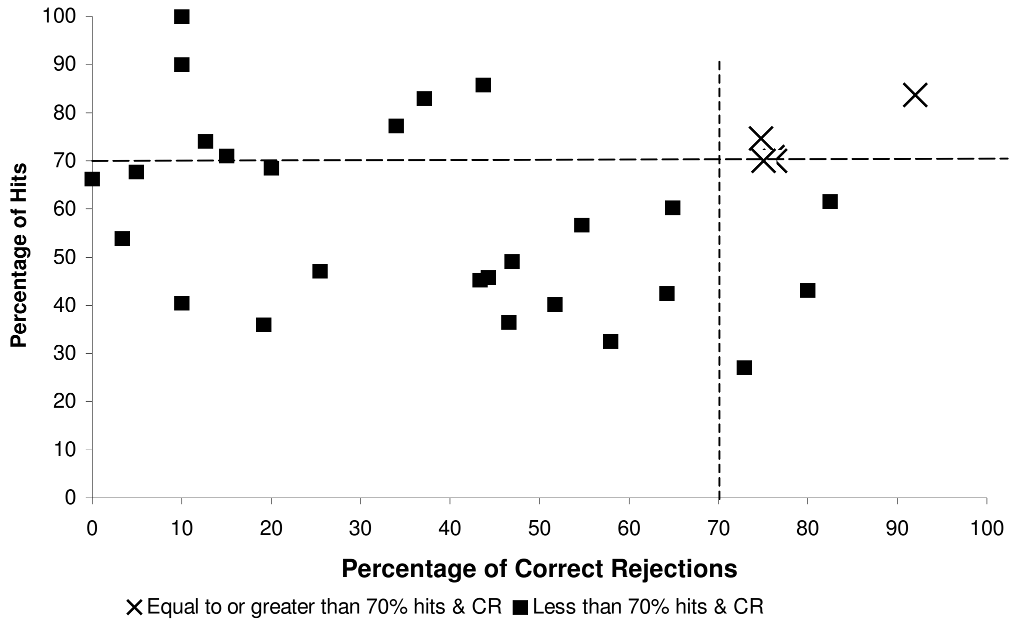


Figure 4 Percentage of correct rejections and percentage of hits for 31 subjects
The crosses represent the subjects for which an estimated CFF was obtained. The black squares represent the subjects for which no estimated CFF could be obtained. Subjects 7x1 and 336x2 had almost identical values thus their data points lay on top of each other.

Figure 4 represents the values of the percentage of correct rejections and percentage of hits (3 Hz) for each of the 31 subjects. The five subjects for which a psychophysical function was obtained are indicated by the crosses while those for which a function was not obtained are represented by the black squares.

Discussion

The initial objective of this experiment was to study the effects of MeHg, and any possible ameliorating effects of DHA on the visual system of the subjects. Several factors lead to this objective becoming unachievable.

Mortality

The first factor was the high mortality rate from urolithiasis, caused by the diet (see appendix B). Once the cause was detected, changing the diet had no effect on the condition or the mortality rate. It is unknown how the condition of urolithiasis affected the behavior of the subjects in the experiment and thus the extent to which it was responsible for the inability to obtain psychophysical functions for the subjects cannot be ascertained.

Response Rate

The low reinforced response rate of many subjects contributed to the low number of psychophysical functions that were generated. Although many subjects made a large number of touches to the sipper tube a high percentage of them were of short duration and thus did not qualify for reinforcement. This is illustrated in Figure 3, which indicates that greater than 50% of the subjects were reinforced at a rate of less than 0.5 reinforcers per minute. Several explanations for this high percentage of short touches are possible, although they are all speculative in nature.

It is possible that some subjects found touching the sipper-tube with their nose mildly aversive. This may be because their nose was moist and thus sensitive to a mild shock, or possibly because the end of the sipper tube was uncomfortable to touch because of its shape. It is also possible that the animals were making contact but the circuit between the nose and the sipper tube was being continuously broken because there was not a good electrical contact between the nose and the sipper tube. If this were the case, it

may manifest itself in the form of many short contacts. Some subjects stopped using their nose to make contact with the sipper-tube, and instead reached in to the cavity where the sipper-tube was located and made contact with one of their front feet. This shows that the contingency of touching the sipper tube was achieved, although in many cases the requirement of a long touch was not.

The effect of short touches to the sipper-tube had a consequence on how often the subjects were brought into contact with the stimuli. The limiting of the opportunity to be exposed to the stimulus LED's through the behavior of emitting very short duration touches also limited the possibility of the subject coming under control of these stimuli as any touch of less than 0.5 seconds duration would have failed to illuminate the LED's.

Psychophysical Functions

Of the data that were analyzed, a psychophysical function was obtained for five subjects. Three factors are common for each subject, reinforcement rate, percentage of correct rejection trials, and percentage of correct hit trials (at 3 Hz). The reinforcement rate for all five subjects was above 1.0 reinforcer per minute, plus the percentage of correct rejection trials and percentage of correct hit trials was at least 70%.

Of the five subjects for which psychophysical functions were obtained the results were consistent for four subjects, which had estimated CFF's ranging from 13.17 Hz to 16.30 Hz while the other subject had an estimated CFF of 9.48 Hz (Table 2). The value for the estimated CFFs was lower than other reports of CFF in rats.

Goldzband and Clark reported the CFF of 12 rats with a mean value of 24 Hz (Goldzban & Clark, 1955) however the results ranged from 31.4 Hz to 22.8 Hz and the luminance of the visual stimuli were not reported. A mean value of 21.54±6.0 Hz was recorded in a study that looked at the CFF in cells in the rats visual cortex by recording single unit responses to flickering stimuli (Wells et al., 2001).

A study that was similar to our experiment looked at the full visual field CFF in rats. Williams et.al; reported that with a log luminance value of –1.3 foot lamberts a CFF of 21 Hz was recorded (Williams et al., 1985). This value converts to log luminance value of -.07 cd/m^2. Test reading taken every second for one minute, at the volumetric center of the operant chambers used in our experiment produced a mean value of 0.5lux illuminance, which calculates to a log luminance value of –3.9cd/m^2. The luminance level in our experimental chambers, therefore are considerably lower than in the Williams experiment. As reported earlier luminance has a bearing on the value of the CFF with lower values of luminance associated with lower CFF values (Grusser & Landis, 1991).

The lower luminance level may have been contributing factors to the low estimated CFFs that were determined from the data, however other factors pertaining to the training frequency may also have been a contributing factor. A more detailed explanation is given in the general discussion.

Given the difference in levels of the luminance of the light sources coupled with an anomaly of the training frequency a value of between 13 Hz and 16 Hz would appear to be a reasonable result. The value of 9.83 Hz that was calculated for the other subject cannot be fully explained, but may have been a function of the training frequency.

Summary

Although this experiment was unsuccessful in testing for the effects of MeHg on the visual system of rats, it did contribute some methodological information. It was established that a psychophysical curve for flicker frequency could be obtained for rats using a positive reinforcing behavioral procedure. The results obtained were similar to those of other studies given the differences in luminance levels and the testing light wavelength frequency. The use of a sipper-tube as a method of requiring a response from the subjects probably contributed to the low number of subjects for which a psychophysical function was obtained. Experiment 2 was conducted to try to overcome the problem of a low response and reinforcer rate in experiment 1.

CHAPTER III

EXPERIMENT 2

Introduction

In experiment one, failure to sustain long responses on the sipper tube appeared to be a major reason for few subjects coming under stimulus control. This was attributed in part to the use of the sipper tube. In the second experiment, the operant was changed from contacting a metal sipper tube to that of breaking an infrared beam, using a nose poke as the required response. Most subjects were able to hold a sustained response, however, stimulus control was not achieved with any subjects.

Method

Subjects

Six Long Evans male rats were housed as in experiment one. None of the subjects had been exposed to MeHg and all were fed a standard rat chow diet. Four of the subjects were experienced in experiment one and had been used to test new versions of the programmed sequences before the experimental animals were exposed to them. These subjects were maintained at a weight of 310 grams to 320 grams. Two subjects were experimentally naive. These animals had been used as breeders for an unrelated experiment. They were maintained at a weight of 360 grams to 380 grams.

Apparatus

The apparatus was the same as in experiment one with the exception that the sipper-tube was removed from the recessed area. The plate through which the sipper-tube had protruded was replaced with a plate that had a vertical slot cut in the center of it. This slot was 12.5mm wide and 25.4mm in height with a radius at the top and bottom. An infrared beam was placed directly behind the plate such that when the subject placed their nose through the hole the beam was interrupted. The breaking of the beam replaced touching the sipper-tube, as the behavioral response that started a new trial.

Training

The naïve subjects were first exposed to a procedure in which the delivery of a sucrose pellet in the hopper was paired with a 0.2 second tone of 4700 Hz. When the subjects were reliably retrieving the pellet from the hopper, on presentation of the tone, training for the main procedure began.

Phase 1 Training long response duration and introduction of flicker trials.

A trial commenced when the subject placed its nose through the slot and broke the infrared beam. At the start of the first trial when the subject broke the infrared beam it was required that the beam be interrupted for 10 msecs. After 10 msec, a brief tone was sounded and a reinforcer was delivered which terminated the trial. The requirement to keep the beam interrupted was then increased by 5% so that on the second trial the beam must be interrupted for 10.5 msecs before the tone sounded and a reinforcer was

delivered. The time requirement was increased by 5% after each reinforcer was delivered until the time requirement was greater than 0.5seconds. This took 34 trials provided the subject did not withdraw earlier than the required time on any of the trials. There were no consequences for an early withdrawal except that no tone sounded and no reinforcer was delivered.

After the requirement of 0.5 seconds had been reached the criteria for the trial changed. When the infrared beam had been continuously interrupted for 0.5 seconds the four LEDs where illuminated. If the subject continued to interrupt the beam, until the end of the required period, a tone would sound and a reinforcer was delivered ending the trial. The required response duration was now increased by 25% after either three consecutive successful trials or six nonconsecutive successful trials. If the subject withdrew from the beam before the required period ended, the LEDs were extinguished and a tone with a frequency of 2900 Hz was sounded for 3 seconds. During this three-second period, a new trial could not be initiated and interrupting the beam had no consequences. The trial was then reset and repeated until it was successfully completed.

When the response requirement exceeded one second the flicker trial began; the LEDs would flicker at a frequency of 3 Hz. Nose withdrawals were reinforced if they occurred within 5 seconds of the start of the flickering stimulus, otherwise, the LEDS were extinguished and the 2900 Hz tone was sounded for three seconds. The same conditions prevailed for this timeout period as stated previously.

As the response requirement reached successive 0.25 second intervals i.e., 1, 1.25, 1.5, 1.75 seconds etc., anyone of the periods, up to the current time requirement, could be

randomly selected as the time requirement to keep the beam interrupted before the LEDs flickered. Thus as the time requirement increased more intervals became available to be selected. Concurrently, as the requirement to keep the beam interrupted reached successive 0.25second intervals, the period for the termination of the interruption of the beam after the flicker was initiated was reduced by 0.25seconds. When the response requirement reached five seconds, the flicker period in which the interruption of the beam had to be terminated had been reduced to one second.

The 3-second tone and the extinguishing of the LEDS was kept in place for both early withdrawal, and for not terminating the interruption of the beam within the required time after the LEDs started flickering. There were no consequences for terminating the interruption of the beam in less than 0.5 seconds.

The minimum period required for the interruption of the beam on the first trial of a new session (start time) was calculated by subtracting the maximum required period of the last trial (end time) of the previous session, from the start time of the previous session, and dividing the difference by 4. The result was then added to the start time of the previous session. The sum became the start time for the first trial of the new session. Thus if the required period of the first trial of the previous session started at 1.12 seconds and the required period of the last trial of the previous session ended at 4.01 seconds then the start time for the first trial of the next session would be $((4.01-1.12)/4)+1.12=1.84$ seconds. The subjects continued training in this program until they had an end time of 5seconds for five consecutive sessions.

The final procedure for phase one was a follows. A trial was initiated by breaking the infrared beam and ended by terminating the interruption of the beam at which time either a reinforcer was delivered or the three-second tone was sounded (unless the period the beam had been interrupted was less than 0.5 seconds). The beam would be interrupted for a sustained period of one to five seconds, in 0.25second intervals, which were randomly selected without replacement, during which the four LEDs were illuminated continuously. After the randomly selected period had expired, the LEDs would flicker at a frequency of 3 Hz. Withdrawal from the beam within one second was then required in order that a reinforcer be delivered. There was a minimum 2second intertrial interval (ITI) between trials during which interruption of the beam had no consequences. The ITI commenced after the delivery of the reinforcer or from the end of the three-second tone. A session terminated after 30 minutes.

Phase 2 Introduction of steady trials.

In phase two of training a steady trial was introduced. In a steady trial, if the beam was continuously interrupted for the period of the selected time requirement, the short tone would sound at the end of that period, simultaneously the LEDs would be extinguished and a reinforcer would be delivered.

The maximum duration started at one second with the release period at 5 seconds as in phase one. The increase in the time requirement and the decrease in the hold requirement remained the same as in the previous phase. After the subjects had reached

the criteria of five consecutive sessions with an end time of five-second period, testing was implemented. Subject 118 died before entering the testing phase.

Results

Phase 1 (Training long response duration and introduction of flicker trials.)

In the first phase of training three of the subjects, 117, 148, and 151, required fewer than 10 sessions to reach the end time requirement of 5 seconds. These subjects advanced their start time by 25% of the difference of the end time to start time of the previous session without a decline in their response rate.

Subjects 115 and 118 could not maintain the response rate at an increase of 25% of the start time per session. Therefore, the increase rate was reduced to 12.5%. Subject 115 took 35 sessions to reach the five-second criterion. For this subject the end time increased the first two sessions, but by the sixth session the end time was declining. The rate of increase for this subject was reduced twice, first to 15% and then to 12.5% in order to keep the subject responding. Subject 116 failed to reach an end time of five seconds even though the increase in the start time was reduced to 12.5%. The end time, for this subject, declined to less than two seconds even after 35 sessions. Table 3 summarizes the number of sessions that each subject required to reach a session end time of 5 seconds.

Table 3 Sessions to End Time Phase 1

Subject	Sessions	% Increase per session
115	35	12.5
117	4	25
118	33	12.5
148	4	25
151	9	25

Sessions equals the number of sessions required to reach 5 second end time.

Phase 2 (Introduction of steady trials.)

Subject 118 died before the start of the second phase of training and subject 116 failed to reach the criteria set to advance to the second phase therefore four subjects entered this segment of training.

Table 4 Sessions to End Time Phase 2

Subjects	1st end time 5 seconds	5th end time 5 seconds
115	-	-
117	13	17
148	10	20
151	24	31

The number of sessions required for each subject to reach criterion in this phase of the training is given in Table 4. Subject 117 achieved criterion the easiest reaching an end time of 5 seconds in the 13th session and reaching criterion in the 17th session. The other subjects required more sessions to achieve the criterion of five continuous sessions with an end time of 5 seconds, subject 148 needing 20 sessions, subject 151 needing 31 sessions, and 115 never achieving criterion.

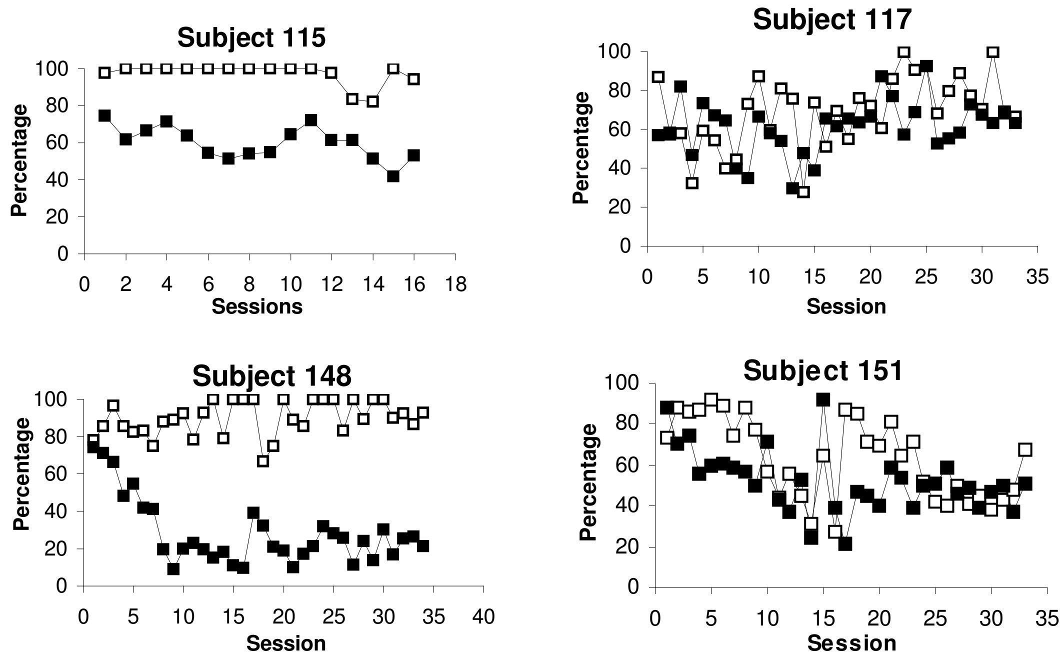


Figure 5 Percentage of correct flicker trials and steady trials
Percentage of hits (open squares), and correct rejections (closed squares) recorded each session.

Figure 5 shows the percentage of correct flicker trials and correct steady trials recorded during the second phase of training. A hit would be recorded when the subject withdrew from the infrared beam during the flicker period. A correct rejection would be recorded during a steady trial when the subject did not withdraw from the infrared beam until the end of the hold period. Three of the four subjects 115,117 and 148 had a hit rate greater than 70%, however, the correct rejection rate was well below the 70%. Therefore, it cannot be assumed that stimulus control was achieved in the steady trials. Subject 117 had a correct rejection rate and a hit rate above 60% but this was not high enough to produce a psychophysical function.

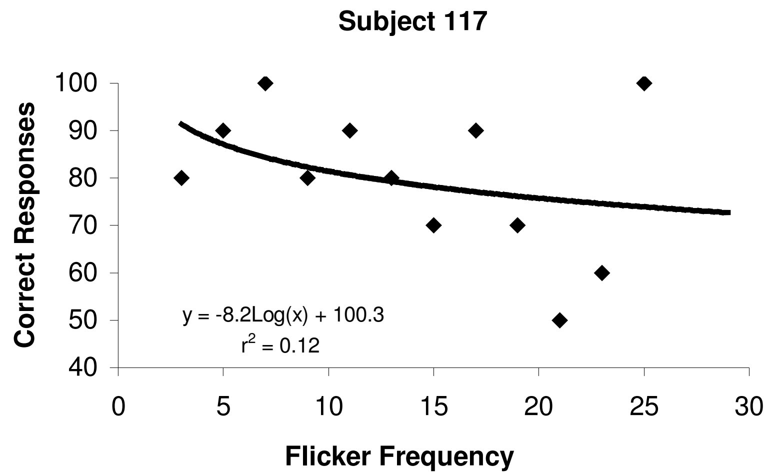


Figure 6 Log Function Subject 117
X-axis represents tested flicker frequency. Y-axis represents percentage of correct flicker trial releases at each tested frequency over 13 sessions. The best fitting curve is calculated using the least square method and fitting a line using the above equation.

Data for subject 117 shows that stimulus control was not attained for this subject. The X-axis of Figure 6 represents the percentage of correct flicker trial releases obtained at different flicker frequencies. None of the frequencies were below 50%. If stimulus control had been achieved, we would expect to see the percentage of correct releases to fall below 50% in the region of 10-25 Hz. Fitting a curve using the least square method the equation [$y = 8.2\log(x) + 100.3$] is obtained. If this were a viable psychophysical curve then the CFF would be approximately 490 Hz. This is an unacceptable value and well beyond the frequency for the CFF of rats. Furthermore, the r^2 obtained for this curve is only 0.1228 giving further evidence that the data obtained does not support a statement that stimulus control had been achieved.

Discussion

The change of method from touching the sipper-tube to breaking the infrared beam was successful in that the percentage of short holds was lower than in experiment one. The mean reinforcer rate in experiment one, when calculated using reinforcers delivered, as a function of trials (touches to sipper-tube) was 5.8%, compared with phase one of experiment two where the mean reinforcer rate is 20.6%. This may indicate that the infrared beam response was less aversive, or easier to sustain than the sipper-tube. The data from the first training sequence indicated that the majority of the subjects were coming under stimulus control of the flickering LEDs.

The method was not successful in obtaining a psychophysical curve. The reason for this is not entirely clear but when considering the results from the first experiment the temporal factor may have played a prominent part in the failure. Given that the salience of a visual stimulus is probably not very high for a rat, plus having to stay immobile for a period i.e., having to stay in one position in order to ensure that the infrared beam stayed interrupted, it is possible that the subjects were responding more to temporal cues than visual cues. The requirement that the subjects hold for a period as well as release at the appropriate time may have overshadowed the requirement that they respond to the flickering or steady LEDs.

The failure of experiments one and two to establish stimulus control and allow the accumulation of any relevant data necessitated a new approach be taken to the problem in experiment three.

CHAPTER IV

EXPERIMENT 3

Introduction

The goal of experiments 1 and 2 was to have the rat position its eyes into a conical receptacle and sustain it there while a light flickered. Four responses were required in this procedure to distinguish among hits, misses, false alarms and correct rejections. This proved to be a difficult response to establish and bring under the appropriate stimulus control. In experiment 3, a simpler approach was attempted.
Rats have been trained too discriminate visual stimuli in an operant chamber by pressing appropriate levers successfully (J. Evenden, 1999; Sanchez et al., 1997; van Haaren & van Hest, 1989; Wyble et al., 2004). However, the discrimination required is either signal and no signal (J. Evenden, 1999), visual and auditory signal (van Haaren & van Hest, 1989; Wyble et al., 2004), or visuo-spatial (Sanchez et al., 1997). The discrimination between a flickering light and a steady light using a two-choice runway (Legg, 1986, 1988; Legg & Turkish, 1983) has also been successfully trained in rats. No reference could be found in which the use of lever pressing as a behavior for discriminating between a flickering visual stimulus and a steady illuminated stimulus has been used, however, there is little reason to think it cannot be achieved.

In this experiments rats were trained to press one of two levers that were either associated with a flickering visual stimulus or a steady visual stimulus.

Method

Subjects

Subjects, four male Long Evans rats approximately one year old, were maintained in accordance with Institutional Animal Care and Use Committee of Auburn University regulations. Standard 9 x 18” x 7.5” plastic boxes with a wire top and solid bottom each housed two subjects that were separated by a diagonal plastic barrier. The subjects were kept on a 12 hour light-dark cycle (lights on at 6.00am), in a temperature and humidity controlled environment, and were fed rat chow once a day, after experimental sessions Their weight was maintained between 330 – 380 grams. Water was freely available in their housing. Two of the subjects had been participants in previous visual discrimination experiments however all four subjects were naive in experiments involving lever pressing.

Apparatus

The apparatus for this experiment was the same as in experiment 2 except the infrared beam was now inoperative.

Training

Autoshaping

The four subjects were exposed to an autoshaping procedure in which the presentation of a retractable lever was paired with a reinforcer. This procedure produced

lever-pressing behavior, which led to the presentation of reinforcers, in all subjects. Subjects were exposed to the procedure for two sessions, one for pressing the left lever and one for pressing the right lever. All achieved the required behavior.

Initial Training

The initial training procedure consisted of two types of trials, steady and flicker. The frequency chosen for the steady trial was 125 Hz. This frequency was selected for two reasons. First, it is considered greater than the CFF of rats. Second, because it was a flickering light rather than a steady light it allowed both conditions, flickering and steady, to have the same average luminosity.

A steady trial commenced with the illumination of the four LEDs flickering at a rate of 125 Hz. A single response (FR1) on the left lever, which was designated the steady LED appropriate lever turned off the LEDs and ended the trial with the presentation of a short tone, which had previously been paired with the delivery of a 45 mg sucrose pellet that was delivered in the hopper. A flicker trial started with the flickering of the LEDs at a rate of 3 Hz. A single response on the right lever, which was designated the flickering LED appropriate lever, turned off the LEDs and ended the trial with the presentation of the tone and the associated reinforcer. There was a 15 second intertrial interval between all trials.

Initially a session lasted for 100 trials. This was later changed to 100 correct trials or 60 minutes, whichever occurred first. When a subject achieved an overall correct response rate of 90% the schedule of reinforcement was increased to a fixed ratio 2 (FR2)

i.e., two presses of the correct lever were required in order to end a trial and receive the reinforcer.

The percentage of correct responses was calculated for flicker trials, steady trials, and all trials combined. A correct response was considered a press on the lever that was associated with the trial that was in progress. The percentage of correct responses was calculated by dividing the number of correct responses emitted during a particular type of trial by the total responses for that trial type and multiplying the result by 100. The overall percentage of correct responses was calculated by dividing the total correct responses for the session by the total responses emitted during the session and multiplying the result by 100. Each time correct responding reached 90% at the current FR value, the FR value was raised by one, until an FR10 was obtained.

Beginning with the FR10 schedule a reset procedure was instituted such that one press on the incorrect lever extinguished the LEDs and ended the trial without a reinforcer being delivered. If the wrong lever was pressed a correction procedure, which consisted of the previous trial being repeated, after the ITI had elapsed, was then initiated. Responding during correction trials was not counted towards the percentage of correct responding.

After the subjects had maintained greater than 90% correct responses at an FR10 for five consecutive sessions, reinforcement rate was reduced to 75% of randomly selected correct trials and the fixed ratio was reduced to FR7. This was found to be the highest FR value that maintained responding with the reduced reinforcer rate. The percentage of correct responses was maintained at 90% for another five trials after which

the correction procedure was removed. After the 90% correct response rate had been maintained for a further three sessions, the reset procedure was removed.

Final Training

The final training procedure was the random presentation of either a flicker trial or a steady trial. Responding on an FR7 schedule of reinforcement on either lever was required to end the trial. The responses need not be consecutive on either lever. If responding on the FR7 schedule was on the trial appropriate lever there was a 0.75 probability of reinforcement. There was no reinforcement for ending the trial on the trial inappropriate lever. A 15 second ITI preceded the presentation of the next trial. No reset or correction procedures were in place. A session ended after responding correctly for 100 trials, or 60 minutes, whichever came first. The criteria before testing could occur was set at five consecutive sessions with correct responding at greater than 80%.

Retraining

Subject 148 initially failed to maintain 80% correct responding that was required in order for testing to commence. In order to bring the subject under better stimulus control the following correction procedure was initiated.

The random selection of flicker or steady trials was changed such that 10 trials of the same type were presented consecutively, commencing with flicker trials. The probability of reinforcement for correct responding was raised to 1.0 and the schedule of reinforcement was kept at FR7. When for correct responding was greater than 80% for

five consecutive sessions the number of consecutive trials of the same type was changed to five. When correct responding again reached greater than 80% random, trials were re-introduced. While correct responding remained at greater than 80%, the probability of reinforcement was gradually reduced to 0.9, 0.8, and 0.75 consecutively. This procedure produced the requirement of maintaining correct responding, by subject 148, at greater than 80%.

Testing

Method of Constant Stimuli

During test sessions 75% of the trials were steady or flicker trials (3 Hz) (i.e., training) and on each trial there was a 0.75 probability of reinforcement for correct responding under an FR7 schedule of reinforcement. Twenty five percent of the trials were probe trials, which consisted of a flicker trial with a flicker frequency randomly selected from 25 frequencies ranging from 4 Hz to 31.25 Hz in approximately 1 Hz steps. No frequency was presented more than once until all frequencies had been selected. Probe trials had a 0.75 probability of being reinforced under an FR7 schedule, irrespective of which lever the responding occurred on. A trial ended after cumulative responding on the FR7 schedule on either lever had occurred. There was a 15 second ITI between trials and a session lasted for 100 correct steady or flicker (3 Hz) trials, or 60 minutes. Testing lasted for 10 sessions and a probability of responding on the flicker lever was calculated for each testing frequency using the accumulated responding across all ten sessions.

Titration Procedure: Ascending

Testing using the method of ascending titration consisted of presenting both flicker and steady trials randomly with the probability of a flicker trial being selected set at 0.75. All correct trials were reinforced.

The initial flicker frequency was set at 3 Hz. Two correct consecutive trials increased the flicker frequency for the next trial by approximately 1 Hz. Each time the subject responded on the flicker appropriate lever at the FR7 schedule for two consecutive trials the frequency was again raised by approximately 1 Hz. Responding on the steady appropriate lever at an FR7 for one trial reduced the flicker frequency by approximately 1 Hz. The flicker frequency never went below 3 Hz. There was a 15 second ITI between trials and the session lasted for 100 correct trials or 60 minutes. Testing was run for two consecutive sessions.

Titration Procedure: Descending

The method of descending titration consisted of presenting both flicker and steady trials randomly with the probability of a steady trial being selected equal to 0.75. The frequency for the flicker trials was set at 3 Hz. Steady trials were assigned a flicker frequency that was initially well above the suspected CFF for rats. All correct trials were reinforced and responding on an FR7 schedule was required. Testing was run for two consecutive sessions.

For the first session, for subjects 140, 145, and 151 the initial flicker frequency for the steady trials was set at 100 Hz. Two correct consecutive trials decreased the

flicker frequency for the next trial while responding on the FLAL for one trial increased the flicker frequency. The flicker frequency never went above 100 Hz. There was a 15 second ITI between trials and the session lasted for 100 correct trials or 60 minutes. The order of reduction of the flicker frequency during the steady trials was 71.43 Hz, 50 Hz, 38.46 Hz, 25 Hz, and then a reduction of approximately 1 Hz for every two consecutive correct steady trials. The initial frequency for the second session was 25 Hz with the reduction for two consecutive correct trials set at approximately 1 Hz. From results obtained from the first descending titration session it was apparent that the CFF was probably below 25 Hz. Starting the session at 100 Hz allowed for less trials at frequencies that were nearer the expected CFF, thus, it was decided to use 25 Hz as the initially frequency. Because of the extra training needed for subject 148 this subject started testing later than the other subjects, therefore, all descending sessions for this subject were initially set at 25 Hz.

The testing frequencies were controlled by the computer program resolution frequency, which was set at 1msec. All testing frequencies were a multiple of 1msec. Table 16 in appendix C shows the flicker frequencies used and the related millisecond units and units per second. Four millisecond units would equal .004 seconds or $1/250^{th}$ of a second. The full flicker cycle consists of two parts, LEDs on and LEDs off. Each part consists of the same time unit thus a full flicker cycle equals two time units, e.g., 125 Hz cycle equals two .004second cycles, one off and one on. Thus, the full cycle would equal 125th of a second or 125 Hz. The example frequency, 125 Hz, was the frequency used for the steady trials. This frequency is assumed to be above the CFF for rats.

Results

Method of Constant Stimuli

Each subject completed ten sessions of testing using the procedure described previously. The flicker appropriate lever responses and the steady appropriate lever responses for each tested frequency of each session were recorded, and the cumulative total for all sessions was recorded. A probability of a flicker appropriate lever response was calculated by dividing the total responses on the flicker appropriate lever by the total responses on both levers, for a particular testing frequency over all ten sessions. A psychophysical function was plotted with the probability of a response on the flicker appropriate lever as the dependent variable. The independent variable was the tested flicker frequencies.

The probability of a response was converted to a Z-score and the standardized result was plotted as a function of the tested flicker frequency. In converting the probabilities into Z scores values of zero probability were given a Z score of –2.33, which is the equivalent Z score of .01 probability. Similarly, probability values of one were given a Z score value of 2.33, which is the equivalent Z score value of a probability value of 0.99. A straight line regression was calculated for the Z-scores of the probability.

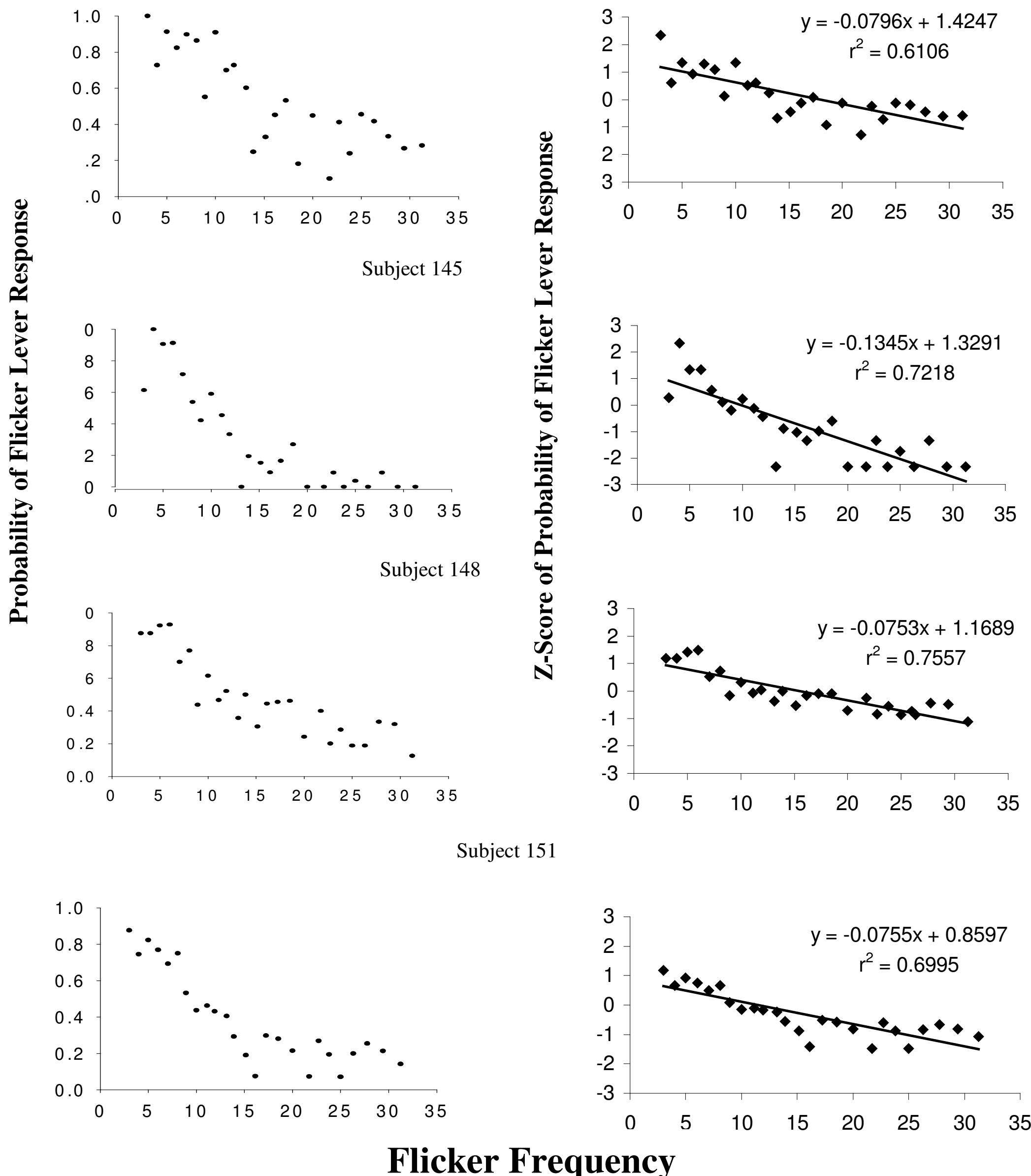


Figure 7 Psychometric functions derived from the Constant Stimuli method
The independent variable is the tested flicker frequencies. The dependent variable for the plots on the left is the probability of a response on the flicker-appropriate lever at a tested frequency. The dependent variable for the plots on the right is the Z-score of the probability of a response on the flicker-appropriate lever at a tested frequency The scatterplot points represent the probability of a response on the flicker-associated lever for all tested flicker frequencies calculated from the total accumulated responding over ten sessions.

A psychometric function for each subject is presented in the left plots of Figure 7. The independent variable is the testing flicker frequency. The dependent variable is the

probability of a response on the flicker appropriate lever. The Z-score of the probability of a response on the flicker appropriate lever is plotted against the flicker frequency in the graphs to the right of Figure 7.

As described in the introduction, variation of biological measurements tends to be normally distributed when the proportions of responses to the value of the stimulus intensity are plotted against the stimulus intensity (Gescheider, 1997). Measurement of critical flicker frequency could be considered a biological measurement because; it is the measurement of a stimulus perceived by the organism. Change in frequency can be considered a change in the intensity of the stimulus. Therefore, the expected distribution curve would be normal. The ogive curve is a cumulative form of the normal distribution and describes how the proportion of cases below a point on the normal distribution increases as the magnitude of the measurement increases (Gescheider, 1997).

If the distribution is normal then the ogive curve has the property that when the probabilities are converted to z scores of a probability of a response on the flicker lever, and are plotted against the frequency of the stimulus, the resultant curve can be described by a straight line (Gescheider, 1997). As the mean of a standardized distribution is zero then the value of the intensity of the stimulus that crosses the line when the Z score is zero will equal the z score of a 0.5 probability of a flicker appropriate lever response. Thus, this value can be considered the threshold of the intensity of the stimulus i.e., the estimated CFF.

Using the general equation for a straight ($y = ax + b$) where (y) equals the Z score of the probability of a response on the flicker appropriate lever, and (x) equals the

flicker frequency. The value of a equals the slope of the line or the discriminability of the subject, and b equals the intercept on the y axis when x equals zero or the theoretical Z score of the probability when the flicker frequency equals zero. The equation can be re-written as ($Z = DF + T_{FF}$) where (Z) equals the Z-score of the probability, (D) equals the discriminability of the stimulus, (F) equals the flicker frequency, and (T_{FF}) equals the theoretical probability when the flicker frequency is zero.

Transposing the equation ($F = \frac{Z - T_{FF}}{D}$).

When the Z-score of the probability equals zero then there is a 50% chance that the subject will respond on the flicker appropriate lever and a 50% chance that they will respond on the steady appropriate lever. Thus, because the value of Z is zero, the equation becomes $\left[F = \left(\frac{-T_{FF}}{D} \right) \right]$.

The function and resultant r^2 values have been inserted in to the plots. The r^2 values and thus the value of the Pearson r correlation coefficient are high and thus support the analysis of the data using the Ogive function. The slope of the regression line is very similar for subjects 140, 148 and 151 indicating that the subjects performed with a similar amount of discrimination when responding to a flicker stimulus. The slope for subject 145 is greater indicating that this subject was able to discriminate the flicker stimulus easier than the other subjects.

Table 5 Mean Frequency (3 Hz)

Subject	T	D	Frequency
140	1.42	-0.08	17.9
145	1.33	-.13	9.9
148	1.17	-0.08	15.5
151	0.86	-0.08	11.4

T = Theoretical Z-Score when frequency equals zero, D = stimulus discriminability. Frequency is the calculated flicker frequency when the Z-Score = 0

Table 5 shows the frequencies at which the probability of a response on the flicker appropriate lever is 50%, for each subject.

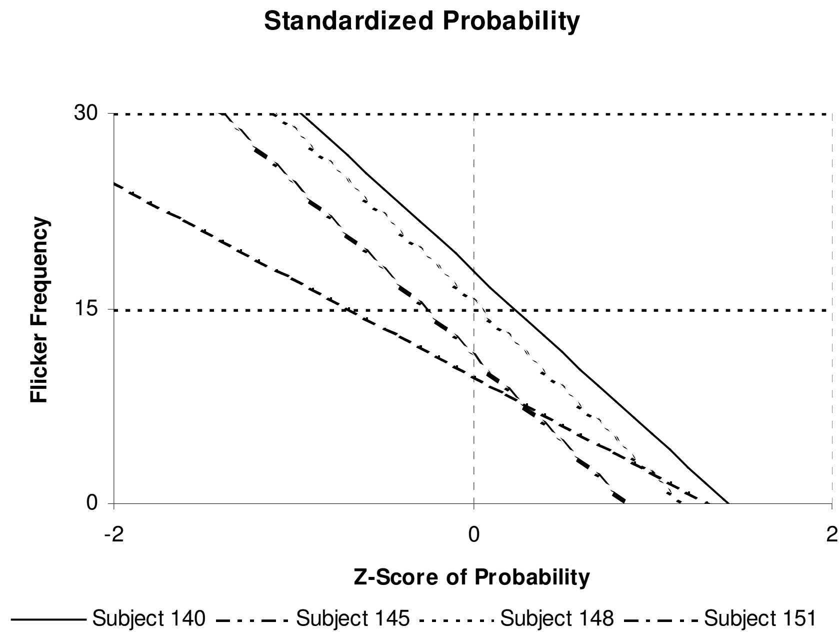


Figure 8 Regression lines of Ogive Functions (3 Hz)

Figure 8 shows the regression lines for each subject plotted from plus to minus two standard deviations on the X-axis, which represents the Z-scores of the probability of a response on the flicker appropriate lever. The regression lines were

plotted using the equations generated from the plots in Figure 7. The point at which the regression line crosses the Y-axis at an X value of zero represents the frequency at which the probability of a response on the flicker appropriate lever is 0.5, for that subject.

Titration

In calculating the estimated CFF using the titration method, the mean of the frequencies where transitions from responding on one lever to responding on the other lever become stable, about a range of frequencies, is considered the estimated CFF. In an ascending frequency session, the flicker frequency starts at a low value and when the subject has responded on the flicker appropriate lever at the correct FR schedule for two consecutive trials then the frequency increases for the next trial. When the subject responds on the steady appropriate lever for one trial then the frequency decreases. This would be considered a transition. When the subject again responds on the flicker appropriate lever for two consecutive trials, the frequency will increase. This would be considered another transition.

During a descending titration session, the opposite would define a transition. The frequency starts at a high value and decreases each time the subject responds at the correct schedule on the steady appropriate lever for two consecutive trials. When the subject responds on the flicker appropriate lever for one trial the frequency will increase for the next trial. When the subject again responds for one trial on the steady appropriate lever the frequency would decrease. Both changes would be considered a transition.

Titration Ascending

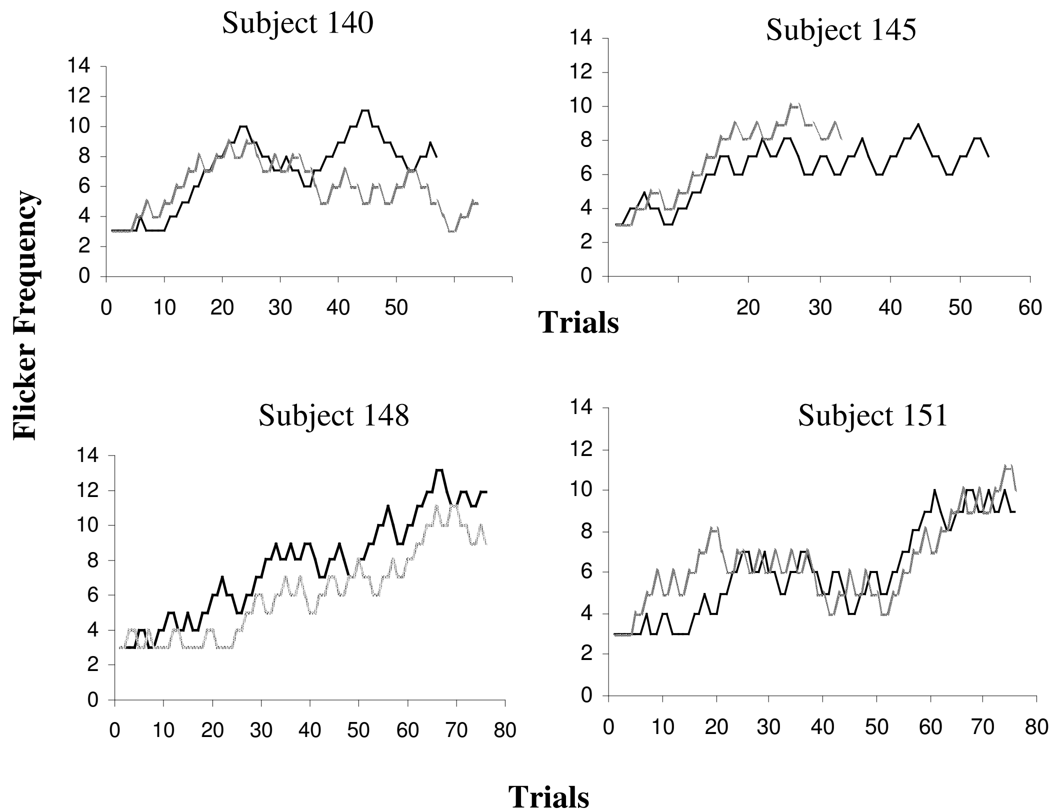


Figure 9 Ascending titration trials.
The X-axis represents the number of test trials in a session. The Y-axis represents the flicker frequencies that were tested. The dark solid line represents the first ascending titration session; the light solid line represents the second ascending titration session.

The results of the first and second ascending titration are illustrated in Figure 9. The independent variable is the number of testing trials and the dependent variable is the flicker frequency. The dark solid line illustrates the first titration session and the light solid line illustrates the second session. Subjects 140 and 145 started making early transitions while subjects 148 and 151 did not start to transitions until later in the session. The range of the transition for subject 140 was large while subjects 145 and 148 had a medium transition range and subject 151 had a small transition range

Table 6 Means of Sessions for Ascending Titration (3 Hz)

	Mean of Transitions (Hz)		
Subject	First Session	Second Session	Overall Mean
140	8.32	6.84	7.58
141	8.71	10.75	9.73
145	11.82	10.23	11.01
148	9.42	9.79	9.61

Table 6 shows the means of the ascending titration sessions, plus the overall mean for the ascending sessions. The overall means ranged from 7.58 Hz for subject 140, to 11.01 for subject 145 with subject 141 having a value of 9.73 Hz and subject 151 having a value of 9.61 Hz.

Titration Descending

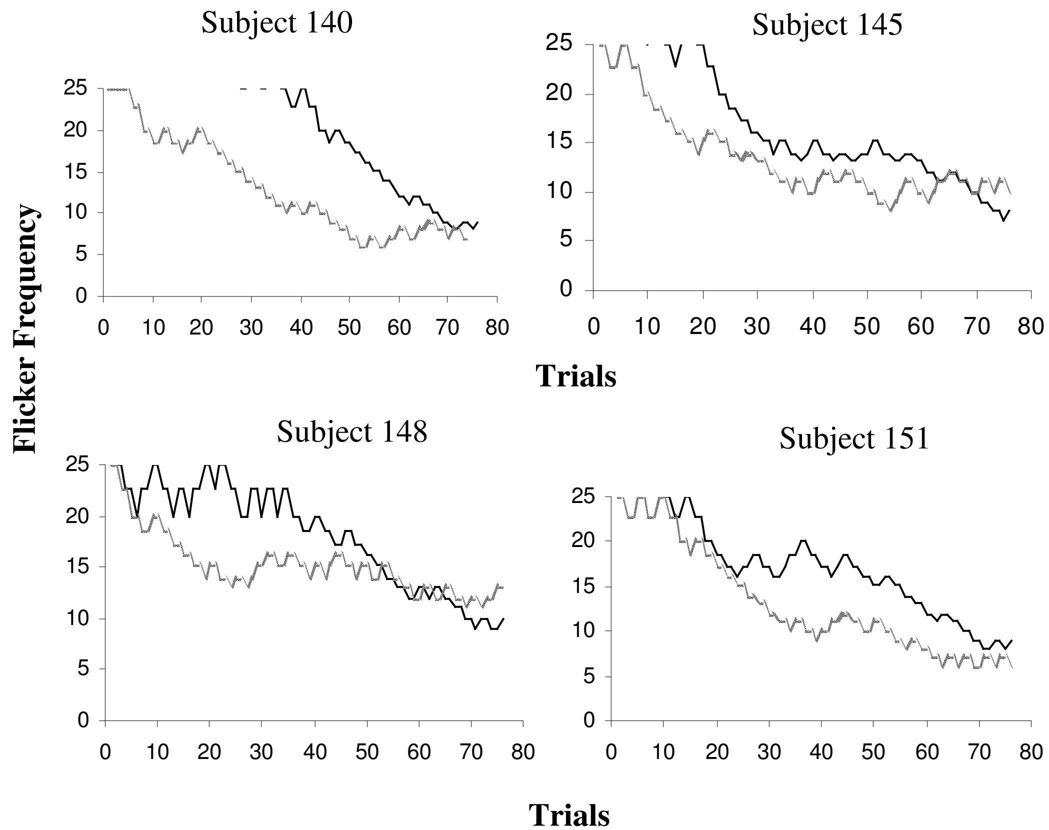


Figure 10 Descending titration trials.
The X-axis represents the number of trials used to test in a session. The Y-axis represents the flicker frequencies that were tested. The dark solid line represents the first descending titration session; the light solid line represents the second descending titration session.

Figure 10 details the changes in the flicker frequency for each subject during the descending titration sessions. Data is shown from 25 Hz on the Y-axis. Data above 25 Hz shows only a progressive decline in the testing flicker frequency, for the three subjects that were tested from 100 Hz. The dark solid line, which represents the first descending titration session, started at 100 Hz for subjects 140, 145 and 151. Because the CFF was

expected to be much lower than this starting frequency, the starting frequency was reduced to 25 Hz for these three subjects on the second session. Subject 148 started both sessions at 25 Hz because testing for this subject started later due to the extended training necessary, as described earlier.

Table 7 Transitions for Descending Titration

	Mean of Transitions (Hz)		
Subject	First Session	Second Session	Overall Mean
140	8.35	7.28	7.82
145	13.06	10.59	11.83
148	11.51	13.83	12.67
151	8.35	8.27	8.31

Data for transitions in the descending titration testing sessions for each subject

Table 7 shows data from the two descending titration sessions. The range of the mean of the transitions for the first session was 8.35 Hz to 13.06 Hz, and for the second session was 7.28 Hz was 6.55 Hz. The overall mean had a range of 4.36 Hz with a high of 12.67 Hz for subject 148 and a low of 7.82 Hz for subject 140. Subject 145 had an overall mean of 11.83 and subject 151 had an overall mean of 8.31.These overall means were comparable with the ascending titration.

Table 8 Mean Frequencies by Method (3 Hz)

Subject	Ascending Titration	Descending Titration	Constant Stimuli	Mean
140	7.58	7.82	17.91	11.1
145	9.73	11.83	9.88	10.48
148	11.03	12.67	15.50	13.07
151	9.61	8.31	11.39	9.77
Mean	9.49	10.16	13.67	11.17

Mean frequencies for each testing method plus the overall mean frequencies by subject and method

Table 8 shows the calculated frequency for the three methods ascending titration, descending titration and constant stimuli. The range of the estimated CFF is 10.33 Hz (subject 140 ascending titration 7.58 Hz, and constant stimuli 17.91 Hz). The means of the subjects estimated CFF range from 9.77 Hz for subject 151 to 13.07 Hz for subject 148. The mean of the different methods range from 9.49 Hz to 13.67 Hz.

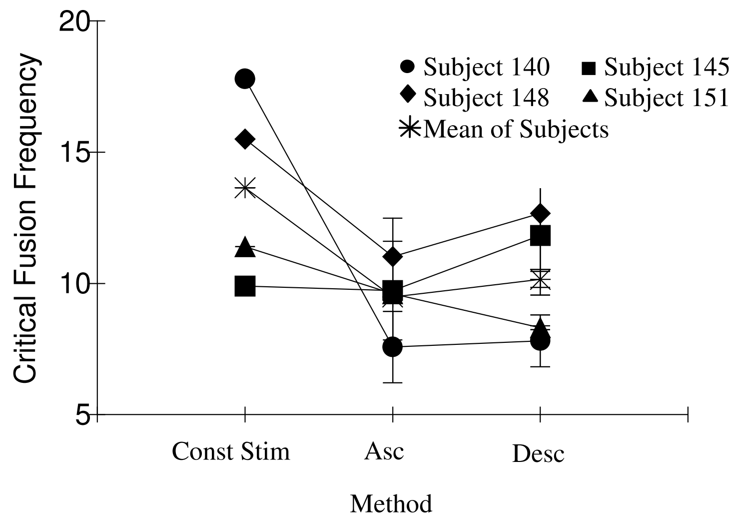


Figure 11 Mean Values for Each Method of Testing
Data points represent mean values for each subject for each method of testing. Error bars are the standard error of the mean. The constant stimuli method shows no error bars because only one set of data is used.

Figure 11 displays the mean values for each method of testing, for each subject and the mean across subjects for each method. The error bars are the standard error. There are no error bars for the constant stimuli method because there was only one set of data for this method, however variance can be estimated by the coefficients of determination (r^2) in Figure 7.

A one-way analysis of variance across and the mean of the subjects CFF's with testing methods as a factor was performed and an ANOVA was performed across the

mean of methods. There was a significant difference for subject 140 [F (2,2) = 40.785, p = 0.02] and 151 [F (2,2) = 86.79, p = 0.011]. There was also a significant difference for the mean of the subjects [F (2,2) = 181.621, p = .005]. Post hoc analysis showed that there was a significant difference between the constant stimuli and the ascending titration (p = 0.022), and the constant stimuli and descending titration (p = 0.023) for subject 140. For subject 151 there was a significant difference between all three methods constant stimuli/ascending (p = 0.031), constant stimuli/descending (p = 0.011), ascending/descending (p = 0.039). Post hoc analysis for the means across methods showed a significant difference between constant stimuli and ascending (p = 0.005), and constant stimuli and descending (p = 0.007).

The method of constant stimuli CFF is calculated by an estimate of the regression. A function is developed to estimate the regression. The function that was used in this analysis was a straight-line function of the standardized scores of the probability of a response on the flicker appropriate lever. This analysis uses the assumption that the distribution is a normal distribution (Gescheider, 1997). If the distribution was not normal then the estimation of the CFF may be inaccurate. Conversely, The titration method calculates the estimated CFF by taking the mean of the transitions of the sessions. In order for this to be accomplished, it is necessary to establish when a steady series of transitions is taking place i.e., when transitions are being continually repeated within a range of testing frequencies. The establishment of this range is somewhat subjective. Figure 10, subject 148 shows a steady series of transitions at the beginning of the session, but the testing frequency then starts to become longer. In contrast, subject 151 in Figure

10 shows an initial downward trend of the frequency, followed by a period of steady transitions. The session ended with a further decline in the testing frequency. In establishing an estimated CFF for titration, the period of longest steady transitions in each session was used. The upper and lower limits of the range were established by the highest and lowest transition within the steady range.

The significant difference in the means of the two methods (ascending/descending titration and the method of constant stimuli) may also be an indication that the two methods were not testing the same event.

Although discrimination between the steady LED stimulus and the flickering LED stimulus was apparent in both methods whether the transition from flicker to steady was what was being tested may be in dispute. The means of the subjects for both titration methods were significantly lower than the mean for the subjects of the constant stimuli method but were not significantly different from each other. Furthermore, Subject 140 had a significant difference among both titration methods and the constant stimuli method but not between the ascending and descending titration methods, and subject 151 had a significant difference among all three methods.

Subject 145 had no significant difference among the methods. This is thought to reflect better discrimination among the stimuli, which is indicated by the slope of the regression line in Figure 7. A slope of zero would indicate that the subject could not discriminate any of the flicker stimuli from a steady stimulus. An increase in a negative slope would be indicative of an ability to correctly distinguish a flickering stimulus from a steady stimulus. The slope for subject 145 was greater than for the other subjects.

Subject 148 did not have any significant difference among the methods although the ability to discriminate was almost equal to subjects 140 and 151. This subject had the highest calculated transition frequency in both titration methods and was mid range in the constant stimuli method. This would explain why there is no significant difference between the methods but it cannot be assumed that the calculated frequencies, from the three methods, represent the true CFF for this subject.

The mean of the calculated frequencies was highest for the method of constant stimuli method than it was for either of the titration methods and the mean of the ascending titration was lower than the mean of the descending titration. This was true for three of the four subjects. The constant stimuli was higher for subjects 140, 148, and 151 than the titration methods and the descending titration was higher for subjects 140, 145, and 148 than the ascending titration.

One of the major differences between methods is the constant stimuli testing method randomly presents stimuli while titration presents the stimuli in either a progressively increasing or decreasing order. The descending titration method interspersed 3 Hz training trials in the session as did the constant stimuli method. The ascending titration method always started close to the training frequency.

The possibility exists that the discrimination that was being tested was between the training frequency (3 Hz) and other frequencies, (including steady trials) and not the discrimination between flickering and steady stimulus. In the Descending titration and constant stimuli methods, the training frequency was always present; therefore, as the testing frequency became increasingly faster discrimination could take place between the

testing and training frequencies. This would have been easier for descending titration than the constant stimuli method because the difference would have been progressively less rather than random, possibly explaining why the constant stimuli results were higher than the descending titration results.

With the ascending titration method the testing always started close to the testing frequency, thus, as the testing frequency became greater an easier discrimination between it and the training frequency was possible.

To test the hypothesis that the discrimination was between the testing frequency and other frequencies a series of training sessions in which the training frequency was progressively faster for each session were performed.

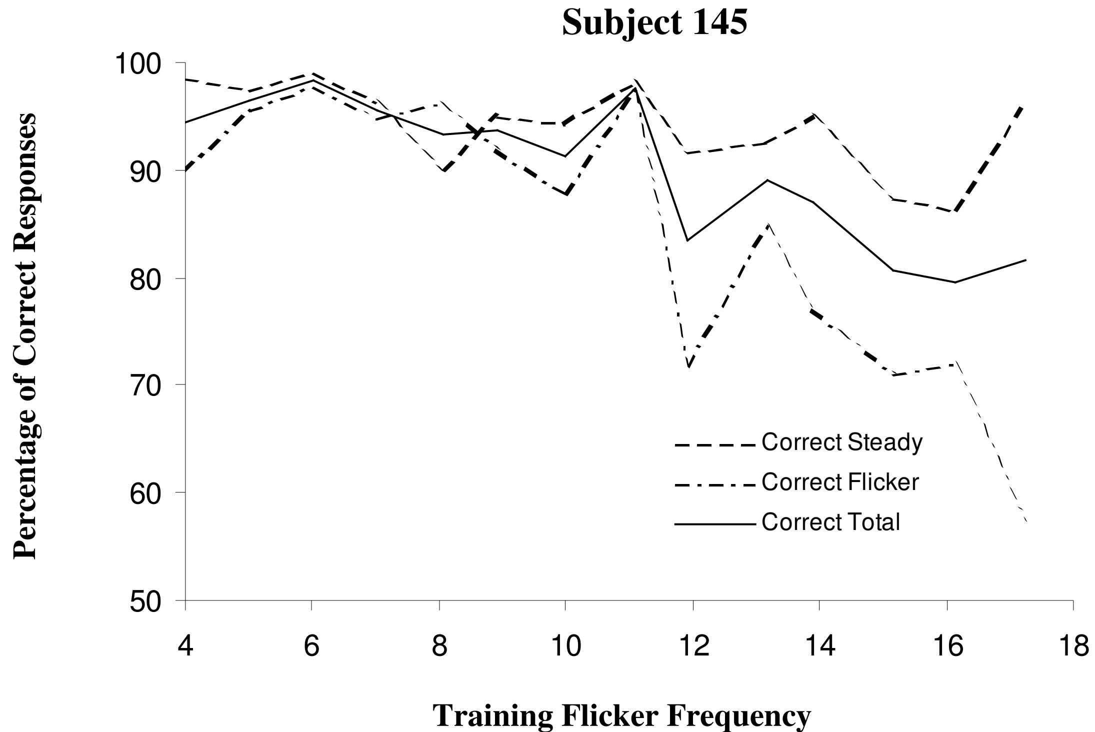


Figure 12 Percentage of Correct Responses
The percentage of correct responses for correct steady responses, correct flicker responses and overall correct responses as a function of the training frequency and the steady frequency for each session.

Figure 12 shows the percentage of correct responses, for subject 145, as the training frequency was increased. Some frequencies were repeated therefore the data is the mean across sessions for any particular frequency. Above a frequency of approximately 11 Hz the percentage of correct responses on the flicker appropriate lever begins to progressively decline. The data for this subject was representative of all subjects. Based on these results the subjects were re-trained at a frequency of 10 Hz until they all met the criteria of 5 consecutive sessions at which correct responding was above 80%.

Results at 10 Hz training.

Testing using the constant stimuli method was conducted over 7 sessions with the data calculated using the same method as the data from the 3 Hz testing sessions. Four sessions of ascending and four sessions of descending titration were run plus an additional four sessions of ascending titration were run with the initial frequency starting at 25 Hz, which was considered above the CFF for the subjects.

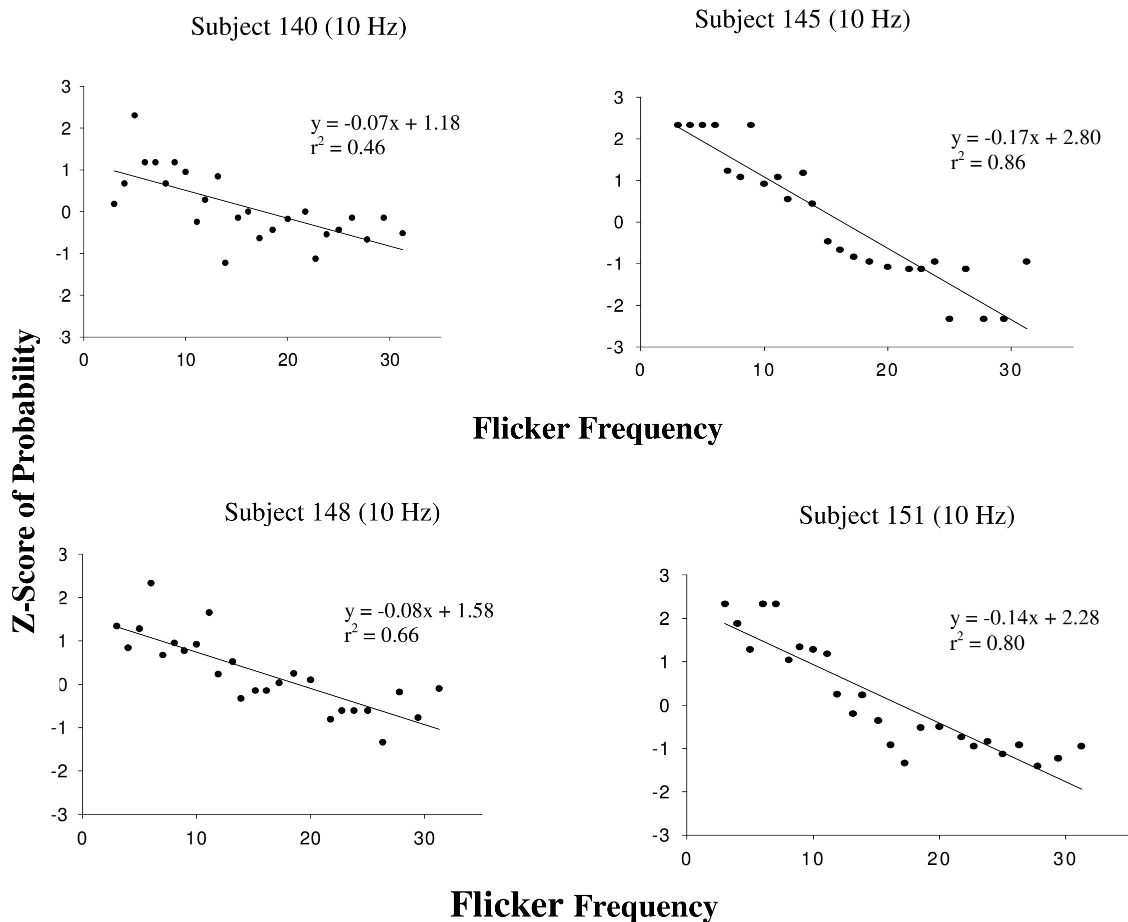


Figure 13 Regression of Z-score of Probability (10 Hz)
Scatterplot shows Z-scores of the probability of a response on the flicker appropriate lever as a function of the flicker frequency. The straight line is the regression line fitted by the least square method.

Figure 13 shows the probabilities of a response on the flicker appropriate lever converted to Z-scores for the seven testing sessions using the constant stimuli method. A probability of one was given a Z-score of 2.33 and a probability of zero was given a Z-score of –2.33 as with the previous analysis.

The value of coefficients of determination was higher than the previous testing, for two subjects (145, 151) and lower for two subjects (140, 148). The slope of the regression line was greater for three subjects (145, 148, 151) suggesting greater discriminability but lower for subject 140.

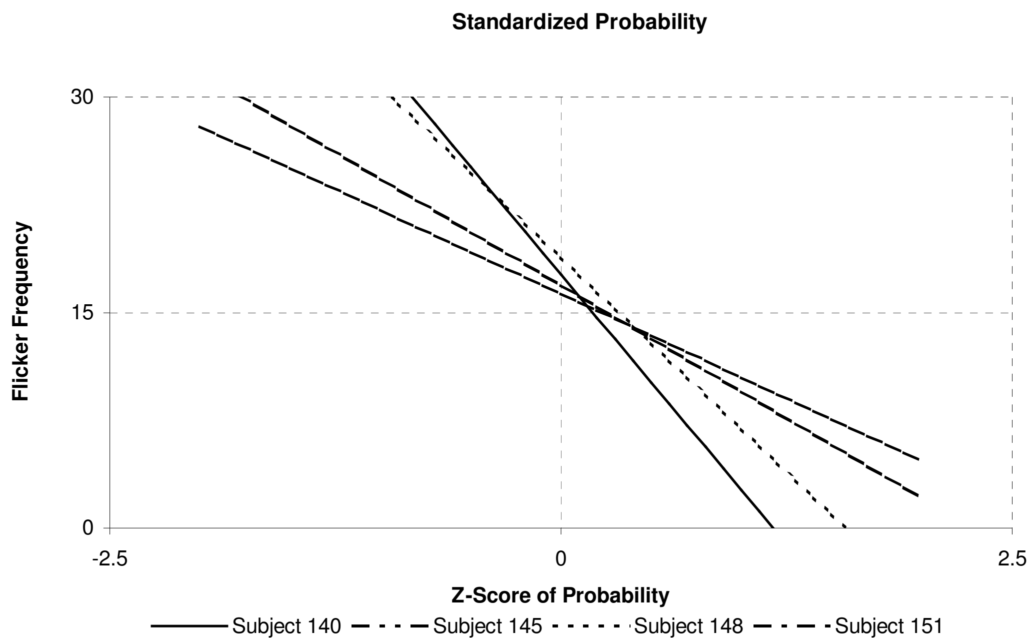


Figure 14 Regression lines of Ogive Functions (10 Hz)

Using the same method as in Figure 8 the regression lines for each subject were plotted from plus to minus two standard deviations on the X axis, which represents

the Z-scores of the probability of a response on the flicker appropriate lever using the equations generated from the plots in Figure 13. The point at which the regression line crosses the Y-axis at an X value of zero represents the frequency at which the probability of a response on the flicker appropriate lever was 50%.

Table 9 Mean Frequency (10 Hz)

Subject	T	D	Frequency
140	1.18	-0.07	17.6
145	2.80	-.17	16.3
148	1.58	-0.08	18.8
151	2.28	-0.14	16.9

T = Theoretical Z-Score when frequency equals zero D = Stimulus discriminability. Frequency is the calculated flicker frequency when the Z-Score = 0

Using the equation $\left[F = \left(\frac{-T_{FF}}{D} \right) \right]$ the estimated critical fusion frequencies were calculated and are presented in Table 9.

Titration

Four ascending sessions, four descending sessions, and four ascending sessions starting at 25 Hz were completed. The same criteria were used as the previous titration sessions with the following exceptions; the descending titration trials started at a frequency of 29.4 Hz and the ascending titration started at a frequency of 6 Hz. Table 10 shows the results of the titration testing sessions.

Table 10 Mean of Transitions for Titration Testing (10 Hz)

Mean of Ascending Transition					
	1st	2nd	3rd	4th	Overall Mean
140	19.6	20.1	17.9	14.9	18.1
145	18.7	23.2	12.5	18.3	18.2
148	14.1	18.2	12.2	13.1	14.4
151	14.2	15.4	20.3	21.1	17.7
Mean of Descending Transition					
	1st	2nd	3rd	4th	Overall Mean
140	14.2	15.7	25.4	19.4	18.7
145	19.0	16.1	10.6	11.7	14.4
148	28.6	15.1	22.1	14.9	20.2
151	18.7	14.8	33.7	17.7	21.2
Mean of Ascending Transition (25 Hz)					
	1st	2nd	3rd	4th	Overall Mean
140	17.7	17.9	14.0	12.7	15.6
145	13.6	19.9	21.2	22.5	19.3
148	14.3	13.8	23.5	29.5	20.3
151	26.7	30.0	29.8	30.4	29.2

The highest mean value for the ascending sessions was 23.2 Hz (subject 145) and the lowest was 12.2 Hz. (subject 148). The mean for the four ascending titration sessions for each subject was subject 140 18.1 Hz, subject 145 18.2 Hz, subject 148 14.4 Hz and subject 151 17.7 Hz. For the descending titration sessions, the highest mean was33.7 Hz and the lowest mean 12.2 Hz. The overall means for the descending titration sessions was 18.7 HZ, 14.4 Hz, 20.2 Hz, and 21.2 Hz for subjects 140, 145, 148 and 151 respectively. Means for the ascending sessions that started at 25 Hz ranged from a high value of 30.0 (subject 151) to a low value of 12.7 Hz (subject 140). The overall mean for subject 140 was 15.6 Hz, for subject 145 19.3 Hz, subject 148 20.3 Hz and for subject 151 29.2 Hz.

Table 11 Data for Testing Sessions (10 Hz)

	Subject 140	Subject 145	Subject 148	Subject 151	Mean
Sessions	13	13	13	13	13
Minimum	12.67	10.55	12.15	14.17	15.41
Maximum	25.44	23.21	29.50	33.73	23.76
Range	12.77	12.65	17.35	19.56	8.35
Overall Mean	17.47	17.20	18.33	22.28	18.82
Std. Error	0.93	1.15	1.63	1.92	0.79
Standard Dev	3.35	4.14	5.87	6.91	2.86

Data for all testing sessions for each subject. All data is in Hz

Table 11 shows the data of all the testing sessions for each subjects, plus the overall mean of all testing sessions for all subjects. A repeated measures ANOVA with testing methods as the factor showed there was no significant difference between subjects. [$F = (3,9)1.643,\ P = 0.248$], neither was there any significant difference within subjects [$F = (4,12)1.495,\ P = 0.224$]. There was no interaction with subjects and methods of testing $(p = 0.106)$.

The ascending sessions starting at 25 Hz produced unexpected results. Of the 16 trials (four per subject), seven ended higher than the starting frequency of 25 Hz. This represented approximately 44% of the ascending 25 Hz sessions. The distribution of these trials was, one for subject 145, two for subject 148 and all four trials for subject 151. Because of these results a second analysis was performed which omitted all of the ascending 25 Hz trials. Two other trials ended at approximately 30 Hz; the second ascending trial for subject 145 and the third descending trial for subject 151. These results are includes in the second analysis.

Table 12 Data without ascending 25 Hz Session

	Subject 140	Subject 145	Subject 148	Subject 151	Mean
N of cases	9	9	9	9	9
Minimum	14.20	10.56	12.16	14.18	15.41
Maximum	25.44	23.21	28.61	33.73	22.96
Range	11.25	12.65	16.46	19.56	7.55
Overall Mean	18.32	16.27	17.46	19.19	17.81
Std. Error	1.13	1.36	1.74	1.98	0.83
Standard Dev	3.41	4.09	5.23	5.95	2.50

Data for all testing sessions except ascending 25 Hz sessions, for each subject. All data is in Hz

Table 12 shows the data of all the testing sessions for each subject, plus the overall mean of all testing sessions for all subjects after the ascending 25 Hz sessions had been removed. By removing these sessions, the variance was reduced for all subjects except subject 140 where the standard deviation increased from 3.35 to 3.41. The range of the mean across subjects was reduced from 5.08 to 2.92. A repeated measure ANOVA showed no significant difference within subjects for the different testing methods [$F = (2,6)\ 0.307, P = 0.746$] and no significant difference among subjects. There was no interaction between subjects and the different methods of testing ($P = 0.615$).

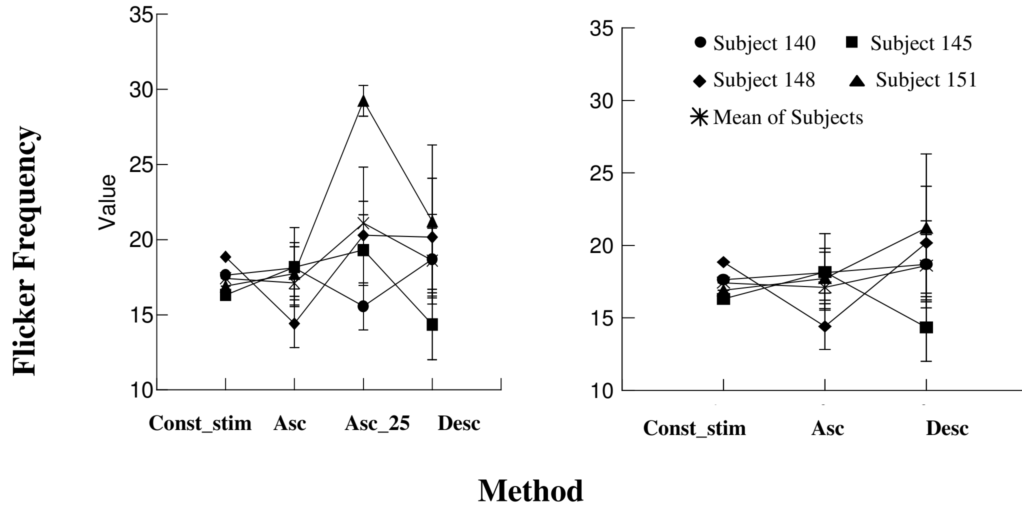


Figure 15 Mean Values for Each Method of Testing
Data points represent mean values for each subject for each method of testing. Error bars are the standard error of the mean. The constant stimuli method shows no error bars because only one set of data is used.

Figure 15 present the means for each subject for each type of testing method and the mean for each method. The left graph shows the means including the ascending 25 Hz method; the right graph has the data from the ascending 25 Hz method eliminated. The greater variability that the ascending 25 Hz sessions add to the data is clearly illustrated in difference between the charts in Figure 15.

The error bars represent the standard error of the mean. There are no error bars for the constant stimuli method because the data for this method represents the flicker frequency at which a 0.5 probability of a response on flicker associated lever will occur, for all data over seven sessions. Variability for the method of constant stimuli is given by the coefficient of determination (r^2) for the functions developed from the data for the four subjects. The values of r^2 are, subject 140 = 0.46, subject 145 = 0.86, subject 148 = 0.66 and subject 151 = 0.80.

Table 13 Mean Frequencies by Method (10 Hz)

Type	Subject_140	Subject_145	Subject_148	Subject_151	Mean
Mean ascending	18.13	18.17	14.40	17.73	17.1
Mean descending	18.69	14.35	20.16	21.21	18.6
Mean titration	18.41	16.26	17.28	19.47	17.9
Constant stimuli	17.64	16.31	18.85	16.91	17.4
Overall mean	18.32	16.27	17.46	19.19	17.8

Mean frequencies for each testing method plus the overall mean frequencies by subject and method.

Table 13 shows the means of the calculated flicker frequencies for each subject for each testing method. The row labeled mean titration is the mean of the ascending and the descending sessions combined. The overall mean is the mean of all testing methods combined. The difference of the mean of the titration methods and the constant stimuli methods is 0.77 Hz for subject 140, 0.05 Hz for subject 145, 1.57 Hz for subject 148 and 2.56 for subject 151. The difference of the combined means of the subjects for the titration means and the constant stimuli means is 0.5 Hz. Because there is no significant difference between the methods either within or between subjects the critical fusion frequencies for theses subjects will be considered the overall mean for all methods for each subject. Therefore, the critical fusion frequencies are; for subject 140, 18.32 Hz, subject 145, 16.27 Hz subject 148, 17.46 Hz and subject 151, 19.19 Hz.

Summary

The change of method from using a temporal means to try to establish stimulus control over the flickering LEDs to one that used a discrimination procedure led to the development of psychophysical functions for all subjects. Once autoshaping to the two

levers was achieved then the establishment of discriminatory behavior was soon established for three subjects. The fourth subject needed subsequent training to establish stimulus control, however, this was achieved by a gradual change from alternating the trial types to presenting a series of random trials. The criterion of 80% correct overall responding was established for all subjects.

The initial results from the 3 Hz-testing phase were significantly different among subjects and methods. A new training frequency was empirically derived by progressively increasing the training frequency until the criteria of 80% overall, correct responding could no longer be achieved. A frequency of 10 Hz was selected as the new training frequency because it was the highest frequency at which all subjects responded at 80% or greater accuracy on both types of stimuli.

A comparison of Figures 8 and 14 gives an indication that the subjects were responding to a differential between a flickering stimulus and a steady stimulus after the 10 Hz training. Figure 8 shows the Z-Score of the probability of responding on the flicker appropriate lever plotted against the testing flicker frequency, for testing under the 3 Hz training sessions. The range of 8 Hz, under the 3 Hz training procedure, may indicate that some subjects were responding to the low training frequency of 3 Hz (subjects 145, 151) while others are responding to a general flickering stimulus (subject 140). Subject 148 may have been responding to both. Figure 14 has the same structure as Figure 8 but displays the data for testing after the 10 Hz training had been implemented. The range has been reduced to 2.5 Hz and the overall mean of the four subjects was higher. Subject 140 had a lower mean frequency (16.6 Hz-16.3 Hz) while the other subjects all had a higher

mean frequency ranging in difference from 6.4 Hz for subject 145 to 3.3 Hz by subject 148. The smaller difference in mean between the four subjects, plus the overall higher mean may indicate that the visual systems of the four subjects are similar and that they were all responding to the same stimuli. The range for the 10 Hz training was 5.08 Hz (22.8 Hz-17.20 Hz) and the difference between the different methods was not significant.

The ascending 25 Hz titration sessions produced unexpected results. For subject 151 the mean transition was approximately 30 Hz (Table 10). When the ascending 25 Hz data was removed from the analysis the mean differences in the types of testing was only 1.5 Hz and the difference in the overall mean for all testing sessions between subjects was 2.92 Hz. Figure 15 shows the difference in variation when the ascending 25 Hz sessions were included and when they were excluded from the analysis.

Although the final analysis included all the ascending, descending and constant stimuli sessions certain session showed the same pattern as the ascending 25 Hz testing sessions. One ascending session (subject 145) and four descending sessions (subject 140-1 session, subject 148-2 sessions, subject 151-1 session) all showed a similar pattern. If these testing sessions had been left out of the analysis the overall means for each subject would be 17.43 Hz, 15.4 Hz, 15.2 Hz and 17.37 Hz for subjects 140, 145, 148, 151 consecutively, which gives a range of 2.23 Hz. The means for the different methods would be 16.77 Hz (ascending titration), 14.8 (descending titration), and 17.43 Hz (constant stimuli), which give a range of 2.63 Hz between the methods.

The final analysis that was used included all the ascending, descending and constant stimuli sessions. The overall means for the types of sessions for each subject were 18.32

Hz for subject 140, 16.27 Hz for subject 145, 17.46 Hz for subject 148, and 19.19 Hz for subject 151, based on the data acquired after 10 Hz training had been implemented. These Figures are probably close to the CFF for these subjects for the stimuli presented to them in this experiment.

General Discussion

This study compared different methods for estimating the critical fusion frequency in rats. The objective was to develop a method that was compatible with longitudinal studies therefore positive reinforcement was used throughout all experiments.

Experiment one established that a psychophysical function could be calculated for a flickering stimulus in rats however the majority of subjects failed to come under stimulus control. Two factors contributed greatly to this. A temporal factor requiring the subject to keep sustained contact with a metal tube was hard to train for the majority of animals. This limited the number of occasions the subjects were exposed to the stimulus contributing to the lack of stimulus control. The second factor was urolithiasis, which contributed to a high mortality rate and had an unknown effect on behavior.

The use of an infrared beam to replace the sipper tube in experiment two helped to establish longer durations but stimulus control was not established and no psychophysical functions were created using this method.

In experiment three a discrimination procedure was introduced and this proved successful in establishing stimulus control of a steady illuminated stimulus and a

flickering stimulus. Psychophysical functions were obtained for all subjects and an estimated CFF was calculated using three different methods of testing. Because the estimated CFF from the different methods were significantly different, and of a lower value than was expected, based on the literature, a new training frequency was empirically derived.

A second set of testing produced new psychophysical functions for all subjects from which new estimated CFFs were calculated. No significant differences among the methods or within the subjects using this method, was found and estimated CFF values were comparable with other values obtained in the literature.

The five psychophysical functions that were generated in experiment 1 had estimated CFFs that were of similar to the values to those generated in experiment three during the 3 Hz training stage. The similarity of these values may be an indication that experiment one, while failing to produce data for the majority of subjects, did establish that discrimination between a flickering light stimulus of one frequency and that of another frequency or a steady stimulus can be made by rats using a positive reinforcement approach. The values obtained in experiment one cannot be considered critical fusion frequencies, for, as was demonstrated in experiment three, the training frequency of 3 Hz was to low to establish a general discrimination between flickering and steady illuminated LEDs. Rather the discrimination was more likely between slow flickering LEDs (3 Hz) and faster flickering LEDs plus steady LEDs.

When the training frequency was raised to 10 Hz, in experiment 3, after a systematic analysis of the effects of training at different frequencies, results from

different testing methods had no significant differences, either between or across subjects. These results justified the training at the higher frequency.

Although the calculated values, from the different methods, were not significantly different from each other, there were unusual discrepancies in the results of some of the test trials. For subject 151 in all four of the ascending 25 Hz sessions and in one of the descending sessions, the flicker frequency increased when it would be expected to decrease. The CFF would be expected to be in the region of 15-20 Hz which means that during the sessions in which the frequency was generally ascending above 25 Hz the subject was pressing the flicker associated lever even though the testing frequency was probably above the flicker threshold. The reason for this phenomenon may be synchronous period doubling.

In a study looking into the phenomenon of synchronous periodic doubling Crevier and Meister recorded the response of a salamander retina to a flickering stimulus that had a square sine wave, using an electroretinogram (ERG). When a flicker frequency of 1 Hz was projected on the eye one response per cycle in the retina was recorded. A frequency of 7 Hz also recorded a response every cycle but a frequency of 13 Hz recorded a response every other cycle. When a frequency of 16 Hz was projected, there is no recognizable order of responses.

As the flicker frequency was increased, the signal that was detected was a fraction of the actual frequency. Thus, the subject would perceive a flicker even though the flicker frequency was above the CFF. In the same study, Human subjects were also tested, using

visual evoked potentials, and the same effect was apparent (Crevier & Meister, 1998). This is the phenomenon known as synchronous periodic doubling.

The mechanism for synchronous period doubling appears to lie in the ganglion cells of the retina. In the study using the salamander retina the ganglion cells spiked once at the onset and twice at the offset phase of a 1 Hz signal. When the frequency was changed to greater than 9 Hz, the spikes changed significantly and were registering only every other cycle. At 13 Hz, the response at the ganglion cells was every 4th cycle. A similar study investigating the same phenomenon in primates conclude

> "That postreceptoral ON and OFF-components contribute substantially to the sine-wave flicker ERG, especially at higher stimulus frequencies. Because of phase cancellation, they mask each other in the net response in a frequency dependent fashion. The photoreceptor contribution is greater than the net postsynaptic component only for frequencies of approximately less than or equal to 10 Hz" (Kondo & Sieving, 2001).

Thus, from evidence in these studies it is possible that the testing sessions that moved the testing frequency above 25 Hz was a consequence of synchronized periodic doubling. Given this it was reasonable to eliminate the ascending 25 Hz sessions, which were the main sessions contributing to this, from the analysis.

Eliminating the ascending 25 Hz sessions brought the range of the mean frequencies across methods for each subject to 2.92 Hz (sd, 1.25), with the highest 19.19 Hz (subject 151) and the lowest 16.27 Hz (subject 145).

If the frequency values that were generated in these experiments, using the visual stimuli that were presented to the subjects, are to be considered the CFF of the subjects then they must be seen to be of comparable value to data produced in other studies.

Goldzband and Clark reported the CFF of 12 rats as far back as 1955, with a mean value of 24 Hz (Goldzban & Clark, 1955) however the results ranged from 31.4 Hz to 22.8 Hz and the luminance of the visual stimuli were not reported. A mean value of 21.54±6.0 Hz was recorded in a study that looked at the CFF in cells in the rats visual cortex by recording single unit responses to flickering stimuli. The luminance value in this experiment was 17 cd/m^2 (Wells et al., 2001).

A study that that looked at the full visual field CFF in rats reported that with a log luminance value of –1.3 foot lamberts a CFF of 21 Hz was recorded (Williams et al., 1985). This value converts to log luminance value of -.07 cd/m^2.

Luminance is the measure of light leaving a surface in a given direction.(Ditchburn, 1976) (p.380). Test reading taken every second for one minute, at the volumetric center of the operant chambers used in our experiment produced a mean value of 0.5 lux illuminance, which calculates to a log luminance value of –3.9cd/m^2. The luminance level in the experimental chambers, therefore are considerably lower than in the Williams experiment. As reported earlier luminance has a bearing on the value of the CFF with lower values associated with lower CFF values (Grusser & Landis, 1991).

Given the difference in luminance values the CFF values obtained in this study appear to be well within the range of values reported elsewhere. The range obtained in these experiments is smaller than Wells et.al., or Goldzban and Clark. Williams et. al., only reported results from individual subjects thus, ranges cannot be compared. The reason for a lower range in our results may be the use of a experimentally derived training frequency rather than one that is arbitrarily used, and may have contributed to

tighter experimental control. This can be seen in the difference in the range of results when a 3 Hz and 10 Hz training frequency was used.

General Summary

A method for determining the critical fusion frequency for rats has been developed using a discrimination procedure and positive reinforcement. The values calculated from the data can be considered close to the CFF of the subjects, for the stimuli used, when compared to other studies looking at the same phenomenon. The method developed incorporates an empirical technique for determining the final frequency that training should take place at in order to eliminate discrimination of the wrong stimuli. Because of the use of positive reinforcement, the method lends itself well to longitudinal studies involving changes in the visual system of rats, and the ease of training makes it possible to use this method for a large numbers of subjects. In particular, the method may be of value in studying the effect on the visual system of toxins such as mercury, which are known to have detrimental effects to vision.

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APPENDIX A

Methylmercury

Mercury (Hg) is a naturally occurring metallic element that is prolific throughout the environment. Mercury is dissipated into the atmosphere by such natural actions as volcanic eruption, degassing of the earths crust, and evaporation from water.

Anthropogenic sources of mercury include industrial pollution, the burning of fossil fuels, mining, refuse incineration and cremation (Gilbert & Grant -Webster, 1995). It is estimated that there may be as much as 80,000 tons of Hg in the atmosphere and a concentration of 0.000003 mg/liter in seawater. In a cubic mile, this translates to 0.1 tons with a total ocean carrying capacity of 46 x 10^6 tons (Daintith, 1996). Mercury is present in several inorganic forms including metallic (Hg^0), mercurous (Hg_2^{2+}), and mercuric (Hg^{2+}) valence states (Daintith, 1996). The dominant form of mercury in the atmosphere is ionic mercury (mercuric) (Hg^{2+}), which can be transformed in to several organic forms of which Methylmercury (MeHg) is one (Council, 2000). According to the International Program of Chemical Safety, as reported by Gilbert and Webster, MeHg is considered one of the six most dangerous chemicals in the World's environment, (Gilbert & Grant - Webster, 1995). When deposition of Hg to water from the atmosphere occurs, transformation to methylmercury (CH^3Hg+) from the inorganic states can transpire by a process known as methylation (Compeau & Bartha, 1985).

Methylation, in which a methyl group (CH^{3-}) is introduced into a molecule (Daintith, 1996), occurs mainly in natural aquatic environments. The mercuric ion (Hg^{2+}), which is much more soluble in water than other ions, can be methylated to give CH^3Hg+,

methylmercury (Chemaly, 2002). Mercurous (Hg_2^{2+}), which is dimeric, that is mercurous contains two molecules of Hg within its molecule, undergoes disproportination in an aqueous solution converting to Hg^0 and Hg^{2+} hence the proportion of Hg^{2+} in water is increased over that in the atmosphere.

It has been proposed that the process of methylation is mediated by sulfate reducing bacteria (SRB). Although the exact procedure of how methylation takes place is not well known, Compeau and Bartha did isolate certain strains of Desulphovibrio Desulfuricans, an SRB, and show that they methylated mercury (Compeau & Bartha, 1985). The methylation process is related to background sulphur in the sediment of bodies of water and appears to take place within a depth of 10 cm of that sediment. Different strains of SRB methylate mercury at different rates. Mercury methylation rates are based on measured sulphate reduction rates in a particular sedimentary area (King, 2001). King et, al; reports that previous studies using pure cultures of SRB have indicated that mercury methylation rates of different phylogenetic groups can vary over three orders of magnitude when normalized to the sulphur reduction rate.

Methylmercury is introduced to the aquatic food chain through the methylation process, probably by the direct consumption of the SRB by plankton, or by direct absorption of MeHg that is dissolved in the water (King, 2001). This step is important because MeHg levels increase by 10^4 in plankton over water concentrations (King, 2001). The plankton are ingested by aquatic species at the lower end of the food chain. About 1.5% of the mercury in sediment and 2% of the mercury in seawater is in the form of MeHg. However approximately 80% of the mercury in fish exists as MeHg,

and fish appear to have a slow rate of elimination. In addition, the amount of MeHg in fish increases with the age and size of the fish (Chemaly, 2002; King, 2001). Species of fish at the lower end of the food chain are in turn ingested by species at the next level of the food chain. This process continues until the top of the food chain is reached. At each level of the food chain, MeHg levels increase by tenfold or less. This process is known as biomagnification. The consumption of fish is the main source of MeHg contamination in the human population.

APPENDIX B

Deaths from urolithiasis

Urolithiasis, a condition resulting from the formation of calculi in the urinary tract, was a major problem in the survival of subjects in experiment one. Deaths from this disorder were attributable to an impurity in the diet, which appeared to promote the formation of the calculi. Changing of the diet, once the condition had appeared in the population did not have any effect on the mortality rate. Males, who comprised approximately 53% of the subjects at the commencement of the training, were affected by this condition more than females by a ratio of 3:1. It should be noted that this problem was not unique to this colony, but has been reported by several laboratories throughout the country.

APPENDIX C

Table 14 Summary of data from 31 subjects.

Subject	Diet	MeHg	Mean Reinforcers per session	Mean% of Correct Rejections	Mean % of Correct Hits (3 Hz)	Reinforcers per minute
2-B	Low DHA	0.0Hg	36.6	76	70	1.22 *
5x2	Low DHA	0.0Hg	29.1	80	43	0.97
7x1	Low DHA	0.0Hg	40.6	76	71	1.35
8x5	Low DHA	0.0Hg	3.8	10	90	0.13
10x2	Low DHA	0.0Hg	13.1	43	45	0.44
12x2	Low DHA	0.0Hg	7.9	0	66	0.26
15-C	Low DHA	0.5 Hg	14.9	47	37	0.50 *
19x2	Low DHA	0.5 Hg	6.6	13	74	0.22
24x2	Low DHA	0.5 Hg	7.2	19	36	0.24
225x2	Low DHA	5.0 Hg	21.8	55	57	0.73
325x2	Low DHA	5.0 Hg	15.2	47	49	0.51
326x2	Low DHA	5.0 Hg	38.3	75	75	1.28
27x2	Low DHA	5.0 Hg	16.6	73	27	0.55
30x4	Low DHA	5.0 Hg	6.1	10	100	0.20
333x2	Low DHA	5.0 Hg	9.6	52	40	0.32
334x1	Low DHA	5.0 Hg	6.9	37	83	0.23
35-A	Low DHA	5.0 Hg	6.0	10	41	0.20
336x2	Low DHA	5.0 Hg	31.8	78	71	1.06
37x1	High DHA	0.0Hg	32.4	92	84	1.08 *
38x1	High DHA	0.0Hg	4.7	5	68	0.16
39x4	High DHA	0.0Hg	6.0	25	47	0.20
41x2	High DHA	0.0Hg	12.5	44	86	0.42
44x2	High DHA	0.0Hg	17.5	44	46	0.58
46x2	High DHA	0.0Hg	16.6	58	33	0.55
51x1	High DHA	0.5 Hg	17.4	64	42	0.58
55x2	High DHA	0.5 Hg	3.8	20	69	0.13
56x2	High DHA	0.5 Hg	6.5	34	77	0.22
57x3	High DHA	0.5 Hg	23.8	65	60	0.79
59x2	High DHA	0.5 Hg	3.6	3	54	0.12
70x2	High DHA	5.0 Hg	5.9	15	71	0.20
72x2	High DHA	5.0 Hg	54.2	82	62	1.81

All data are mean values from last 10 sessions for the 28 surviving subjects plus 3 subjects that died. MeHg represents the concentration of methylmercury administered to the Dam of the subject. A correct rejection was defined as holding to the end of the steady LED period. A correct hit was defined as releasing within 1 second of the start of the flicker during a flicker trial.
* Death from Urolithiasis

Table 15 Mean percentage of hits at tested frequencies.

Mean Percentage of Hits					
	Subjects				
Flicker Frequency	2-B	7x1	326x2	336x2	37x1
3	90	80	90	90	90
5			93		83
7	100	83	84	60	84
9		47	85		83
11	95	42	74	80	68
13		43	63	60	60
15	57	22	70	63	48
17	35	25	44	22	40
19	37	45	20	25	27
21	55	20	40	25	34
23	53	33	45	40	40
25	38	30	40		20
27	32	38	40		20
29	25	25	28		
31	23	30	38		
33	45	40	40		
35	50		20		
37	25	20	10		
39	30	20	15		10
41		20	15		30
43		30	15		40
45			23	30	20
47		20	20	20	10
49		30	27	0	16
Value of "a"	76.7	63.3	83.3	90	80

Mean percentage of hits at each tested frequency for five subjects whose data was analyzed. Row “a” equals the difference between the maximum and minimum mean values for each subject.

Table 16 Time Units per Frequency

Units of 1msecs	Units/sec	Flicker Frequency (Hz)
4	250.00	125.00 *
5	200	100.00
7	142.86	71.43
10	100	50
13	76.92	38.46
16	62.50	31.25
17	58.82	29.41
18	55.56	27.78
19	52.63	26.32
20	50.00	25.00
21	47.62	23.81
22	45.45	22.73
23	43.48	21.74
25	40.00	20.00
27	37.04	18.52
29	34.48	17.24
31	32.26	16.13
33	30.30	15.15
36	27.78	13.89
38	26.32	13.16
42	23.81	11.90
45	22.22	11.11
50	20.00	10.00
56	17.86	8.93
62	16.13	8.06
71	14.08	7.04
83	12.05	6.02
100	10.00	5.00
125	8.00	4.00
166	6.02	3.01

* 125 Hz was the frequency used for the steady trials.