CHARACTERIZATION OF DRUG REWARD IN AN INVERTEBRATE MODEL SYSTEM USING OPERANT CONDITIONING PARADIGMS

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ABSTRACT

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Natural reward circuits are fundamental components of learning as they allow the experience of an event to be associated with a perception of its value. By promoting affective states of eagerness and directed purpose, natural reward also serves as an essential generator of all forms of motivated behavior. Drugs of abuse are able to artificially trigger both the circuitry for reward and the incentive labeling of surrounding cues, as they lead to abnormal learning processes in taxa ranging from planarians to humans. Crayfish, with their modularly organized nervous systems and confirmed vulnerabilities to human drugs of abuse, have recently emerged as a valid model for the study of addiction. Confirmed drug effects in crayfish include psychostimulant properties, sensitization, withdrawal, reinstatement, and drug reward in conditioned place preference paradigms. Here we extend this work with an operant, self-administration paradigm to obtain direct measures of drug reward, along with a characterization of dose response and time course of reward conditioning. In a spatially contingent task individuals learned that entry into a specific substrate quadrant will deliver a bolus of drug. The use of yoked controls allowed quantification of unconditioned drug effects where the drug is presented in a non-contingent fashion. With application of amphetamine close to the brain, crayfish significantly increased operant responding as they readily learned to navigate the paradigm. Infusion into the general circulation followed a similar pattern but proved less effective. The establishment of an effective self-administration paradigm in crayfish provides a unique, comparative perspective on the neural mechanisms of drug-sensitive reward and the phylogenetically conserved vulnerabilities to addictive plant alkaloids.

Keywords: Addiction, Amphetamine, Invertebrate Reward, Crayfish, Operant Learning, Instrumental Learning

The field of addiction research has recently shown increased interest in the use of invertebrate systems. The crayfish model is at the forefront of this movement, with its accessible, modular nervous system, and demonstrated sensitivity to drugs of abuse. The present study of invertebrate reward employs an instrumental conditioning paradigm to investigate the stimulation of exploratory drive by amphetamine injection directly into the head ganglion. To obtain amphetamine reward, treated animals were required to execute side-specific antennal movements. Yoked controls, receiving the drug on the same temporal pattern as the treated animal, but independent of their own antennal movements, provide a measure of the unconditioned psychostimulant effects. The effect of reward contingency on changes in behavior was assayed at three different drug doses. Comparison of the levels of operant responding in treatment versus yoked controls revealed an increase in operant responding at the highest dose tested (1.0 μg/infusion). Moreover, dose dependent stimulation of antennal movements, behaviors typically associated with active exploration, suggests that amphetamine enhances exploratory drive, most likely through the activation of the seeking system, and supports the use of antennal measurements as a sensitive assay for drug-associated psychostimulation.

Keywords: Addiction, Amphetamine, Crayfish, Exploration, Invertebrate Reward

Dedication

To my mother

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1. Introduction

Brain reward pathways mediate pleasure states in response to natural rewards e.g. food, sex, and contact comfort (Kelley & Berridge, 2002). This reward circuitry may also be co-opted by drugs of abuse and repeated pharmacological activation of this pathway precipitates addiction in vulnerable individuals (Robinson & Berridge, 2000; Hyman & Malenka, 2001). As the individual progresses through the addictive cycle, initial drug use fueled by euphoric effects is replaced by compulsive drug- seeking and -taking, even in the face of harmful consequences (Koob & Le Moal, 2005; Kalivas & Volkow, 2005). Addictive drugs also alter the functioning of pathways underlying learning and memory (Gould, 2010). As a result, stimuli associated with drugs assume salience and have the capacity to trigger a craving for the drug; a feature critical in relapse.

Although the various stages of addiction have been successfully modeled in a range of mammalian systems (Deneau et al., 1969; Bergman et al., 1989; Spealman et al., 1989; Collins et al., 1983; Sanchis-Segura & Spanagel, 2006), there has recently been an increased interest in the use of invertebrate systems. Advantages of this 'simpler systems' approach (Wolf & Heberlein, 2003; Burne et al., 2011; Søvik & Barron, 2013) include a nervous system consisting of relatively few, often larger, neurons. These allow for the use of neurophysiological, anatomical, and biochemical approaches, providing an ease of experimental manipulation that is difficult to match with mammalian models. Invertebrate and vertebrate models (humans included) are united by the conserved nature of the cellular and molecular pathways that subserve functions critical to survival (Hen, 1992, 1993; Tierney, 2001; Tierney et al., 2003;

Porzgen et al., 2001; Vernier et al., 1995, 1997).

Decapod crustaceans, such as crayfish and lobsters, have proven to be excellent models in neuroethological studies owing to their anatomical and physiological characteristics (Clarac & Pearlstein, 2007). With a nervous system that responds to human drugs of abuse and contains fewer than a thousand, individually identifiable, monoaminergic neurons, crayfish are a suitable model for the identification of neural substrates underlying invertebrate drug reward. Amphetamine (Alcaro et al., 2011), cocaine (Nathaniel et al., 2012) and morphine (Nathaniel et al., 2010) have been shown to act as psychostimulants, which sensitize with repeated exposure (Dziopa et al., 2011; Nathaniel et al., 2010; Nathaniel et al., 2012). Moreover, amphetamine and cocaine stimulate learning of drug paired substrate (Panksepp & Huber, 2004; Nathaniel et al., 2009) in a conditioned place preference paradigm (CPP). Discontinuation of the drug produces withdrawal (Huber et al., 2011; Nathaniel et al., 2009), and a single priming dose is sufficient to reinstate the drug induced CPP (Nathaniel et al., 2009).

Although CPP effectively captures the reinforcing nature of a drug, it is an indirect assessment of the drug's affective properties, relying on behavioral responses to the conditioned stimuli. A more direct measure of the motivation to obtain drugs and of drug reward derives from an operantly-controlled self-administration paradigm. Under this paradigm, the subject controls drug delivery by performing a predetermined, instrumental task (Gardner, 2000; Belin et al., 2009; Deroche-Gamonet et al., 2004). Systemic drug administration contingent on an operantly-conditioned behavior requires rapid delivery and precise timing of drug application. This necessitates surgical implantation of an indwelling cannula, a significant challenge for most small invertebrate models (Søvik & Barron, 2013),

which limits the dissection of invertebrate drug-sensitive reward.

The present paper aimed to establish an operant self-administration paradigm in crayfish, using a fully automated, spatially explicit approach in which crayfish controlled infusions of amphetamine through an indwelling cannula, by seeking out, and entering a particular substrate in an experimental arena. Towards this goal, the study characterized behavioral responses to (1) novelty - using locomotor responses of untreated individuals in a newly encountered environment; (2) non- contingent drug administration - with dose-response curves to quantify basic psychostimulant effects of the drug; (3) contingent drug administration - by measuring changes in the extent of conditional responses as the individual gains knowledge about the pairing, and (4) different sites of delivery – by injecting the drug systemically versus near the brain.

2. **Materials and Methods**

Animal maintenance and surgery

Crayfish (*Orconectes rusticus*) were wild caught from the Portage River near Bowling Green, Ohio, USA (41.377965,-83.475812). Animals were maintained under controlled environmental conditions in an aerated community tank with stable water temperature (20°C), pH7, 12:12h light:dark cycle, and fed twice a week with rabbit chow. Three days prior to the experiment, intermolt males with all appendages intact were selected and isolated in perforated plastic containers (Ø: 140mm, ht: 70mm). These were placed in holding trays supplied with continuously circulating, filtered, aerated water from the community tank. Surgery was conducted after animals were anesthetized (20 mins in ice). Cannulae were implanted so as to deliver the drug directly over the supra-esophageal ganglion (Experiment 2) or into the pericard (Experiment 3), at sites identified previously through dissections. A 26.5 gauge needle

was used to drill through the exoskeleton at the selected point, a 30 mm deactivated fine bore fused silica cannula (Agilent, od 250 µm, id = 50 µm) was introduced through the opening to a depth of 2 mm, and secured with cyanoacrylate adhesive and bonding material. Following surgery animals were returned to their housing containers and allowed to recover overnight.

Experimental design and injection protocol

Experiment 1 aimed to characterize the behavior patterns that crayfish exhibit in novel environments. To this end, the levels of locomotory behavior and preference for textural cues were quantified in each of five trials by placing an individual in the center of a circular polyethylene arena, (Ø: 50 cm, ht: 25 cm) divided into four quadrants (two hard and two soft) with alternate quadrants presenting the same substrate. Measures were obtained for each 20 minute time segment within a 3- hour sample period. Information about the spatiotemporal preference of animals was obtained using a second group of thirteen animals. Preference for a particular substrate was quantified by measuring the time spent on the two substrates (hard and soft). The stability of substrate preference over time was assessed using a within subject design and three separate trials. Between trials, the arena was rinsed with dechlorinated, conditioned water to remove chemical cues.

Experiments 2 and 3 aimed to assess changes in operant responding that result from conditioning the operant behavior using drug reward. Different doses of d-amphetamine were tested for their ability to support self-administration across two anatomical locations, namely, the supra- esophageal ganglion (experiment 2 Doses:100 ng, 300 ng and 1µg/infusion) and the pericard (experiment 3 Doses:100 ng, 300 ng, 1µg, 3 µg and 10 µg/infusion).

Self-administration employed a master-yoke triadic design in which subjects were randomly assigned to one of three groups: Master, Yoke, or Control. Master animals received

drug reinforcement contingent on their movement into a selected substrate using a balanced design with an equal number of animals rewarded on hard and soft substrates. Yoked subjects received drug reinforcement on the same time schedule as the master animal to which it was yoked, and independent of its own response. Yoked controls thus provide a measure for unconditioned responses to amphetamine, and by comparison, a measure of potential learned responses in the master animal. Control animals received the saline vehicle only, contingent on its movement (as in the master group).

On the test day, a 0.5 m length of deactivated, fine-bore, fused silica capillary (Agilent, deactivated needle materials, od 250µm, id = 160µm) was connected to a 1ml glass syringe (Exmire CMA) fitted to a microdialysis pump (NE-1010, New ERA Pump Systems) via a small section of Tygon microbore tubing (Fisher Scientific, id = 250µm). After the capillary was primed with saline/drug solution it was connected to the cannula stub implanted in the crayfish with Tygon microbore tubing. Each drug dose was evaluated using 6 master-yoke pairs (i.e., 3 rewarded on soft substrate, and 3 on the hard substrate).

Behavioral analyses

The operant behavior was considered to be met when the animal entered the selected substrate. The reinforcement schedule was continuous, with every operant behavior meeting criteria (active response) resulting in the infusion of 5 µl amphetamine solution or saline control, lasting approximately 1 s. Once a valid response had occurred, a time out period of 5 s was instituted during which any further instrumental response went unrewarded. The drug application was started when the focal animal was placed into the experimental chamber, and tracking continued for a total of 3 hours. The animal's movements in the experimental arena were viewed using a camera (Sony HDR-HC5 HDV 1080i) centered overhead. The analog

video signal was digitized via an A/D converter (Canopus ADVC-110) connected to an Apple Macintosh computer (iMac; with 2.66GHZ Intel Core 2 Duo, OSX 10.7.4).

A collection of freeware programming functions for the analysis of behavior (available at http://iEthology.com/) were employed to extract locomotory movement data from the live video stream. The location of the animal was expressed as a pair of numerical coordinates in a 2D cartesian plane, obtained at a sample rate of 2 Hz from frames digitized at DV-video resolution (720x480 pixels). Instances of operant behavior activated a microinfusion pump for automated drug delivery via a custom robotic interface (usb/serial adapter DB-9RS-232). The automated mechanisms for the recognition of operant acts and for the activation of drug reward ensured consistent application over the course of the trial. Following the conclusion of the experiment, movement descriptors were extracted including distance travelled, mean speed, and frequency of operant behavior. Conditioned changes in operant responses (experiments 2 and 3) were quantified by comparing operant responses between treatment and yoke animals in each dose category for acquisition of amphetamine self-administration. An index of operant learning (LI) was calculated as the ratio of operant responses made by an animal to the total distance travelled. This total distance calculated, included large scale movements, above a threshold value of 3.5 pixels to prevent the inclusion of non-specific behaviors like grooming. LI thus provides an indicator of motivation to obtain the drug rather than a measure of overall increase in activity. The test phase was binned into 20 min segments and a learning index calculated for each.

Statistical analyses

Statistical analyses were conducted using JMP (Version 10.0, SAS Institute Inc., Cary, NC). Levels of significance were set at $p \leq 0.05$ for all tests. Descriptive statistics for time spent, distance traveled, mean speed, and operant behavior were binned into 20 minute time

segments and are reported in Table 2 and 3. Substrate preference was assessed using two-tailed, paired t tests. The effect of contingent drug administration on LI, was tested with a repeated measures design. Since LI values were neither normal (Shapiro-Wilk W test, $p << .001$) nor homoscedastic (Brown-Forsythe Test, $p << .001$), a more conservative approach was adopted and ranked values of this variable were used.

3. **Results**

Locomotor responses in a novel environment

Locomotor responses to novelty were quantified using distance travelled by animals in a novel test arena. Initial time segments following the animal's placement in the arena, were characterized by high levels of activity. Crayfish exhibited a strong tendency to explore by following the walls of the tank with frequent movements of antenna and instances of rearing up against the perimeter walls. Transitions from one substrate to another within the arena often involved a momentary pause with entrance into the new quadrant preceded by exploratory contacts with the antennae. Crayfish (n =5) exhibited a significant decrease in locomotion over a time course of three hours (F [8, 32] = 3.84, $P < 0.01$)) (fig. 1).

Untrained crayfish allowed to choose between a soft, textured substrate and a hard, smooth surface, demonstrated a natural preference for the former (fig. 2). Animals chose to spend a majority of their time (59.96% ± 3.90%) on the soft substrate compared to the hard surface (trial 1: paired t [12] = 2.55, $P < 0.05$). This preference for soft substrates was stable, being maintained in subsequent replicate trials; 2 (59% ± 4.43% of total time on soft, paired t [11] = 2.19, $P < 0.05$), and 3 (66.78% ± 3.78% of total time spent on soft substrate; paired t [10] = 4.43, $P < 0.01$)).

Unconditioned effects of amphetamine

Distance travelled by individuals in the yoked group was used to assess the unconditioned effects of amphetamine. Animals in the yoked set received the drug independent of their own behavioral responses and thus provided a measure for the unconditioned psychostimulant effects of the drug. The three different dose categories of 0.1 μg/infusion (16.74 ± 1.12), 0.3 μg/infusion (13.45 ±1.12) and 1 μg/infusion (12.83 ± 1.09) were comparable in terms of the total number of injections received. The effect of dose on distance travelled over the duration of the trial, was examined using a repeated measures design. Levels of locomotion did not differ significantly across different dose categories (F [2, 15] = 0.03, P = 0.76). Motor activity decreased over time (F [8, 8] = 12.63, $P << 0.001$). The interaction effect of time by dose was non-significant (F [16, 16] = 0.57, P = 0.86)

Effect of reward conditioning on operant responding

Treatment and yoke sets for each dose category (n=6/group) were compared on the basis of Learning Index (LI) score. LI scores were computed as a measure of operant behavior (fig.4). An increase in the LI scores of treatment relative to the yoke group was observed, most prominently at the highest drug dose of 1.0 μg/infusion. Effect of reward contingency over the duration of conditioning session was significant when examined using a repeated measures design (Treatment x Time interaction: F [8, 3] = 68.29, $P < 0.05$). LI scores of the treatment animals in this dose group increased rapidly after just 1.5 h, whereas LI scores of the yoke remained unchanged across the trial. Increase in LI scores of treatment groups relative to the yoke were also observed for both the intermediate- (0.3 μg/infusion) and the lowest doses (0.1 μg/infusion), but were not statistically significant. LI scores of treatment and yoke groups appeared to be more similar when operant tasks were rewarded with lower doses of

amphetamine, indicative of a dose dependent increase in strength of reward conditioning. Maximal increase in LI scores relative to the yoke were observed for the treatment individuals being rewarded contingently with the highest dose.

Effect of injection site on reward conditioning

The effect of drug delivery site on reward strength was examined by also injecting the drug systemically using a protocol similar to that previously described for the brain injections. LI scores of treatment and yoked sets were compared for each dose category (fig.5) using a repeated measure design. At amphetamine doses of 0.1 μg, 0.3 μg and 1.0 μg/infusion, LI scores of animals receiving the drug contingently tended to be higher than that of their yoked counterparts. For comparable dose categories, systemic amphetamine injection of produced less distinct differences in LI scores between the treatment and yoke groups. The 3.0 μg and 10 μg/infusion doses were restricted to pericardial administration. When injected into the brain, these doses were observed to precipitate aberrant motor responses, including tail flips and excessive grooming that prevented animals from engaging in normal locomotion.

Anatomical Site	Dose (μg/infusion)	Treatment x Time	Time
Brain	0.1	F [8, 3] = 5.83, P = 0.28	F [8, 3]= 14.23, P = 0.10
	0.3	F [8, 3] = 7.15,P = 0.23	F [8, 3] = 5.25, P =0.31
	1.0	F [8, 3] = 54.10, P = 0.02	F [8, 3] = 68.29, P = 0.01*
Hemolymph	0.1	F [8, 1] = 5.83, P =0.56	F[8, 1] =14.23, P =0.40
	0.3	F[8, 3] =7.28, P =0.22	F[8, 3] =2.02, P =0.66
	1.0	F[8, 1] =4.56, P =0.56	F[8, 1] =39.53, P =0.34

	3.0	F[8, 1] =2.48, P =0.89	F[8, 1] =2.49, P =0.89
	10.0	F[8, 3] =1.71, P =0.73	F[8, 3] =1.50, P =0.77

Table1. Results of repeated measures ANOVA comparing learning index scores of treatment versus yoked animals. The two sets differed in their experience of reward contingency. Individuals in the treatment group received the drug reward contingent on their behavior. The yoked set received the drug injections on a schedule identical to that of the treatment animals and thus independent of their own behavioral response. Revised *p* values determined using Bonferroni's correction for multiple comparison tests to maintain overall α levels of 0.05 significance.

4. **Discussion**

The present paper demonstrates the ability of amphetamine to support self-administration under an operant conditioning paradigm in an invertebrate system. We showed that free moving, behaving crayfish learn to self-inject amphetamine under continuous reinforcement schedules. The ability of amphetamine to act as a reinforcer in the crayfish nervous system was previously demonstrated using a conditioned place preference paradigm (CPP) (Panksepp & Huber, 2004). Amphetamine-evoked CPP appeared after just a single exposure, was persistent, and displayed prompt reinstatement. With the establishment of this self-administration paradigm we provide more direct evidence for the rewarding action of drugs in a system with the potential to further disentangle the mechanisms underlying drug reward in the crayfish model.

Under a spatially contingent, operant conditioning paradigm, crayfish selectively engage in tasks paired with drug infusions. Levels of operant responding in treatment animals

exceeded that of their yoked counterparts that received amphetamine infusions on the same temporal pattern but in a manner unrelated to their own behavior, as well as those of the vehicle control group that received contingent injections of saline. Amphetamine generated response was clearly dose dependent. At the lowest dose of 0.1 μg, the response of treatment animals was similar to that of their yoked partners. A distinction between the treatment and yoke animals was apparent at the 0.3 μg dose level, but failed to achieve statistical significance. For 1.0 μg, the highest dose included in our study, the greatest separation in response levels was observed between the two groups. Treatment animals displayed an increased propensity to engage in task paired with the drug reward following just 120 min of exposure to reward contingency. Studies that have assayed multiple unit doses for acquisition of self- administration have demonstrated that rate of acquisition (van Ree et al., 1978; Carroll & Lac, 1997) motivation to obtain the drug (Gerrits & van Ree, 1995), and probability of acquisition (van Ree et al., 1978; Carroll & Lac, 1997) are positively correlated with the unit dose.

Towards the end of the session, operant responding showed a tendency to decrease. This suggests that there is a ceiling for amphetamine intake. This upper limit is likely to be affected by both the total amount of drug injected and the unit dose per injection. Plateauing amphetamine intake after a period of self-administration, is indicative of a decrease in reinforcement efficacy either because the amount of amphetamine injected established internal levels of the drug that reached satiation, or that generate aversive states when exceeded. Previous studies conducted in our lab indicated that amphetamine at higher doses (5 mg/kg) increases the occurrence of tail flips and convulsions (Alcaro et al., 2011). Since tail flips are primarily an escape response of crayfish mediated by a monoaminergic circuit (Glanzman &

Krasne, 1983), amphetamine most likely generates aversive states at higher doses that impose a constraint on drug intake.

Dose response curves obtained in this study show large error bars for the treatment group compared to the yoke and vehicle control groups. Response to drugs are characterized by large inter- individual differences in both humans and animals (de Wit et al., 1986; Piazza et al., 1998; Marinelli, 2005). Although self-administration may be acquired with relative ease by some individuals, others tend to be more resistant. Individuals who are highly sensitive to the effects of the drug are also more susceptible to the drug's reinforcing properties and are at greater risk for entering the addictive cycle (Piazza, et al., 1989; Piazza et al., 2000). Since our *O. rusticus* sample is derived from a wild population, the error bars reflecting between-subject variability in the acquisition of operant responding are likely to be large.

We examined the unconditioned effect of amphetamine injections on locomotor movements using the conditioning arena as an open field. Crayfish placed in a novel arena show enhanced levels of locomotion and antennal movements. Novel stimuli appear to facilitate exploratory behaviors that under normal scenarios, are essential for gaining access to natural rewards. As crayfish become habituated to their environment, a reduction in locomotion is observed and animals tend to settle along the perimeter walls of the test arena. In contrast, amphetamine has been demonstrated to increase motor activity and stereotypy in mammals (Fog, 1969; Schiorring, 1971; Segal & Mandell, 1974; Hoebel et al., 1983). We characterized the unconditioned effects of the drug using distance travelled by crayfish in the yoke set under each dose category. No significant differences in levels of locomotion were observed between the different dose categories or relative to the saline controls. Lack of amphetamine induced increase in measures of locomotion for crayfish has previously been observed (Panksepp &

Huber, 2004). Unchanged locomotory response levels could arise if animals spent more time in tactile exploration of the arena. In crayfish, exploration of surroundings is strongly dependent on mechanoreception using active movements of the antenna (Basil & Sandeman, 2000; Koch, Patullo, & Macmillan, 2006). Therefore, it is possible that stimulation of the appetitive motivational states by amphetamine results in increased tactile investigation using sensory appendages rather than locomotion.

To investigate the role of microinjection sites in reward conditioning we tested whether self-administration of amphetamine into the pericard would be supported using a similar framework of operant conditioning. Although a wide range of doses were tested, less obvious changes in performance of the treatment groups relative to the yoke were observed. Unlike brain injections, amphetamine delivered into the hemolymph appears to be less effective for the acquisition of drug self-administration. It has previously been demonstrated that administration of d-amphetamine directly into the crayfish brain is more effective than pericardial injections at enhancing exploratory behaviors (Alcaro et al., 2011). This finding may be interpreted based on the rate hypothesis of psychoactive drug action. The hypothesis states that faster the drug reaches the brain and starts to act, the greater the reinforcing effects and abuse liability (Nelson et al., 2006). A number of study have reported enhanced subjective responses with higher infusion speeds (Abreu et al., 2001; Nelson et al., 2006). When given the option of choosing between identical doses of i.v. cocaine injections delivered with different infusion speeds, most rats preferred the faster infusion rates (Schindler et al., 2009). Increase in motivation to acquire the drug is observed with rapid time frame of delivery (Minogianis et al., 2013). The route of administration influences the reinforcing effects of drug by altering the speed with which the drug reaches the brain (Volkow et al., 2000). Application

of the drug directly over the supraesophageal ganglion minimizes the time delay between operant response and the reinforcing drug injections thus increasing the effectiveness of the conditioning paradigm.

The presence of drug sensitive circuits in the crayfish brain supports an evolutionarily conserved adaptation that facilitates exploration, appetitive motivation, and learning (Ikemoto & Panksepp, 1999; Alcaro et al., 2011) in both natural and abnormal contexts (e.g. behaviors such as drug seeking, self-administration, and relapse under the influence of addictive drugs). Exploration of their surrounding by crayfish, relies on tactile information received from the antennae and antennules (McMahon et al., 2005, Patullo & Macmillan, 2006) and is conveyed to the olfactory lobe (Mellon, 2000). Modulated by serotonin (Sandeman & Sandeman, 1987; Sandeman et. al., 1988) and dopamine (Tierney et al., 2003) transmission, the olfactory lobe plays an active role in exploration and is likely to play an important role in amphetamine and other drug mediated reward (Nathaniel et al., 2012).

The establishment of an automated, operantly conditioned self-administration paradigm in crayfish sets the stage for more complex studies of invertebrate reward, including (1) mechanisms of reward, (2) how the appetitive/seeking disposition is implemented in a relatively simple neural system, and (3) how such a disposition is targeted by the rewarding action of drugs of abuse. This neuroethological work will contribute an expanded evolutionary, and comparative understanding of a key component in learning, and of natural reward as an important life-sustaining process.

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Time Segment	Treatment	Yoke
Saline		
0	15568.24 ± 1679.72	
20	12289.95 ± 2170.59	
40	10909.87 ± 3053.52	
60	7123.54 ± 1605.	
80	5130.47 ± 1202.72	
100	3164.02 ± 875.83	
120	5419.11 ± 1437.54	
140	3842.51 ± 1283.43	
160	5769.10 ± 1440.81	
Dose 0.1 µg		
0	16158.96 ± 1857.57	15022.52 ± 2454.68
20	12628.29 ± 1421.67	10762.43 ± 1929.27
40	12771.77 ± 1417.07	8797.90 ± 1328.91
60	10440.95 ± 2360.67	6137.05 ± 1383.60
80	10031.76 ± 2276.54	5996.24 ± 1382.33
100	8330.80 ± 2322.31	5588.57 ± 1277.31
120	9180.68 ± 2228.53	4803.28 ± 1002.05
140	6231.08 ± 2166.41	4789.84 ± 975.12
160	4752.52 ± 1096.37	3263.26 ± 711.66
Dose 0.3µg		

0	12250.06±1950.51	16404.49±2640.10
20	9259.06±2198.61	10440.63±1830.23
40	6478.60±2654.29	10216.23±1984.97
60	6020.42±852.65	8370.55±1906.39
80	3986.69±878.95	7710.52±1562.04
100	5062.39±1667.30	6831.35±2332.43
120	5387.13±3552.26	6206.90±1357.58
160	3356.41±1174.52	5822.76±1655.83
Dose 1.0 µg		
0	10298.48 ± 2189.31	15473.27 ± 2583.71
20	8192.89 ± 1866.67	10969.27 ± 3612.78
40	7243.04 ± 1322.27	10993.98 ± 3156.96
60	6256.14 ± 1119.97	9248.83 ± 3119.25
80	5970.63 ± 1159.02	6594.40 ± 1671.67
100	5309.98 ± 1529.85	4009.99 ± 1404.70
120	4172.72 ± 1085.70	2804.02 ± 1504.74
140	4430.44 ± 1214.40	3172.39 ± 934.90
160	5366.07 ± 1522.87	2267.83 ± 869.44

Table 2. Descriptive statistics (mean ± S.E.M) for measures of movement (in pixels) for crayfish of master, yoke and vehicle control groups, during each 20min time segment of the experiment, when drug infusions were delivered near the supraesophageal ganglion.

Time Segment	Treatment	Yoke
Saline		
0	17785.85 ± 1437.70	
20	10030.45 ± 2493.87	
40	7459.06 ± 2143.75	
60	6285.88 ± 2191.19	
80	5023.25 ± 2269.58	
100	5051.99 ± 2239.99	
120	4212.95 ± 2139.13	
140	4451.81 ± 1897.48	
160	4579.23 ± 1966.56	
Dose 0.1 µg		
0	13791.05 ± 2393.45	14076.03 ± 3065.93
20	7503.23 ± 1765.12	8753.02 ± 3052.37
40	4421.73 ± 1253.18	7574.61 ± 2804.84
60	3603.81 ± 1134.56	7101.70 ± 2435.01
80	3642.43 ± 898.55	5197.72 ± 1826.11
100	2348.05 ± 941.95	5567.84 ± 1992.21
120	2556.01 ± 1530.04	5782.48 ± 2167.62
140	3599.36 ± 1944.93	5371.43 ± 1575.63
160	2184.92 ± 868.24	5365.31 ± 2132.08

Time Segment	Treatment	Yoke
Dose 0.3 µg		
0	17071.78 ± 2931.43	16464.97 ± 2427.65
20	12415.77 ± 2735.66	11835.37 ± 2711.46
40	10766.78 ± 3225.81	7951.59 ± 2337.11
60	6063.40± 1839.88	6901.10 ± 1609.74
80	6499.05 ± 2580.91	5913.74 ± 1334.29
100	9562.42 ± 5771.91	4764.68 ± 1362.35
120	9904.55 ± 6543.22	3740.80 ± 579.39
140	2933.07 ± 1607.64	4768.29 ± 1302.94
160	2394.13 ± 1085.37	3652.91 ± 1341.83
Dose 1.0 µg		
0	13202.63 ± 1758.82	12075.71 ± 1192.57
20	9723.46 ± 1436.08	10797.30 ± 2356.38
40	5782.46 ± 1286.49	8447.03 ± 1886.21
60	5532.58 ± 1889.08	7881.16 ± 1979.72
80	3923.84 ± 1746.47	7854.63 ± 1999.18
100	4265.61 ± 2030.84	5528.21 ± 1953.25
120	3736.81 ± 2655.71	6379.87 ± 1042.80
160	4601.14 ± 2908.21	5500.96 ± 1883.72

Dose 3.0 µg		
0	13036.13 ± 2476.46	11362.73 ± 1847.86
20	9504.28 ± 2069.61	7767.08 ± 2585.93
40	5289.55 ± 2043.26	6320.47 ± 2069.93
60	5335.15 ± 1353.34	5861.42 ± 1670.64
80	4296.66±1587.62	4798.55 ± 662.41
120	3689.74±1455.39	4228.77 ± 799.11
140	2840.75 ± 1852.92	4208.09 ± 1010.18
160	3154.47 ± 2332.28	3621.43 ± 836.19
Dose 10.0 µg		
0	10495.45 ± 3862.45	7578.97 ± 890.89
20	6572.89 ± 2370.84	4668.00 ± 916.27
40	5384.55 ± 1688.75	5351.58 ± 1908.79
60	4641.68 ± 1729.57	6545.31 ± 2217.32
120	3625.88 ± 638.47	5213.92 ± 1687.11
140	5884.30 ± 1527.13	5674.85 ± 2079.90
160	4522.66 ± 602.46	5955.78 ± 1447.44

Table 3. Descriptive statistics (mean ± S.E.M) for measures of movement (in pixels) for crayfish of master, yoke and vehicle control groups, during each 20min time segment of the experiment, when drug infusions were delivered into the pericard.

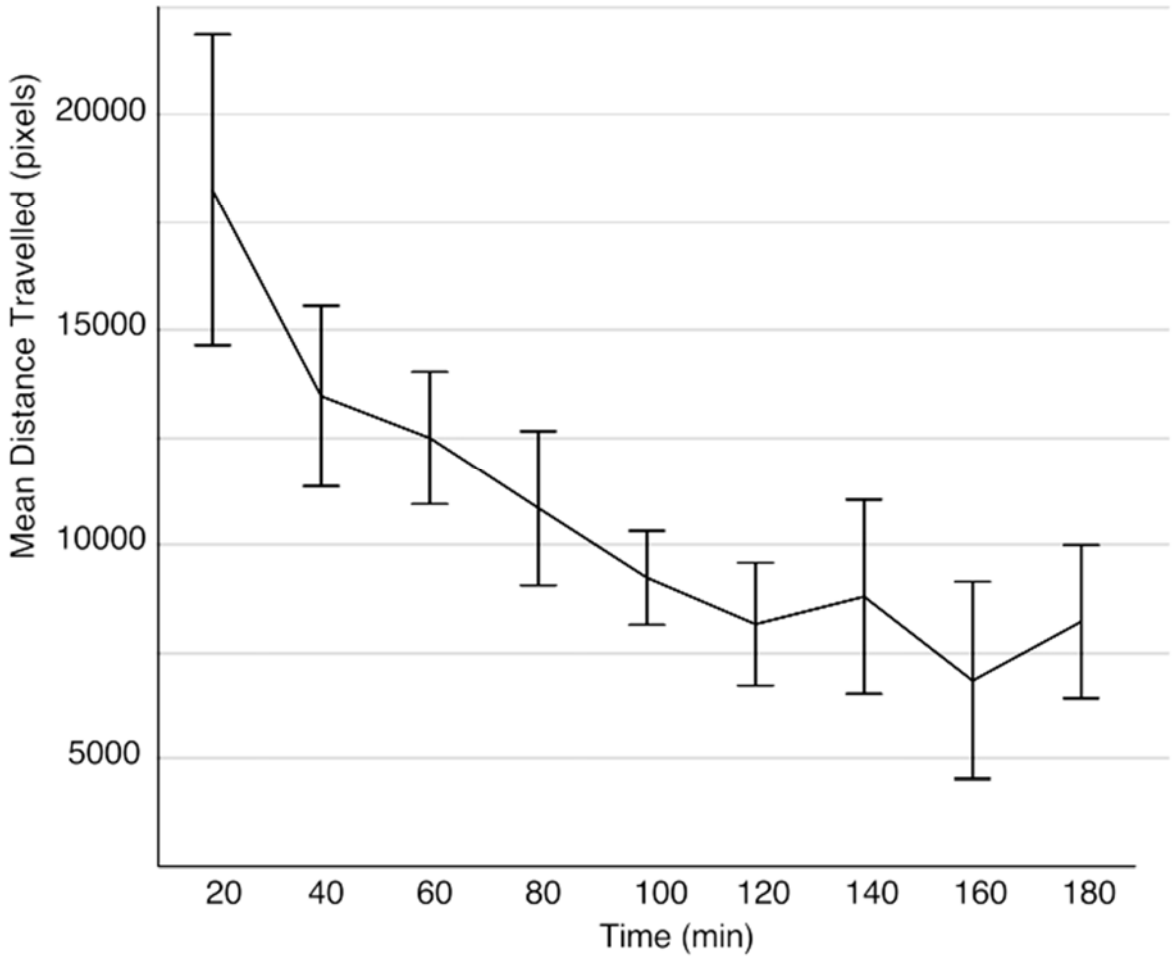

Fig 1. Summary statistics for mean distance travelled over time. Crayfish (n =5) exhibited a significant decrease in locomotion over a time course of three hours (F [8, 32] = 3.84, P < 0.01)) Initial time segments show higher levels of locomotion that gradually decrease, indicating an increased familiarization with the test environment.

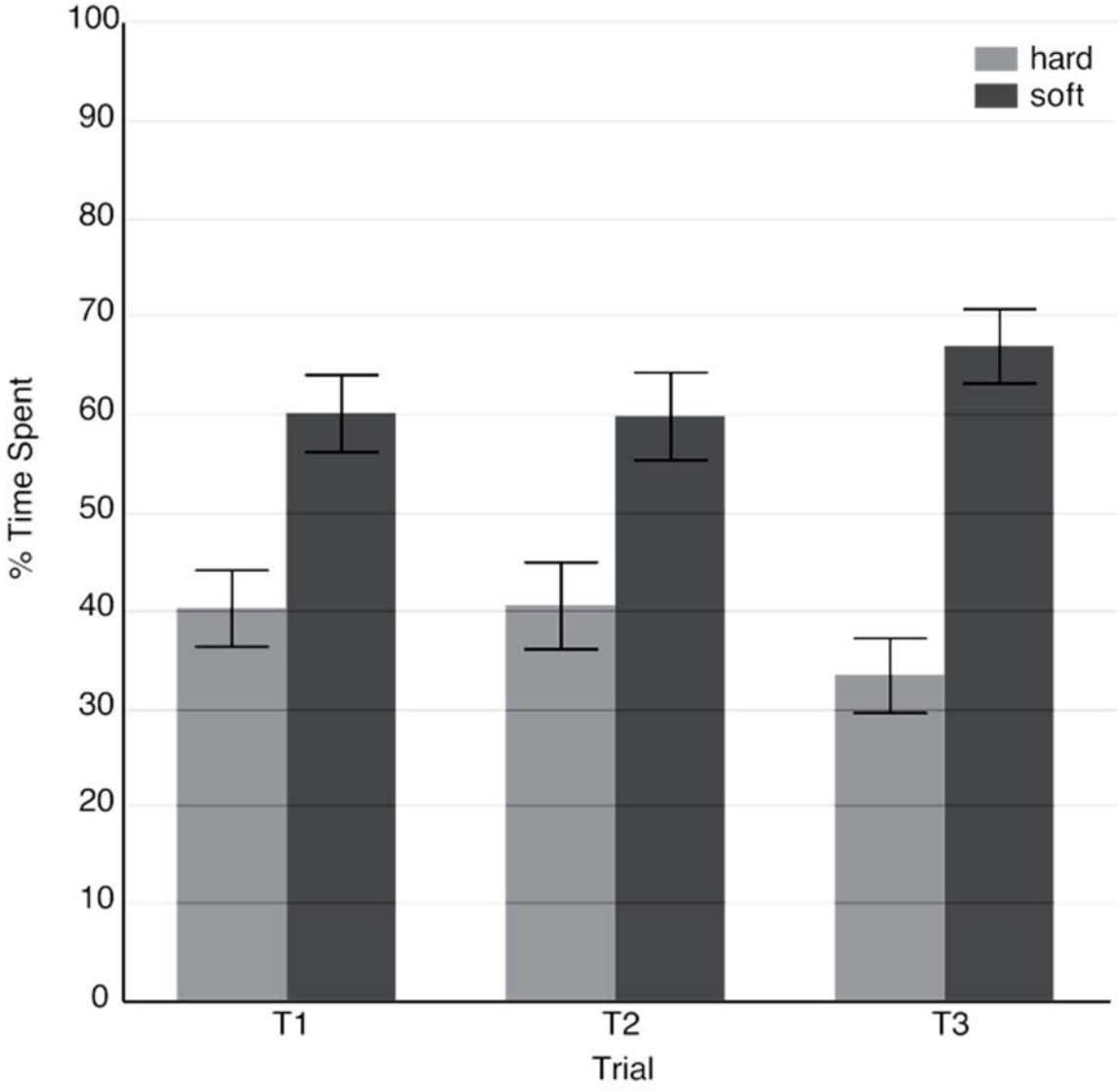

Fig 2. Crayfish (n=13) exhibited a population-level preference for the soft textured quadrants of the conditioning arena, across 3 consecutive trials. Over a period of 5h, crayfish spent significantly more time on the soft textured substrate than predicted by chance alone. Reported are the time (mean±S.E.M) spent on the hard and the soft substrates for each trial. The preference for the soft substrate was stable and observed across all three days of testing (Trial 1: paired $t\,[12] = 2.55$, $p < 0.05$, Trial 2: paired $t\,[11] = 2.19$, $p < 0.05$, and Trial 3 paired $t\,[10] = 4.43$, $p < 0.01$).

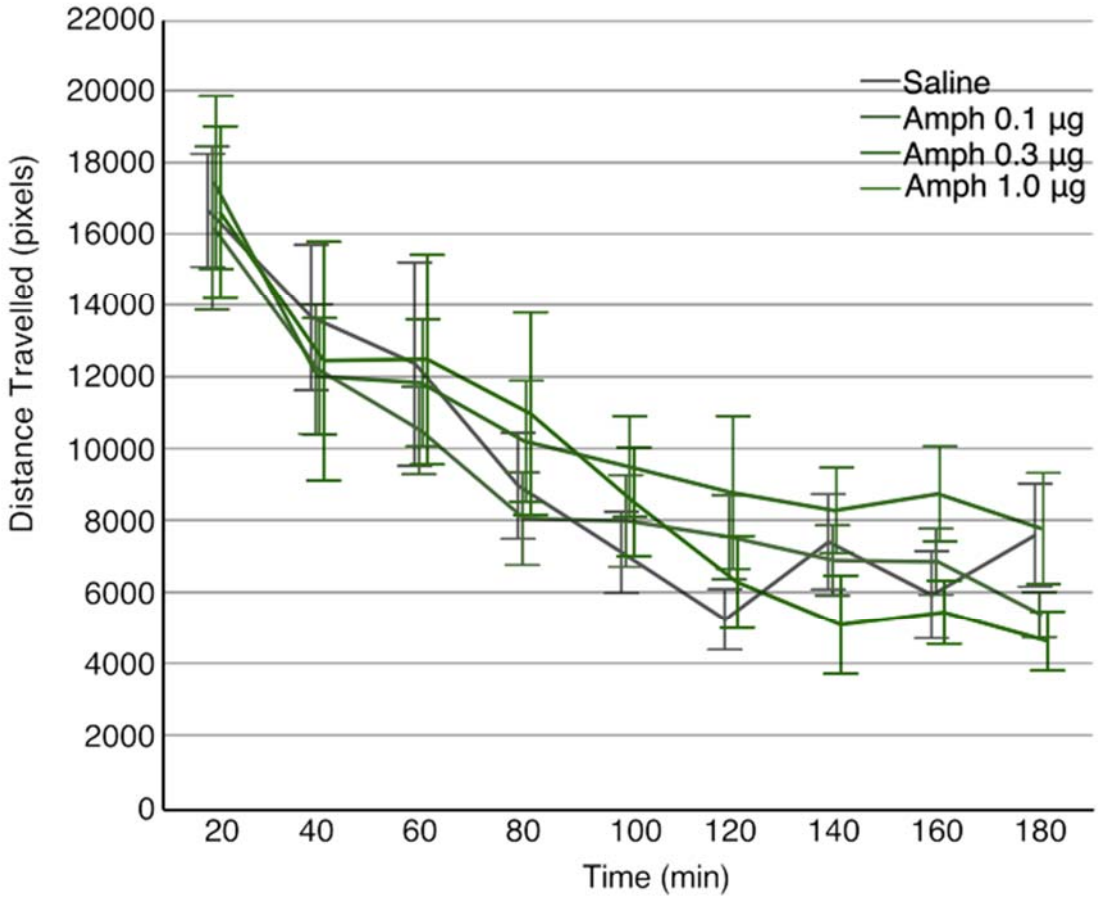

Fig 3. Non contingent injections of amphetamine does not affect levels of locomotion. Yoke animals for all three dose categories (0.1 µg, 0.3 µg and 1 µg, n = 6 for each dose category) show similar levels of locomotion. Movements of the animals in the vehicle control group were indistinguishable from those of the yoke animals. Individuals in all groups show a decrease in locomotion over time that bottomed out after 80 min ($F[8, 8]=12.63$, $P<<.001$).

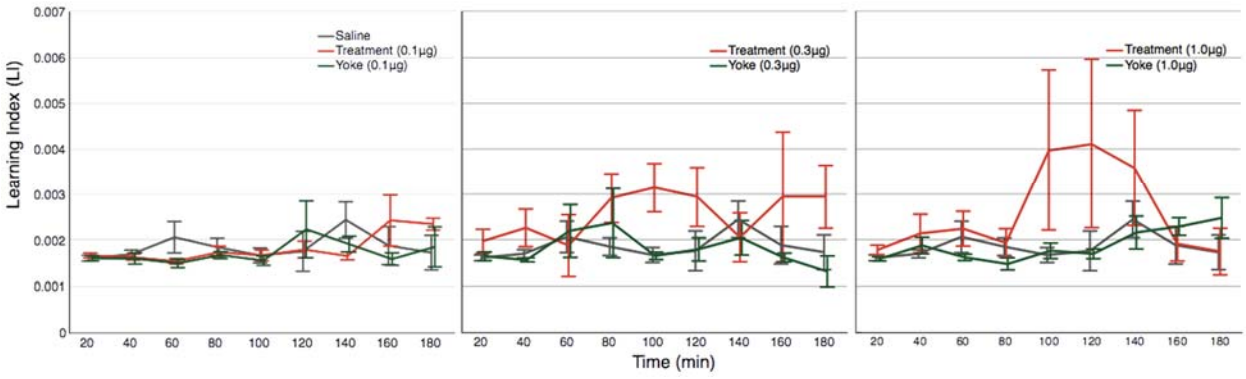

Fig 4. Crayfish learning a spatial operant task with contingent drug administration delivered at the supra-esophageal ganglion. Treatment animals receiving drug reward contingent to their behavior show levels of learning. Yoke animals given non-contingent amphetamine injections fail to learn. Vehicle controls receiving response contingent injections of only saline display levels of operant responding similar to yoke animals. Reward strength is dose dependent and increases with higher doses of amphetamine for 0.1 µg (a), 0.3 µg (b), and 1.0 µg (c). Learning in crayfish self-administering 1.0 µg/infusion amphetamine were significantly higher than their yoked counterparts. Operant responding by treatment group show an increase after 80 mins of exposure to reward conditioning compared to the yoke group (Treatment x Time interaction: $F[8, 3]= 68.29$, $P<0.05$).

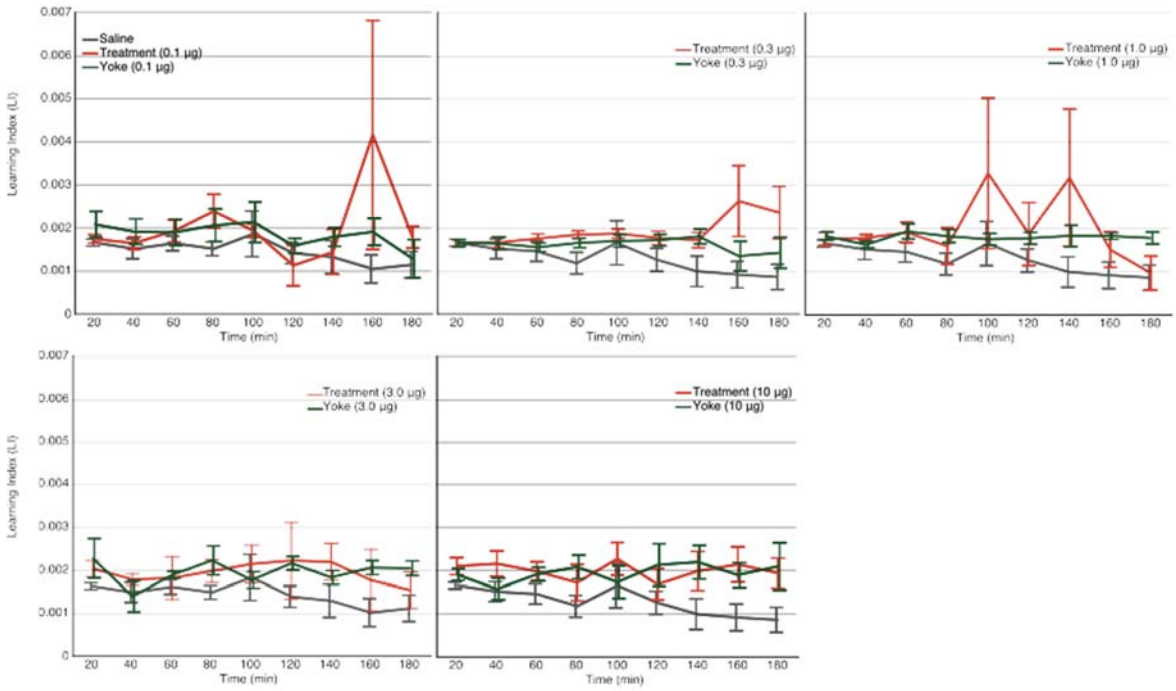

Fig 5. Crayfish learning a spatial operant task with contingent drug administration delivered into the pericardial sinus. Treatment animals in the first three dose categories (0.1µg (a), 0.3 µg (b), and 1.0 µg (c)) show levels of earning. Yoke animals given non-contingent amphetamine injections fail to learn, at all doses tested. Vehicle controls receiving response contingent injections of only saline display levels of operant responding similar to yoke animals. Operant responding for amphetamine at doses of 3.0 µg (d) and 10.0 µg (e), are similar for both treatment and yoke groups. Learning of the operant paradigm by crayfish receiving behavior contingent injections of amphetamine into the pericard is less robust than animals receiving the drug into the supra-esophageal ganglion.

CHAPTER II: PSYCHOSTIMULANT EFFECTS OF AMPHETAMINE ON CRAYFISH ANTENNAL MOVEMENTS DURING INSTRUMENTAL LEARNING

1. **Introduction**

Animals have an inherent tendency to explore their surroundings. This gain in knowledge of local conditions drives learning and enables them to predict events, and adapt to changing environmental circumstances (Gibson, 1988). Efficient behavioral search strategies purportedly emerge from an incentive motivational SEEKING system (Panksepp et al., 2002). Increased exploration is observed when cues are paired with salient stimuli in both natural (Bindra & Campbell, 1967) and experimental contexts, such as rewarding electrical brain stimulation (Clarke & Trowill, 1971). Similarly, the rewarding effects of psychostimulant drugs, such as cocaine, amphetamine and apomorphine, elicit a range of exploratory behaviors from stereotypies indicating exaggerated responsiveness to sensory input e.g. repetitive sniffing/whisking, to locomotor hyperactivity (Iwamoto, 1984), and have been widely assessed using locomotor activity under paradigms such as open field tests (Conn, 2013).

The psychomotor stimulant theory of addiction (Wise & Bozarth, 1987) suggests that the rewarding effects of a drug, along with increases in locomotor responses, share the same underlying neuronal mechanisms. Several lines of evidence support this theory. Addictive agents such as amphetamine, cocaine, and opioids elicit forward locomotion by activating the dopaminergic circuitry, which is also involved in brain stimulation reward (Calabrese, 2008). Additionally, individuals exhibiting enhanced motor activity in novel environments also display increased sensitivity to drugs of abuse, acquire self-administration more readily, and show greater susceptibility to entering the addictive cycle (Piazza et al., 1989; Marinelli & White, 2000). Thus, the psychostimulant effects of a drug appear to serve as a strong predictor

of its rewarding properties.

Invertebrate taxa are increasingly popular models for analyzing the neurobiological underpinnings of complex neuropsychiatric disorders, such as addiction, because of the multiple advantages of the 'simpler systems' approach (Wolf & Heberlein, 2003; Burne et al., 2011; Søvik & Barron, 2013). Enhanced locomotion, exploration and consummatory behaviors in response to drugs have been reported for nematodes (Morgan & Sedensky, 1995; Feng et al., 2006), planaria (Raffa & Martley, 2005), slugs (Wong et al., 1991), and flies (McClung & Hirsh, 1999; Bainton et al., 2000; Singh & Heberlein, 2000; Dimitrijevic et al. 2004). Although *Drosophila (*Kaun et al., 2012) and *Caenorhabditis elegans* (Schafer, 2004) are amongst the more widely used models for the identification of behavioral, molecular, and genetic pathways mediating drug effects, additional benefits may accrue from the use of larger invertebrate taxa such as crayfish.

The general advantages of crayfish as a model for drug reward are well-known, ranging from a relatively simple and modularly arranged nervous system, detailed knowledge of their neurochemical substrates, to the expression of a complex behavioral repertoire (Huber, 2005). Moreover, they are highly responsive to human drugs of abuse (Huber, 2005), exhibit psychostimulant effects to amphetamine (Alcaro et al., 2011), cocaine (Nathaniel et al., 2012), and morphine (Nathaniel et al., 2010), show learning of drug-associated environmental cues (Panksepp & Huber, 2004; Nathaniel et al., 2009), as well as withdrawal (Huber et al., 2011; Nathaniel et al., 2009), and relapse (Nathaniel et al., 2009).

In their natural environment, crayfish rely heavily on olfactory and tactile cues detected using the antennae and antennules for moving about and orienting (Basil & Sandeman, 2000). Receptors located on the antennae permit crayfish to detect objects in its vicinity with the

movements of the two antenna being largely independent of each other (Zeil, et al., 1985; Sandeman, 1989; Bruski & Dunham, 1990). Antennae thus serve an important role in navigation and exploration in the animal's habitat (Koch et al., 2006; Patullo & Macmillan, 2006). Since antenna movements are likely to have a strong attentional component, and are selectively engaged by the animal, the behavior should offer a suitable substrate for modification with response reinforcer contingencies.

The current study evaluated the effect of amphetamine on exploratory movements of crayfish antenna within an operant contingency framework. Using an automated, operant paradigm, individuals were allowed to control infusions of amphetamine through an indwelling cannula by movements of their antenna. Using antennal movements as an indicator to assess changes in exploratory drive, this paradigm explores the effect of amphetamine injections when delivered in a response contingent manner. Towards this goal, the study quantified antennal movements to characterize (1) baseline levels in untreated animals, (2) unconditioned effects of amphetamine injection in relation to the amount of drug delivered, and (3) learning of the operant contingency when movements of the antenna were paired with drug reward.

2. **Materials and Methods**

Animal maintenance and surgery

Orconectes rusticus crayfish (n=42) were wild caught from the Portage river near Bowling Green, Ohio, USA (41.377965,-83.475812), maintained under controlled environmental conditions in an aerated community tank at stable conditions (20°C, pH 7, 12:12h light:dark cycle), and fed twice a week with rabbit chow. Three days prior to the experiment, intermolt males with all appendages intact were selected and individually isolated

in perforated plastic containers (Ø: 140mm, ht: 70mm). These were placed in holding trays supplied with continuously circulating, filtered, aerated water from the holding tanks. Surgery was conducted after animals were anesthetized (20 mins in ice). A cannula was implanted directly over the supra-esophageal ganglion at a site identified previously through dissections. A 26.5 gauge needle was used to drill through the exoskeleton at this point, a 30 mm deactivated fine bore fused silica cannula (Agilent, od 250 µm, id = 50 µm) was introduced through the opening to a depth of 2 mm, and secured with cyanoacrylate adhesive and bonding material. Following surgery animals were returned to their housing containers and allowed to recover overnight.

Experimental design and injection protocol

The experimental arena was a glass aquarium (0.5 X 0.26 X 0.3 m) filled with aerated, conditioned water at room temperature. A nylon hex nut was glued to its dorsal carapace, which was then attached to a fixed acrylic holder such that the animal rested with its legs comfortably on a platform surface. To capture the antennal motions each antenna was color tagged with a glass bead attached approximately 10 mm from the proximal end using cyanoacrylate glue. Movement of both antennae were simultaneously recorded at 2 fps, using a digital camcorder (Sony HDR-HC5 HDV 1080i) positioned directly in front of the animal, and lit from overhead with a fluorescent lamp (15W/120V).

Self-administration employed a master-yoke triadic design in which subjects were randomly assigned to one of three groups: Master, Yoke, and Control. Master animals received drug reinforcement contingent on the movement of a selected antenna in a balanced design with an equal number of animals rewarded on left and on right antennae. Yoked subjects received drug reinforcement on the same time schedule as the master animal to which

it was yoked, and independent of its own response. Yoked controls thus provide a measure of the unconditioned effects of amphetamine administration, and by comparison, a measure of potential learned responses in the master animal. Control animals received the saline vehicle only, contingent on its antennal movement (as in the master group). Amphetamine solutions were prepared immediately before each experiment by dissolving D-amphetamine sulphate (FW: 368.5; Sigma, St. Louis: A 5880) in 125mM NaCl (isotonic crayfish saline), to a final concentration of 100 ng, 300 ng, or 1 μg/5 μl.

On the test day, a 0.5 m length of deactivated, fine-bore, fused silica capillary (Agilent, deactivated needle materials, od 250 μm, id = 160 μm) was connected to a 1ml glass syringe (Exmire CMA) fitted to a microdialysis pump (NE-1010, New ERA Pump Systems) via a small section of Tygon microbore tubing (Fisher Scientific, id = 250 μm). After the capillary was primed with saline/drug solution it was connected to the cannula stub implanted in the crayfish with Tygon microbore tubing. Each dose was evaluated using 6 master-yoke pairs (i.e., 3 paired with left antenna, 3 with right antenna). The test animal was attached to the holder and, following a 10 min acclimation period, antennal movements were tracked and rewarded continuously for a 3 hour period.

The criterion for operant behavior was met when the selected antenna's movement exceeded an angular speed of 50°/s. The reinforcement schedule was continuous, with every operant behavior meeting criteria (active response) resulting in the infusion of 5 μl amphetamine solution or saline control, lasting approximately 1s. A collection of freeware programming functions for the analysis of behavior (available at http://iEthology.com/) were employed to extract antennal movement data from the live video stream. Antennal position was expressed as a pair of numerical coordinates, calculated with reference to the antennal base in a

2D cartesian plane. Displacement of the bead was measured frame by frame.

Statistical analyses

Statistical analyses were conducted using JMP (Version 10.0, SAS Institute Inc., Cary, NC). Levels of significance were set at $p \leq 0.05$ for all tests. Descriptive statistics for time spent, distance traveled, mean speed, and operant behavior were binned into 30 minute time segments and are reported in Table 1. The effect of time in contingent drug administration on measures of (log- and z-transformed) antenna displacement, was tested with a repeated measures design. A SAS Proc Mixed model was used to test the effect of drug amount on distance traveled. For this analysis, data from each treatment yoke pair was combined and treated as a single unit and entered as random effect. Time and drug amount were entered as fixed effects in this model.

3. **Results**

Changes in antennal movement in response to reward contingency

Conditioned psychostimulant effects were anticipated in the movement of the master animal's rewarded antenna, which has exclusive control over the infusion pump. Following the introduction of response-contingent drug delivery, treated crayfish showed increased measures of antennal movement. Initial tests indicated homogeneity of responses across the entire experiment between the two antennae within treatment (master) animals, as no difference was evident between the controlling antenna and the one that was not. Data for both antennae were subsequently pooled within each animal. Movement enhancing effects of amphetamine were dose-dependent, with animals treated at the 1.0 μg dosage level performing the greatest number of antennal sweeps (fig. 1c). Although not significant (F [6, 5] = 0.68, p = 0.68), there was a strong tendency towards increased antennal movement in the master animals, compared to their

yoked controls. At low and intermediate doses of 0.1 (F [6, 5] = 0.22, p = 0.95) and 0.3 µg/infusion (F [6, 5] = 0.71, p = 0.66), respectively, the treatment and yoked individuals displayed similar levels of antennal displacement and no clear distinction between the two groups emerged over the conditioning period (fig. 1a and 1b).

Psychostimulant effects of amphetamine on antennal movement

In the absence of significant conditioned effects (see above), antenna movements of master and yoked individuals were pooled and compared with saline controls for an analysis of unconditioned effects of psychostimulants. Whereas saline-treated crayfish displayed a gradual drop in antennal movements over the duration of the experiment, amphetamine infusion tended to result in higher levels of antennal movements (F [6, 17] = 0.55, p = 0.76). This increase was dose dependent, with increased measures of antenna movements associated with times of greater drug delivery (t [115] = 8.47, P <.0001*;* fig. 2).

4. **Discussion**

Invertebrate systems have been effective in modeling a number of components of the addiction cycle, from incentive learning, to loss of control over intake, drug use in the face of aversive conditions, and relapse after a period of abstinence (Kusayama & Watanabe, 2000; Nathaniel et al., 2009; Huber et al., 2011; Rawls et al., 2011; Raffa et al., 2013; Devineni & Heberlein, 2013). However, self-administration paradigms have been more difficult to establish as the rapid application of drugs via injection into the circulation is made difficult by small body size of most invertebrates (Søvik & Barron, 2013). This study aimed first to measure psychostimulant effects during instrumental learning in crayfish, and to then assess self- administration to complement existing conditioned place preference (CPP) and measures of unconditioned effects of drug (Alcaro et al., 2011).

Results of the present study extend previous work on drug-activation of reward in crayfish in several respects. First, the increase in antennal movements observed with amphetamine infusion confirms the psychostimulant effect of this drug in *Orconectes rusticus* using an independent and novel experimental paradigm. Since a crayfish's exploration of its surroundings relies heavily on tactile cues detected by antennae and antennules and processed in the olfactory lobe, such increase is indicative of an activation of brain circuits that promote exploratory and approach behaviors in the natural environment (McMahon et al., 2005). It is also consistent with the motor effects observed in the operant spatial conditioning task (chapter 1, this study), as well as studies using psychostimulant effects in conditioned place preference paradigms (Nathaniel et al., 2010; Alcaro et al., 2011; Nathaniel et al., 2012).

Findings from the study indicate crayfish were unable to associate side-specific antennal movement with the delivery of drug reward. While previous studies have indicated that the movements of the two antenna are largely independent of each other (Sandeman, 1985) it is possible that a certain degree of coupling between the movement of the two antennae exists. Interestingly, a strong coupling of leg and antennal movements has been reported (Sandeman & Wilkens, 1983), and since legs were not immobilized, the effect of such interactions may have impacted the animal's performance in this learning task. A left right antenna asymmetry where the two antenna execute movements that differ in 3D space could also affect the outcome of our conditioning paradigm (Tobo et al., 2012; Frasnelli et al., 2012).

The non-contingence of drug-reward with the actions of the yoked controls was designed to provide a measure for the unconditioned effects of the drug against which learning of reward contingency was evaluated. However, non-independence of the psychostimulant effects on the

two antennae created inadvertent contingencies sufficient to obscure detection of learning under the experimental conditions used. Whereas individuals treated at the 1.0 μg level exhibited a larger number of antennal displacements than both yoked and saline controls, the difference did not reach levels of statistical significance. A clearer indication of reward conditioning may emerge with a different choice of operant task. The learning of reward contingency may have been impeded by the target behavior being already too frequent prior to conditioning. Under this scenario, choice of antennal movements that have lower spontaneous frequency would be beneficial. An alternative strategy could involve the placement of objects within the range of the two antenna that the animal can explore, with reward delivery made contingent on the increase in frequency of contacts. A similar strategy has been successfully employed for side-specific conditioning of honeybee antenna (Kisch & Erber, 1999; Kisch & Haupt, 2009).

These results suggest that future work might also benefit from modification of drug dosages and reward schedules. Antennal displacement at the 1.0 μg dosage was relatively steep, increasing ca. 30% over the first hour of reinforcement, and then dropping back to initial levels over the subsequent two hours. Altering the continuous schedule of reinforcement used in the present study to an intermittent schedule of reinforcement has the potential to better capture the effectiveness of the reinforcer (Panlilio & Goldberg, 2007). By enforcing a schedule where a certain number of responses are required to obtain a unit dose of reward, the cumulative effect of the drug experienced by the animal is likely to be controlled.

The successful establishment of an operant self-administration paradigm in crayfish provides a suitable framework for more complex studies of invertebrate reward. In addition to reward strength, the establishment of self-administration paradigm will allow us to obtain measures of withdrawal and reinstatement. Furthermore, characterization of the substrates and

pharmacology of drug-associated behaviors, becomes an approachable goal following the development of an operant conditioning paradigm. Regions of the CNS associated with reward processing can be identified and pharmacological manipulations can be utilized to determine the underlying neurochemistry. A likely target of amphetamine and other drugs is the dopamine and serotonin modulated (Sandeman & Sandeman, 1987; Sandeman et al., 1995; Schmidt, 1997) olfactory lobe of crayfish (Alcaro et al., 2011).

Finally, technological and quantitative tools for an automated classification and comprehensive analyses of behavior have been limited. The application of machine vision-based learning has recently allowed the development of efficient and reliable assessment of appetitive and consummatory components of behavior in real-time (Anderson & Perona, 2014). The present study continues these advances in computational ethology providing an optimized setup for drug delivery and automation of learning paradigms for real-time collection of fine-scale behavioral metrics associated with addiction.

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Time Segment	Treatment	Yoke
Saline		
30	18301.40 ± 1522.15	
60	17400.93 ± 1781.31	
90	17139.71 ± 1635.36	
120	15529.02 ± 1746.57	
150	16269.23 ± 1829.42	
180	15132.12 ± 1463.98	
210	14750.87 ± 1880.30	
Dose 0.1 µg		
30	20221.87 ± 2092.09	22616.65 ± 3661.66
60	19414.34 ± 2460.37	24488.06 ± 5196.70
90	16295.28 ± 2106.34	22135.77 ± 4556.61
120	15396.10 ± 2260.19	21622.11 ± 4862.39
150	13093.62 ± 1801.51	17557.41 ± 3031.21
180	14006.10 ± 1553.73	17051.33 ± 2827.82
210	11256.42 ± 1453.09	14475.18 ± 2808.51

Time Segment	Treatment	Yoke

Dose 0.3 µg		
30	22853.61 ± 5712.46	24133.42 ± 3625.78
60	22085.28 ± 6136.83	23740.34 ± 3615.94
90	18630.59 ± 4240.34	23153.76 ± 3525.49
120	18015.36 ± 4024.34	24436.80 ± 3476.04
150	19810.42 ± 4243.49	25092.44 ± 3871.23
180	16526.70 ± 2875.24	24198.41 ± 4517.94
210	16393.52 ± 3670.08	23527.28 ± 4048.26
Dose 1.0 µg		
30	23090.30 ± 3657.99	15741.82 ± 1640.94
60	35506.45 ± 8594.32	17545.36 ± 2486.32
90	28643.90 ± 5253.79	16641.26 ± 2208.66
120	28265.73 ± 4533.05	17140.51± 2491.34
150	25006.45 ± 3373.50	16736.92 ± 2204.67
180	19397.08 ± 1834.08	15008.02 ± 1990.83
210	20305.74 ± 1528.13	14745.62 ± 2075.09

Table 1. Descriptive statistics for measures of antennal movement (in pixels) for crayfish of master, yoke and vehicle control groups, during each 30min time segment of the experiment.

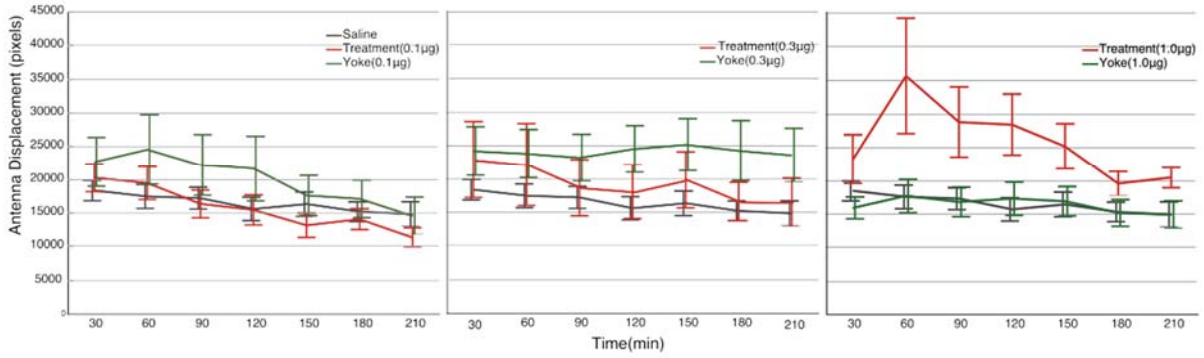

Fig.1. Amphetamine injections increase antennal movements under an operant conditioning paradigm. Movements of antenna for each 30 min period are shown as mean±sem for individuals receiving saline/drug delivered at the supra-esophageal ganglion. Increase in antenna movement in treatment group relative to the yoke set indicated a dose dependent trend. Maximum increase was observed for the highest dose of 1 µg (F [6, 5] = 0.68, p = 0.68, fig.1c). Treatment and yoke animals at 0.1 µg (F [6, 5] = 0.22, p = 0.95, fig.1a) and 0.3 µg (F [6, 5] = 0.71, p = 0.66, fig.1b) had similar levels of antennal movements.

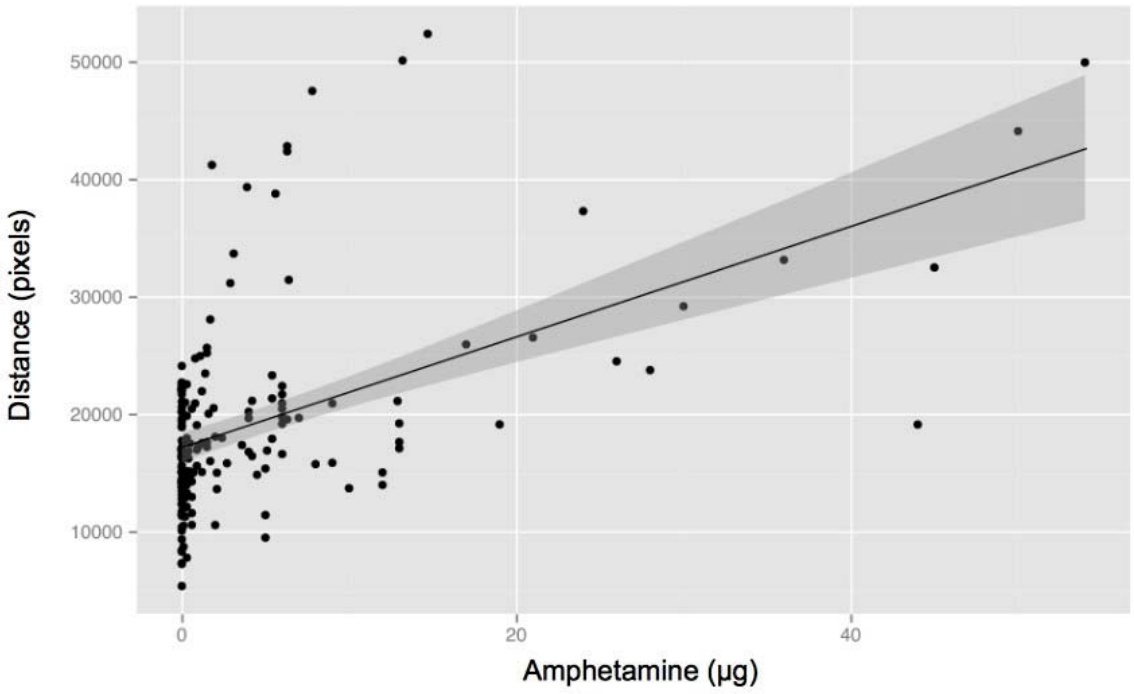

Figure 2. The psychostimulant effect of amphetamine administration on antennal movements, under an operant conditioning paradigm. Data points represents mean distance travelled by the antenna of control individuals or master-yoke pair (treated as a single unit) for a given amount of drug; both variables represented for each 30 min interval of the trial. An increase in antenna movements is observed with higher levels of amphetamine administered ($t\,[115] = 8.47$, $p < .0001$).