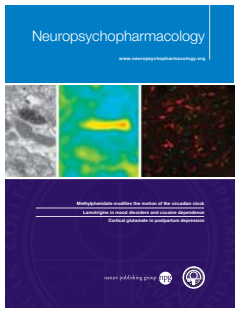
ACCEPTED ARTICLE PREVIEW

Accepted Article Preview: Published ahead of advance online publication



Reinforcing Effects of Binary Mixtures of Common bath salts Constituents: Studies with 3,4-Methylenedioxypyrovalerone (MDPV), 3,4-Methylenedioxymethcathinone (Methylone), and Caffeine in Rats

Brenda M Gannon, Kayla I Galindo, Melson P Mesmin, Kenner C Rice, Gregory T Collins

Cite this article as: Brenda M Gannon, Kayla I Galindo, Melson P Mesmin, Kenner C Rice, Gregory T Collins, Reinforcing Effects of Binary Mixtures of Common bath salts Constituents: Studies with 3,4-Methylenedioxypyrovalerone (MDPV), 3,4-Methylenedioxymethcathinone (Methylone), and Caffeine in Rats, *Neuropsychopharmacology* accepted article preview 5 July 2017; doi: 10.1038/npp.2017.141.

This is a PDF file of an unedited peer-reviewed manuscript that has been accepted for publication. NPG are providing this early version of the manuscript as a service to our customers. The manuscript will undergo copyediting, typesetting and a proof review before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers apply.

Accepted manuscript

Received 13 March 2017; revised 26 June 2017; accepted 29 June 2017; Accepted article preview online 5 July 2017

© 2017 Macmillan Publishers Limited. All rights reserved.

ACCEPTED ARTICLE PREVIEW

Reinforcing effects of binary mixtures of common *bath salts* constituents: studies with 3,4-methylenedioxypyrovalerone (MDPV), 3,4-methylenedioxymethcathinone (methylone), and caffeine in rats

Brenda M. Gannon, Ph.D.[1], Kayla I. Galindo, M.A.[1], Melson P. Mesmin[1], Kenner C. Rice, Ph.D.[2], Gregory T. Collins, Ph.D.[1,3]

1 – Department of Pharmacology, University of Texas Health Science Center at San Antonio, San Antonio, TX

2 – Molecular Targets and Medications Discovery Branch, NIDA and NIAAA, Bethesda, MD

3 – South Texas Veterans Health Care System, San Antonio, TX

Corresponding Author: Gregory T. Collins, Ph.D.
University of Texas Health Science Center at San Antonio
Department of Pharmacology
7703 Floyd Curl Dr – MC# 7764, San Antonio, TX 78229
E-mail: CollinsG@uthscsa.edu

Accepted manuscript

Running Title: Reinforcing effects of *bath salts* mixtures in rats

© 2017 Macmillan Publishers Limited. All rights reserved.

ACCEPTED ARTICLE PREVIEW

Abstract

Bath salts use is associated with high rates of abuse, toxicity, and death. *Bath salts* preparations often contain mixtures of drugs including multiple synthetic cathinones (e.g., 3,4-methylenedioxypyrovalerone [MDPV] or 3,4-methylenedioxymethcathinone [methylone]) or synthetic cathinones and caffeine; however, little is known about whether interactions among *bath salts* constituents contribute to the abuse-related effects of *bath salts* preparations. This study used male Sprague Dawley rats responding under a progressive ratio schedule to quantify the reinforcing effectiveness of MDPV, methylone, and caffeine, administered alone and as binary mixtures (n=12 per mixture). Each mixture was evaluated at four ratios (10:1, 3:1, 1:1, and 1:3) relative to the mean ED_{50} for each drug alone. Dose-addition analyses were used to determine the predicted, additive effect for each dose pair within each drug mixture. MDPV, methylone, and caffeine each maintained responding in a dose-dependent manner, with MDPV being the most potent and effective, and caffeine being the least potent and effective of the three *bath salts* constituents. High levels of responding were also maintained by each of the *bath salts* mixtures. Although the nature of the interactions tended towards additivity for most *bath salts* mixtures, supra-additive (3:1 MDPV:caffeine, and 3:1 and 1:1 methylone:caffeine) and sub-additive (3:1, 1:1, and 1:3 MDPV:methylone) interactions were also observed. Together, these findings demonstrate that the composition of *bath salts* preparations can impact both their reinforcing potency and effectiveness, and suggest that such interactions among constituent drugs could contribute to the patterns of use and effects reported by human *bath salts* users.

Accepted manuscript

© 2017 Macmillan Publishers Limited. All rights reserved.

ACCEPTED ARTICLE PREVIEW

Introduction:

Abuse of “designer drugs” such as synthetic derivatives of cathinone has become a serious public health problem worldwide. These synthetic cathinones were marketed as safe and legal alternatives to illicit stimulants and are often sold as *bath salts* preparations; however, increases in poison control center calls and emergency room visits (Drug Abuse Warning Network, 2013) has led to the Drug Enforcement Administration placing 13 of the most commonly detected synthetic cathinones under Schedule I regulations. Frequently sold as powders or capsules, *bath salts* are often administered via intravenous (IV), oral, or nasal routes with users frequently administering multiple doses within a session (Forrester *et al,* 2012; Baumann *et al,* 2013; Johnson and Johnson, 2014). Analysis of *bath salts* preparations obtained in the US suggests that 3,4-methylenedioxypyrovalerone (MDPV) and 3,4-methylenedioxymethcathinone (methylone) were two of the most widely available and abused synthetic cathinones when these drugs first emerged (Spiller *et al,* 2011; Shanks *et al,* 2012; Seely *et al,* 2013). Analogous to other abused stimulants, MDPV and methylone interact with dopamine, norepinephrine, and serotonin transporters (DAT, NET, and SERT; respectively) where MDPV functions as a DAT-selective cocaine-like transporter *inhibitor* and methylone functions as a non-selective amphetamine-like transporter *substrate* (Baumann *et al,* 2013; Eshleman *et al,* 2013; Simmler *et al,* 2013).

Bath salts preparations frequently contain multiple psychoactive compounds, including mixtures of multiple synthetic cathinones or a synthetic cathinone and caffeine, and the composition of these preparations varies not only with regard to the identity and purity of the psychoactive ingredient(s), but also varies across time within a single “brand” (Davies *et al,* 2010; Spiller *et al,* 2011; Shanks *et al,* 2012; Caudevilla-Galligo *et al,* 2013; Seely *et al,* 2013; Zuba and Byrska, 2013). Caffeine is also commonly identified in combination with other stimulant drugs (e.g., cocaine, 3,4-methylenedioxymethamphetamine [MDMA], and methamphetamine) (Goh *et al*, 2008; Lapachinske *et al*, 2015; Vidal Gine *et al*, 2016). One

Accepted manuscript

© 2017 Macmillan Publishers Limited. All rights reserved.

ACCEPTED ARTICLE PREVIEW

study that analyzed the contents of seized drug preparations found that MDPV, methylone, and caffeine were three of the most frequently detected constituents, regardless of formulation (e.g., pills, powders, etc.), and that 65-80% of pills containing both MDPV and caffeine (Seely *et al,* 2013). Perhaps relatedly, human *bath salts* users report effects that range from highly pleasurable (e.g., euphoria and alertness) to aversive (e.g., agitation, paranoia, tachycardia, and death) (Ross *et al,* 2011; Spiller *et al,* 2011; Forrester *et al,* 2012; Johnson and Johnson, 2014). Although *bath salts* preparations typically contain more than one psychoactive ingredient, most preclinical research has focused on characterizing the effects of individual cathinones, rather than modeling *bath salts* as drug mixtures.

Thus, the primary goal of the current study was to determine whether the reinforcing effects of MDPV and methylone are altered when they are administered as binary mixtures with other common *bath salts* constituents (i.e., MDPV+caffeine, methylone+caffeine, and MDPV+methylone). Although the mechanism of action of caffeine (adenosine A1 and A2A receptor antagonist) differs from stimulants like MDPV and methylone, caffeine has cocaine-like and methamphetamine-like discriminative stimulus effects (Garrett and Griffiths, 2001; Justinova *et al, 2003; 2009;* Collins *et al,* 2016). Additionally, pretreatment with caffeine has been shown to increase/enhance cocaine self-administration in both rats and monkeys (Horger *et al,* 1991; Schenk *et al,* 1994; Comer and Carroll, 1996). Although these findings suggest that caffeine might do more than mimic the effects of drugs such as cocaine or methamphetamine, relatively little is known about the reinforcing effects of drug mixtures containing cocaine and caffeine. Similarly, despite the fact that *bath salts* preparations often contain caffeine, it is currently unknown whether caffeine alters the reinforcing effects of synthetic cathinones, such as MDPV and methylone.

Dose-addition analyses are a powerful approach to determining the nature of drug-drug interactions (e.g., additive, supra-additive, or sub-additive) when both drugs produce the same pharmacologic effect. Because the composition of *bath salts* preparations varies greatly and

Accepted manuscript

© 2017 Macmillan Publishers Limited. All rights reserved.

ACCEPTED ARTICLE PREVIEW

because the nature of drug interactions is known to vary depending upon the proportion at which the constituents are mixed, each binary *bath salts* mixture was evaluated at four fixed-dose ratios (10:1, 3:1, 1:1, and 1:3) relative to the ED_{50} of each drug. Dose-addition analyses were used to test the general hypothesis that the reinforcing effects (potency and effectiveness) of *bath salts* mixtures containing multiple synthetic cathinones or a synthetic cathinone and caffeine are greater than would be predicted for an additive interaction.

Methods:

Subjects

Male Sprague-Dawley rats (275-300 g) were obtained from Harlan (Indianapolis, IN) and maintained in a temperature and humidity controlled environment, on a 10/14-h dark/light cycle. All rats were singly housed and had free access to tap water and Purina rat chow throughout the study. All experimental procedures were conducted in accordance with the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio, and the Eighth Edition of the Guide for Care and Use of Laboratory Animals (National Research Council, 2011).

Surgery

Rats were prepared with chronic indwelling catheters in the left femoral vein under 2% isoflurane anesthesia as previously described (Gannon *et al,* 2017a). Penicillin G (60,000 U/rat) was administered subcutaneously immediately following surgery to prevent infection, and all rats were allowed 5–7 days to recover before commencement of experiments. Catheters were flushed daily with 0.2 ml saline (before operant sessions) and 0.5 ml heparinized saline (100 U/ml, after operant sessions).

Apparatus

Accepted manuscript

© 2017 Macmillan Publishers Limited. All rights reserved.

ACCEPTED ARTICLE PREVIEW

All operant sessions were conducted in standard operant conditioning chambers (Med Associates, St Albans, VT) located inside sound attenuating cubicles. A set of green, yellow, and red LED lights was located above each of two levers, and a white house light was located at the top center of the opposite wall. A variable speed syringe driver delivered drug solutions through Tygon® tubing connected to a stainless steel fluid swivel and spring tether held in place by a counter-balanced arm.

Self-Administration:

Training

Rats were trained to respond under a fixed ratio (FR) 1 schedule of reinforcement for MDPV (0.032 mg/kg/inf) or methylone (0.32 mg/kg/inf) during daily 90-min sessions. A yellow LED above the active lever (left or right; counterbalanced) signaled drug availability, and completion of the response requirement resulted in a drug infusion (0.1 ml/kg over ~1-sec) and initiated a 5-sec timeout (TO) signaled by the illumination of the red, yellow and green LEDs above the active lever and the houselight. Responding on the inactive lever and responding during timeouts (active and inactive lever) were recorded but had no scheduled consequence. This schedule (FR1:TO 5-sec) was in place for 10 sessions, and all rats met acquisition criteria (>20 infusions and >80% of the responses emitted were on the active lever for two, consecutive days). Subsequently, the response requirement increased to an FR5 for at least an additional 10 sessions, and upon meeting stability criteria (± 20% of the mean number of infusions for 3 consecutive days, with no increasing or decreasing trend) all rats were transitioned to a progressive ratio (PR) schedule of reinforcement.

Reinforcing effectiveness of individual *bath salts* constituents

For the remainder of the experiment, responding was maintained under a PR schedule of reinforcement under which response requirements for each successive infusion incremented

Accepted manuscript

© 2017 Macmillan Publishers Limited. All rights reserved.

ACCEPTED ARTICLE PREVIEW

according to the following equation: Ratio=[5e^(inf#*0.2)]−5. The maximum session duration was 12 h, but sessions were terminated if a ratio was not completed within 45 min (i.e., 45-min limited hold). Two groups of rats were trained to respond for 0.032 mg/kg/inf MDPV, with one group (n=12) used to evaluate MDPV (0.0032-0.32 mg/kg/inf), caffeine (0.1-1.78 mg/kg/inf) and their binary mixtures, and the other group (n=19) used to evaluate MDPV (0.0032-0.32 mg/kg/inf), methylone (0.1-1.78 mg/kg/inf), and their binary mixtures. A third group of rats (n=15) was trained to respond for 0.32 mg/kg/inf methylone and was used to evaluate methylone (0.032-1.78 mg/kg/inf), caffeine (0.1-1.78 mg/kg/inf), and their binary mixtures. The first dose evaluated was 0.032 mg/kg/inf MDPV (MDPV-trained rats) or 0.32 mg/kg/inf methylone (methylone-trained rats), with all remaining doses evaluated in a random order. Each dose (or dose pair) was available for at least two consecutive sessions and until responding met stability criteria (±2 infusions from the previous session). All doses (dose pairs) for a particular drug (drug mixture) were evaluated prior to evaluating the next drug (drug mixture). Dose-response curves of individual constituents were established twice (once before and once after evaluation of drug mixtures) to determine whether responding changed over time. Because the study design required that 12 rats complete all portions of the study, and because toxicity was observed when rats were responding for methylone, 7 rats were added to the MDPV+methylone group, and 3 rats were added to the methylone+caffeine group.

Accepted manuscript

Drug Mixtures:

Composition of binary *bath salts* mixtures

Bath salts mixtures were constructed using the concept of dose-equivalence (see Tallarida and Raffa, 2010). Accordingly, dose-response curves for individual subjects were first normalized to the dose condition that maintained the greatest number of infusions (i.e., E_{max}) and saline, with the number of infusions maintained by saline serving as the 0% effect level, and the difference in the number of infusions maintained by the E_{max} and saline serving as the 100%

© 2017 Macmillan Publishers Limited. All rights reserved.

ACCEPTED ARTICLE PREVIEW

effect level. Normalized dose-response curves were then fit using a linear regression of the data spanning the 20%-80% effect levels (no more than one data point >80% and one data point <20%) to obtain ED_{50}s, slopes, and y-intercepts for each pair of constituent drugs (i.e., MDPV and caffeine, methylone and caffeine, or MDPV and methylone). Mixtures were designed at four ratios (10:1, 3:1, 1:1, and 1:3) relative to the mean ED_{50} for the group (i.e., dose of each drug that maintained 50% of the maximal effect level). Since caffeine failed to maintain 50% of the maximum effect of either MDPV of methylone, the dose that maintained 50% of the maximal effect of caffeine (0.56 mg/kg/inf) was used to determine the composition of MDPV+caffeine and methylone+caffeine mixtures. In order to fully evaluate the reinforcing effects of the *bath salts* mixtures, each fixed ratio mixture (e.g., 10:1, 3:1, 1:1, or 1:3) included a series of fixed dose pairs that spanned the 0-100% predicted effect levels (see Supplementary Table 1 for more details).

Accepted manuscript

Dose-Addition Analyses

Predicted additive effect levels for each dose pair were calculated by first converting the unit dose of each constituent into dose-equivalents of the training drug (e.g., caffeine into MDPV equivalents for the MDPV+caffeine mixtures) using the following function (eq. 1):

$$Dose B_{eq}A = [(Slope^{B} * Dose^{B}) + (int^{B} - int^{A})] / Slope^{A} \quad (1)$$

where $Slope^{A}$ and $Slope^{B}$ are the slope parameters and int^{A} and int^{B} are the y-intercepts derived from the linear portion of the dose-response curves of drugs A (the training drug) and B, respectively, and $Dose^{B}$ is the unit dose of drug B that is present in each dose pair. Summing the unit dose of drug A and the $B_{eq}A$ allows each dose pair to be expressed in terms of the total dose equivalents of drug A. The total equivalent dose for each dose pair was then used to calculate the predicted effect for an additive interaction using the following function (eq. 2):

$$\text{Predicted Additive Effect Level} = (Slope^{A} * \text{Total } Dose_{eq}A) + int^{A} \quad (2)$$

© 2017 Macmillan Publishers Limited. All rights reserved.

ACCEPTED ARTICLE PREVIEW

where Total $Dose_{eq}A$ is the total dose equivalents of drug A (i.e., $Dose^{A}$ + $DoseB_{eq}A$ from eq. 1). Predicted additive dose-response curves were determined for individual subjects for each drug mixture, and are graphically represented as the mean (± SEM) of the total drug equivalents (mg/kg/inf) and the mean (± SEM) predicted effect level (normalized to the E_{max} for Drug A) for each fixed-dose pair of each drug mixture.

Statistical Analyses

Dose-response curves for individual constituents were analyzed by one-way repeated measures ANOVA with post-hoc Dunnett's tests. All dose-response curves were analyzed by linear regression to obtain measures of potency (ED_{50}) and effectiveness (E_{max}) in individual subjects. For each constituent drug, the E_{max} (± SEM) values were compared between groups (i.e., MDPV in MDPV+caffeine and MDPV+methylone groups, caffeine in MDPV+caffeine and methylone+caffeine groups, and methylone in MDPV+methylone and methylone+caffeine groups) of rats using a student t-test, while significant differences in ED_{50}s between groups were indicated by non-overlapping 95% confidence intervals. For each *bath salts* mixture, potency (observed ED_{50} / predicted ED_{50}) and effectiveness (predicted E_{max} / observed E_{max}) ratios were calculated for individual subjects at each of the four dose ratios (10:1, 3:1, 1:1, and 1:3). Dose ratios for which the 95% confidence interval did not include 1 were considered to be significantly different than additive, with ratios less than 1 indicative of a supra-additive interaction, greater than 1 indicative of a sub-additive interaction, and inclusive of 1 indicative of an additive interaction.

Accepted manuscript

Results:

Reinforcing Effects of Individual Constituents

Figure 1 (top row) shows the number of infusions maintained by the *bath salts* constituents alone for rats that responded for MDPV ($F[5,55]=231.0$, $p<0.001$; post-hoc 0.01-

© 2017 Macmillan Publishers Limited. All rights reserved.

ACCEPTED ARTICLE PREVIEW

0.178 mg/kg/inf p<0.05) and caffeine (F[4,44]=15.1, p<0.001; post-hoc 1-1.78 mg/kg/inf p<0.05) (left panels), methylone (F[5,55]=60.0, p<0.001; post-hoc 0.1-1.78 mg/kg/inf p<0.05) and caffeine (F[4,44]=23.5, p<0.001; post-hoc 1-1.78 mg/kg/inf p<0.05) (middle panels) and MDPV (F[5,70]=259.3, p<0.001; post-hoc 0.01-0.178 mg/kg/inf p<0.05) and methylone (F[4,56]=96.6, p<0.001; post-hoc 0.32-1.78 mg/kg/inf p<0.05) (right panels). Normalized dose-response curves for the self-administration of bath salts constituents are shown in the lower two panels of Figure 1. All three *bath salts* constituents maintained responding in a dose-dependent manner; however, differences in potency (rank order: MDPV > methylone = caffeine) and effectiveness (rank order: MDPV > methylone > caffeine) (maximum number of infusions) were observed. Because each drug was evaluated in two groups of rats, between group comparisons of maximum number of infusions earned (E_{max}) and potency (ED_{50}) were also made. Although the E_{max} for MDPV (26.4 ± 1.0 for MDPV+caffeine and 26.3 ± 1.0 for MDPV+methylone) and caffeine (8.7 ± 0.4 for MDPV+caffeine and 8.3 ± 0.5 for methylone+caffeine) were comparable between groups (Table 1), methylone maintained significantly less responding (t=2.77, p<0.05) in rats from the methylone+caffeine group (14.8 ± 0.8) than in rats from the MDPV+methylone group (18.1 ± 0.9). Differences in potency were not observed for any constituent. For the majority of the drugs, slope, ED_{50}, and E_{max} values did not differ for dose-response curves generated before and after evaluation of mixtures; however, for rats from the MDPV+caffeine group, caffeine's effects (E_{max} and slope) were significantly decreased upon redetermination (data not shown).

Self-administration of methylone alone (1.0 and 1.78 mg/kg/inf) was lethal in 2 of 19 MDPV-trained rats and 1 of 15 methylone-trained rats.

Reinforcing Effects of Binary Mixtures of MDPV and Caffeine

Predicted and observed dose-response curves for mixtures of MDPV+caffeine are shown in Figure 2 (top row). When combined at dose ratios of 10:1, 3:1, 1:1, and 1:3, mixtures

Accepted manuscript

© 2017 Macmillan Publishers Limited. All rights reserved.

ACCEPTED ARTICLE PREVIEW

of MDPV+caffeine maintained responding in dose-dependent manner. With the exception of the 3:1 MDPV:caffeine ratio, the dose-response curves obtained for mixtures of MDPV+caffeine did not depart from the predicted additive dose-response curves. Although the 3:1 mixture of MDPV+caffeine was significantly more potent than predicted (Figure 3; top row, left panel), none of the mixtures of MDPV and caffeine differed from predictions for a strictly additive interaction with regard to their reinforcing effectiveness (Figure 3; top row, right panel). The E_{max} ± SEM and the final ratio completed ± SEM for each mixture of MDPV+caffeine are reported in Table 1.

Reinforcing Effects of Binary Mixtures of Methylone and Caffeine

Predicted and observed dose-response curves for mixtures of methylone and caffeine are also shown in Figure 2 (middle row). As with mixtures of MDPV+caffeine, dose-dependent increases in responding were observed for all fixed-dose ratios of methylone+caffeine. Although each of the mixtures exhibited additive interactions with respect to potency (Figure 3; middle row, left panel), when evaluated at 3:1 and 1:1 ratios of methylone:caffeine, the two largest dose pairs of each mixture consistently maintained more responding than predicted for an additive interaction. Indeed, the effectiveness ratios for the 3:1 and 1:1 mixtures of methylone+caffeine were significantly smaller than 1, indicating a supra-additive interaction between methylone and caffeine with regard to their reinforcing effectiveness (Figure 3; middle row, right panel). The E_{max} ± SEM and the final ratio completed ± SEM for each mixture of methylone+caffeine are reported in Table 1.

Self-administration of the largest dose pair of the 3:1 mixture of methylone+caffeine (0.80 mg/kg/inf methylone + 0.79 mg/kg/inf caffeine) was lethal in 2 out of 14 rats.

Reinforcing Effects of Binary Mixtures of MDPV and Methylone

Accepted manuscript

© 2017 Macmillan Publishers Limited. All rights reserved.

ACCEPTED ARTICLE PREVIEW

As observed with other binary *bath salts* mixtures, mixtures of MDPV and methylone maintained dose-dependent increases in self-administration, regardless of the ratio at which the cathinones were mixed (Figure 2; bottom row). Although the reinforcing effects of small dose pairs of MDPV+methylone appeared to be additive in nature, larger dose pairs at the 3:1, 1:1, and 1:3 ratios of MDPV+methylone tended to maintain less responding than predicted for an additive interaction. Indeed, although substantial variability was observed among the potency ratios for the MDPV+methylone group, the 3:1 mixture of MDPV+methylone exhibited a sub-additive interaction with regard to potency; additive interactions were observed for all other mixtures (Figure 3; bottom row, left panel). With regard to reinforcing effectiveness, an additive interaction was observed for the 10:1 ratio, whereas the effectiveness ratios for the 3:1, 1:1, and 1:3 mixtures of MDPV+methylone were all significantly greater than 1, indicative of a sub-additive interaction (Figure 3; bottom row, right panel). The E_{max} ± SEM and the final ratio completed ± SEM for each mixture of MDPV+methylone are reported in Table 1.

The self-administration of MDPV+methylone mixtures was associated with lethality in 5 of 17 rats. For the 1:1 mixture, 1 of 14 rats died at the 4th dose pair (0.03 mg/kg/inf MDPV + 1.23 mg/kg/inf methylone), and 2 of 4 rats died at the 5th dose pair (0.05 mg/kg/inf MDPV + 2.19 mg/kg/inf methylone). At the 1:3 mixture, 1 of 14 rats died at the 4th dose pair (0.015 mg/kg/inf MDPV + 1.85 mg/kg/inf methylone), and 1 of 2 rats died at the 5th dose pair (0.03 mg/kg/inf MDPV, 3.3 mg/kg/inf methylone). Due to the high incidence of lethality (50%), the largest dose pairs of the 1:1 and 1:3 mixtures of MDPV+methylone were not evaluated in all subjects.

Accepted manuscript

Discussion:

Bath salts preparations typically contain mixtures of drugs including multiple synthetic cathinones or synthetic cathinones and caffeine, and use of these preparations is associated with high rates of abuse, toxicity, and death. Despite this, little is known about how the

© 2017 Macmillan Publishers Limited. All rights reserved.

ACCEPTED ARTICLE PREVIEW

composition of these *bath salts* preparations impacts their abuse-related and toxic effects. As such, the current study evaluated the reinforcing effects of three common *bath salts* constituents (i.e., MDPV, methylone, and caffeine) and used dose-addition analyses to characterize the nature of the interaction(s) between binary *bath salts* mixtures comprising two synthetic cathinones (i.e., MDPV+methylone) or a synthetic cathinone and caffeine (i.e., MDPV+caffeine, and methylone+caffeine). The present study provides evidence that the composition of *bath salts* preparations can significantly impact both their reinforcing potency and effectiveness, with mixtures of MDPV+caffeine (3:1) being more potent than predicted for an additive interaction, mixtures of methylone+caffeine (3:1 and 1:1) being more effective than predicted for an additive interaction, and mixtures of MDPV+methylone (3:1, 1:1, and 1:3) being less effective and less potent (3:1) than predicted for an additive interaction.

Consistent with literature describing the reinforcing effects of MDPV (Aarde *et al,* 2013; Watterson *et al,* 2014; Schindler *et al,* 2015; Gannon *et al,* 2017a; Gannon *et al,* 2017b), methylone (Watterson et al, 2013; Creehan *et al,* 2015; Vandewater *et al,* 2015; Nguyen *et al,* 2016), and caffeine alone (Collins *et al,* 1984; Briscoe *et al,* 1998), MDPV was the most effective, whereas caffeine was the least effective of the *bath salts* constituents. Importantly, although numerous studies have used PR schedules to compare relative reinforcing effectiveness of MDPV and methylone to other stimulants (e.g., cocaine, methamphetamine, MDMA), this is the first to directly compare these two common *bath salts* constituents. Using the final ratio completed as an index of relative reinforcing effectiveness, MDPV (1123.2 ± 114.1) was a ~6-fold more effective reinforcer than methylone (190.4 ± 39.4) and a ~45-fold more effective reinforcer than caffeine (24.7 ± 1.9). Although previous reports suggest that the reinforcing effects of IV caffeine are dubious and short-lived (Atkinson and Enslen, 1976; Briscoe *et al,* 1998), in the present study, caffeine maintained low but consistent levels of responding when substituted from a MDPV or methylone baseline, regardless of the order that

Accepted manuscript

© 2017 Macmillan Publishers Limited. All rights reserved.

ACCEPTED ARTICLE PREVIEW

the doses were evaluated. However, upon re-evaluation at the end of the study, caffeine appeared to be less effective in the MDPV-trained rats.

Even though caffeine was found to be a weak reinforcer, supra-additive interactions were observed when caffeine was self-administered in combination with more effective reinforcers. For instance, when mixed at a 3:1 ratio with MDPV, a highly effective reinforcer, caffeine produced a supra-additive interaction with regard to reinforcing potency, suggesting that *bath salts* preparations containing MDPV and caffeine can produce a full MDPV-like effect with significantly less MDPV. Conversely, when mixed at a 3:1 or 1:1 ratio with methylone, a moderately effective reinforcer, caffeine produced a supra-additive interaction with regard to reinforcing effectiveness, suggesting that the reinforcing effects of *bath salts* preparations containing methylone and caffeine would be significantly greater than *bath salts* preparations containing only methylone. Although these studies were not designed to identify the mechanism(s) that underlie interactions between caffeine and the synthetic cathinones, caffeine (through its antagonism of adenosine A_{2A} receptors) can increase dopamine D_2 receptor signaling, which has been hypothesized to play a role in caffeine's ability to potentiate the psychostimulant effects of other indirect dopamine receptor agonists (e.g., cocaine) (Ferré, 2016). Although it is unclear why the effects of caffeine differed in *bath salts* mixtures containing caffeine+MDPV (increased potency) and caffeine+methylone (increased effectiveness), it is a possibility that a ceiling effect limited our ability to detect enhancements in the effectiveness for mixtures of MDPV+caffeine, and that supra-additive interactions would have been observed if different methods for quantifying reinforcing effectiveness had been employed (e.g., demand curve analyses). Alternatively, it is also possible that differences in the types of supra-additive interactions are related to differences in the mechanism of action for MDPV (highly selective inhibitor of DAT) and methylone (non-selective substrate at DAT, NET, and SERT) (Baumann *et al,* 2013; Simmler *et al,* 2013). Clearly, more in-depth investigations into the mechanisms that underlie these interactions are warranted.

Accepted manuscript

© 2017 Macmillan Publishers Limited. All rights reserved.

ACCEPTED ARTICLE PREVIEW

Whereas supra-additive interactions were observed for *bath salts* mixtures containing caffeine, sub-additive interactions were observed when MDPV and methylone were combined. Although the 3:1 mixture of MDPV+methylone was found to be less potent than predicted for an additive interaction, 3:1, 1:1, and 1:3 mixtures of MDPV+methylone were all found to exhibit sub-additive interactions with regard to reinforcing effectiveness. This finding contrasts a report that identified a supra-additive interaction between mephedrone (a monoamine transporter substrate) and MDPV with regard to excitatory hDAT currents, and proposed that this could result in mixtures of substrates and inhibitors producing effects greater than either drug alone (Cameron *et al,* 2013). However, it is important to note that methylone, and MDPV to a lesser extent, also have actions at NET and SERT that likely impact the reinforcing effects of these drugs/drug mixtures that are not captured in an isolated *in vitro* system. Interestingly, as the proportion of methylone in the MDPV+methylone mixtures increased, the maximal effect observed became more similar to the maximal effect produced by methylone alone (see Figure 1, bottom right panel). Thus, one interpretation of these findings is that the actions of methylone at SERT may be decreasing the reinforcing effectiveness of the MDPV+methylone mixture, a notion that is supported by the demonstration that increases in serotoninergic activity are associated with decreases in stimulant self-administration by nonhuman primates (Wee *et al*, 2005; Wee and Woolverton, 2006).

However, because these sub-additive interactions were observed at larger dose pairs, it is also possible that decreases in reinforcing effectiveness resulted from the onset of other/adverse effects that limited the ability of the rats to respond at high rates. Indeed, the self-administration of these large dose pairs was lethal in 5 rats. Although the mechanism of this toxicity is unclear, it is likely related to the fact that methylone is a mechanism-based inhibitor of CYP2D6, the enzyme primarily responsible for the metabolism of methylone (Pederson *et al,* 2013; Elmore *et al,* 2017). However, because lethality has not previously been reported for rats self-administering methylone (e.g., Watterson et al, 2013; Creehan *et al,* 2015; Vandewater *et*

Accepted manuscript

© 2017 Macmillan Publishers Limited. All rights reserved.

ACCEPTED ARTICLE PREVIEW

al, 2015; Nguyen *et al,* 2016), and because the majority of the deaths (7 of 10) were associated with the self-administration of *bath salts* mixtures containing methylone (5 with methylone+MDPV, and 2 with methylone+caffeine), pharmacodynamic interactions between the toxic effects of *bath salts* constituents cannot be ruled out. Importantly, methylone has also been associated with toxicity and death in human *bath salts* users, particularly when used in preparations that also contain MDPV (e.g., Spiller *et al,* 2011; Pearson *et al,* 2012). Since MDPV maintains very high levels of responding without overt signs of toxicity, and toxicity was only observed in the presence of methylone, it is likely that the toxicity observed with mixtures of MDPV+methylone was the result of MDPV maintaining high rates of responding that also resulted in large cumulative doses of methylone.

Abuse and toxicity associated with the use of *bath salts* has become a major public concern in the past decade. While it has been demonstrated that some common constituents of these preparations (e.g., MDPV) are more effective than other drugs of abuse when self-administered alone (e.g., Gannon *et al,* 2017a), *bath salts* preparations are often mixtures of multiple drugs. The present study is the first to directly assess the reinforcing effects of mixtures of common *bath salts* constituents. There were four main findings: (1) MDPV is a more potent and effective reinforcer than methylone which is equipotent but more effective than caffeine; (2) *bath salts* mixtures containing caffeine can function as more potent (MDPV+caffeine) and more effective (methylone+caffeine) reinforcers than expected based on the effects of the constituents alone; (3) *bath salts* mixtures containing MDPV+methylone are less effective reinforcers than expected; and (4) *bath salts* mixtures containing methylone appear to be more toxic than methylone alone. Although most *bath salts* mixtures exhibited strictly additive interactions, these findings indicate that supra-additive interactions can occur between drugs with different mechanisms of action (i.e., MDPV+caffeine, and methylone+caffeine) and sub-additive interactions can occur between drugs that act primarily at monoamine transporters but differ in transporter selectivity (i.e., MDPV+methylone). Taken together, these studies

Accepted manuscript

© 2017 Macmillan Publishers Limited. All rights reserved.

ACCEPTED ARTICLE PREVIEW

demonstrate that the composition of *bath salts* preparations can significantly impact their abuse-related and toxic effects, and suggest that such interactions could contribute to the patterns of use and/or the adverse effects reported by human *bath salts* users.

Accepted manuscript

© 2017 Macmillan Publishers Limited. All rights reserved.

ACCEPTED ARTICLE PREVIEW

Funding and Disclosure:

This research was supported by grants from the National Institutes of Health and the National Institute on Drug Abuse (R01DA039146 [GTC], T32DA031115 [BMG]). The work of the Molecular Targets and Medications Discovery Branch was supported by the Intramural Research Programs of the National Institute on Drug Abuse and the National Institute of Alcohol Abuse and Alcoholism. The Authors have no conflicts of interest to disclose

Accepted manuscript

© 2017 Macmillan Publishers Limited. All rights reserved.

ACCEPTED ARTICLE PREVIEW

References:

Aarde SM, Huang PK, Creehan KM, Dickerson TJ, Taffe MA (2013) The novel recreational drug 3,4-methylenedioxypyrovalerone (MDPV) is a potent psychomotor stimulant: self-administration and locomotor activity in rats. *Neuropharmacology* 71:130–140.

Atkinson J, Enslen M. (1976) Self-administration of caffeine by the rat. *Arzneimittelforschung.* 26:2059–2061.

Baumann MH, Partilla JS, Lehner KR, Thorndike EB, Hoffman AF, Holy M, *et al* (2013) Powerful cocaine-like actions of 3,4-methylenedioxypyrovalerone (MDPV), a principal constituent of psychoactive bath salts products. *Neuropsychopharmacology* 38:552–562.

Brandt SD, Sumnall HR, Measham F, Cole J (2010) Analyses of second-generation 'legal highs' in the UK: Initial findings. *Drug Test Analysis* 2:377–382.

Briscoe RJ, Vanecek SA, Vallett M, Baird TJ, Holloway FA, Gauvin DV (1998) Reinforcing effects of caffeine, ephedrine, and their binary combination in rats. *Pharmacol Biochem Behav* 60:685–693.

Cameron KN, Kolanos R, Solis E Jr, Glennon RA, De Felice LJ (2013) Bath salts components mephedrone and methylenedioxypyrovalerone (MDPV) act synergistically at the human dopamine transporter. *Br J Pharmacol* 168(7):1750-1757.

Caudevilla-Gálligo F, Ventura M, Iciar BI, Ruiz I, Fornís I (2013) Presence and composition of cathinone derivatives in drug samples taken from a Drug Test Service in Spain (2010–2012). *Human Psychopharmacol*. 28: 341–344.

Collins RJ, Weeks JR, Cooper MM, Good PI, Russell RR (1984) Prediction of abuse liability of drugs using IV self-administration by rats. *Psychopharmacology.* 82:6–13.

Collins GT, Abbott M, Galindo K, Rush EL, Rice KC, France CP (2016) Discriminative Stimulus Effects of Binary Drug Mixtures: Studies with Cocaine, MDPV, and Caffeine. *J Pharmacol Exp Ther* 359(1):1-10.

Accepted manuscript

© 2017 Macmillan Publishers Limited. All rights reserved.

ACCEPTED ARTICLE PREVIEW

Comer SD, Carroll ME (1996) Oral caffeine pretreatment produced modest increases in smoked cocaine self-administration in rhesus monkeys. *Psychopharmacology (Berl)* 126(4):281-285.

Creehan KM, Vandewater SA, Taffe MA (2015) Intravenous self-administration of mephedrone, methylone, and MDMA in female rats. *Neuropharmacology* 92:90-97.

Davies S, Wood DM, Smith G, Button J, Ramsey J, Archer R, *et al* (2010) Purchasing "legal highs" on the Internet--is there consistency in what you get? *Q J Med* 103:489–493.

Drug Abuse Warning Network (2013) "Bath salts" drugs were involved in over 20,000 drug-related emergency department visits in 2011. Substance Abuse and Mental Health Services Administration.

Elmore JS, Dillon-Carter O, Partilla JS, Ellefsen KN, Concheiro M, Suzuki M, *et al* (2017) Pharmacokinetic profiles and pharmacodynamics effects for methylone and its metabolites in rats. *Neuropsychopharmacology* 42(3):649-660.

Ferré S (2016) Mechanisms of the psychostimulant effects of caffeine: implications for substance use disorders. *Psychopharmacology (Berl)* 233(10):1963-1973.

Forrester MB (2012) Synthetic cathinone exposures reported to Texas poison centers. *Am J Drug Alcohol Abuse* 38(6):609-615.

Gannon BM, Galindo KI, Rice KC, Collins GT (2017a) Individual differences in the relative reinforcing effects of 3,4-methylenedioxypyrovalerone (MDPV) under fixed and progressive ratio schedules of reinforcement in rats *J Pharmacol Exp Ther* 361:181-189.

Gannon BM, Rice KC, Collins GT (2017b) Reinforcing effects of 3,4-methylenedioxypyrovalerone (MDPV), α-pyrrolidinopentiophenone (α-PVP) and their enantiomers in rats. *Behav Pharmacol* (In Press)

Garrett BE, Griffiths RR (2001) Intravenous nicotine and caffeine: subjective and physiological effects in cocaine abusers. *J Pharmacol Exp Ther* 296: 486–494.

Accepted manuscript

© 2017 Macmillan Publishers Limited. All rights reserved.

ACCEPTED ARTICLE PREVIEW

Goh CY, van Bronswijk W, Priddis C (2008) Rapid nondestructive on-site screening of methylamphetamine seizures by attenuated total reflection fourier transform infrared spectroscopy. *Appl Spectrosc* 62:640-648.

Horger BA, Wellman PJ, Morien A, Davies BT, and Schenk S (1991) Caffeine exposure sensitizes rats to the reinforcing effects of cocaine. *Neuroreport* 2:53–56.

Johnson PS and Johnson MW (2014) Investigation of "bath salts" use patterns within an online sample of users in the United States. *J Psychoactive Drugs* 46:369–378.

Justinova Z, Ferré S, Barnes C, Wertheim CE, Pappas LA, Goldberg SR, et al (2009) Effects of chronic caffeine exposure on adenosinergic modulation of the discriminative-stimulus effects of nicotine, methamphetamine, and cocaine in rats. *Psychopharmacology (Berl)* 203(2):355-367.

Justinova Z, Ferré S, Segal PN, Antoniou K, Solinas M, Pappas LA, et al (2003) Involvement of adenosine A1 and A2A receptors in the adenosinergic modulation of the discriminative-stimulus effects of cocaine and methamphetamine in rats. *J Pharmacol Exp Ther* 307 (3):977-986.

Lapachinske SF, Okai GG, dos Santos A, de Bairros AV, Yonamine M (2015) Analysis of cocaine and its adulterants in drugs for international trafficking seized by the Brazilian Federal Police. *Forensic Sci Int* 247:28-53.

National Research Council (2011) Guide for the Care and Use of Laboratory Animals, 8th ed., National Academy Press, Washington, DC.

Nguyen JD, Grant Y, Creehan KM, Vandewater SA, Taffe MA (2016) Escalation of intravenous self-administration of methylone and mephedrone under extended access conditions. *Addiction Biol* [Epub ahead of print].

Pearson JM, Hargraves TL, Hair LR, Massucci CJ, Frazee CC III, Garg U, et al (2012) Three fatal intoxications due to methylone. *J Anal Toxicol* 36(6):444-451.

Accepted manuscript

© 2017 Macmillan Publishers Limited. All rights reserved.

ACCEPTED ARTICLE PREVIEW

Pederson AJ, Petersen TH, Linnet K (2013) In vitro metabolism and pharmacokinetic studies on methylone. *Drug Metab Dispos* 41(4):1247-1255.

Ross EA, Watson M, Goldberger B. (2011) “Bath salts” intoxication. *N Engl J Med*. 365:967–968.

Schenk S, Valadez A, Horger BA, Snow S, Wellman PJ (1994) Interactions between caffeine and cocaine in tests of self-administration. *Behav Pharmacol* 5(2):153-158.

Schindler CW, Thorndike EB, Goldberg SR, Lehner KR, Cozzi NV, Brandt SD, and Baumann MH (2016) Reinforcing and neurochemical effects of the “bath salts” constituents 3,4-methylenedioxypyrovalerone (MDPV) and 3,4-methylenedioxy-N-methylcathinone (methylone) in male rats. *Psychopharmacology (Berl)* 233(10)1981–1990.

Seely KA, Patton AL, Moran CL, Womack ML, Prather PL, Fantegrossi WE, *et al* (2013) Forensic investigation of K2, Spice, and “bath salt” commercial preparations: a three-year study of new designer drug products containing synthetic cannabinoid, stimulant, and hallucinogenic compounds. *Forensic Sci Int* 233:416–422.

Shanks KG, Dahn T, Behonick G, Terrell A (2012) Analysis of first and second generation legal highs for synthetic cannabinoids and synthetic stimulants by ultra-performance liquid chromatography and time of flight mass spectrometry. *J Anal Toxicol* 36:360–371.

Simmler LD, Buser TA, Donzelli M, Schramm Y, Dieu L-H, Huwyler J, *et al* (2013) Pharmacological characterization of designer cathinones in vitro. *Br J Pharmacol* 168:458–470.

Spiller HA, Ryan ML, Weston RG, Jansen J (2011) Clinical experience with and analytical confirmation of *bath salts* and “legal highs” (synthetic cathinones) in the United States. *Clin Toxicol* 49:499–505.

Tallarida RJ and Raffa RB (2010) The application of drug dose equivalence in the quantitative analysis of receptor occupation and drug combinations. *Pharmacol Ther* 127:165–174.

Accepted manuscript

© 2017 Macmillan Publishers Limited. All rights reserved.

ACCEPTED ARTICLE PREVIEW

Vandewater SA, Creehan KM, Taffe MA (2015) Intravenous self-administration of entactogen-class stimulants in male rats. *Neuropharmacology* 99:538-545.

Vidal Giné C, Ventura Vilamala M, Fornís Espinosa I, Gil Lladanosa C, Calzada Álvarez N, Fitó Fruitós A, *et al* (2016) Crystals and tablets in the Spanish ecstasy market 2000-2014: Are they the same or different in terms of purity and adulteration? *Forensic Sci Int* 263:164-168.

Watterson LR, Hood L, Sewalia K, Tomek SE, Yahn S, Johnson CT, *et al* (2013) The reinforcing and rewarding effects of methylone, a synthetic cathinone commonly found in “bath salts”. *J Addict Res Ther* Suppl 9:002.

Watterson LR, Kufahl PR, Nemirovsky NE, Sewalia K, Grabenauer M, Thomas BF, *et al* (2014) Potent rewarding and reinforcing effects of the synthetic cathinone 3,4-methylenedioxypyrovalerone (MDPV). *Addiction Biol* 19:165–174.

Wee S, Anderson KG, Baumann MH, Rothman RB, Blough BE, Woolverton WL (2005) Relationship between the serotonergic activity and reinforcing effects of a series of amphetamine analogs. *J Pharmacol Exp Ther* 313:848-854.

Wee S, Woolverton WL. (2006) Self-administration of mixtures of fenfluramine and amphetamine by rhesus monkeys. *Pharmacol Biochem Behav* 84:337–343.

Zuba D, Byrska B (2013) Prevalence and co-existance of active components of “legal highs.” *Drug Test Anal* 5:420–429.

Accepted manuscript

© 2017 Macmillan Publishers Limited. All rights reserved.

ACCEPTED ARTICLE PREVIEW

FIGURE LEGENDS

Figure 1: Dose-response curves for the self-administration of MDPV (squares), caffeine (circles), and methylone (triangles) under a progressive ratio schedule of reinforcement (n=12 per group). *Abscissa: First and second rows*- "SAL" represents data obtained when saline was available for infusion, whereas doses refer to the unit dose of each drug available for infusion expressed as mg/kg/inf on a log scale. *Third row*- Doses refer to the MDPV (first and third columns) or methylone (middle column) equivalents available for infusion expressed as mg/kg/inf on a log scale. *Ordinate: First row*- Total infusions ± SEM obtained during the session. *Second and third rows*- Percent of the maximal effect ± SEM, normalized to the drug in each pair that maintained the most responding (100%) and saline (0%).

Accepted manuscript

© 2017 Macmillan Publishers Limited. All rights reserved.

ACCEPTED ARTICLE PREVIEW

Figure 2: Dose-response curves for the self-administration of binary mixtures of MDPV+caffeine (top row), methylone+caffeine (middle row), or MDPV+methylone (bottom row) (n=12 per group). Each mixture was tested at four fixed-dose ratios [10:1 (left column), 3:1 (left-center column), 1:1 (right-center column), 1:3 (right column)] relative to the ED_{50} of the drug in each pair that maintained the most responding. Experimentally determined dose-response curves (white circles) represent the mean (± SEM) for twelve rats. Predicted, additive dose-response curves (gray, dashed lines) represent the mean (± SEM) for twelve rats. *Abscissa:* Doses refer to total MDPV (top and bottom rows) or methylone (middle row) equivalents available for infusion expressed as mg/kg/inf on a log scale. *Ordinate:* Percent of the maximal effect, normalized to the drug in each pair that maintained the most responding (100%) and saline (0%) as shown in Figure 1.

Accepted manuscript

© 2017 Macmillan Publishers Limited. All rights reserved.

ACCEPTED ARTICLE PREVIEW

Figure 3: Potency ratios (observed ED_{50} / predicted additive ED_{50}, left column) and effectiveness ratios (predicted additive E_{max} / observed E_{max}, right column) for binary mixtures of MDPV+caffeine (top row), methylone+caffeine (middle row), and MDPV+methylone (bottom row) (n=12 per group). Ratios for individual subjects are depicted by the gray dots. The group means and 95% confidence intervals are depicted by the vertical black line and error bars, respectively.

Accepted manuscript

© 2017 Macmillan Publishers Limited. All rights reserved.

ACCEPTED ARTICLE PREVIEW

Tables:

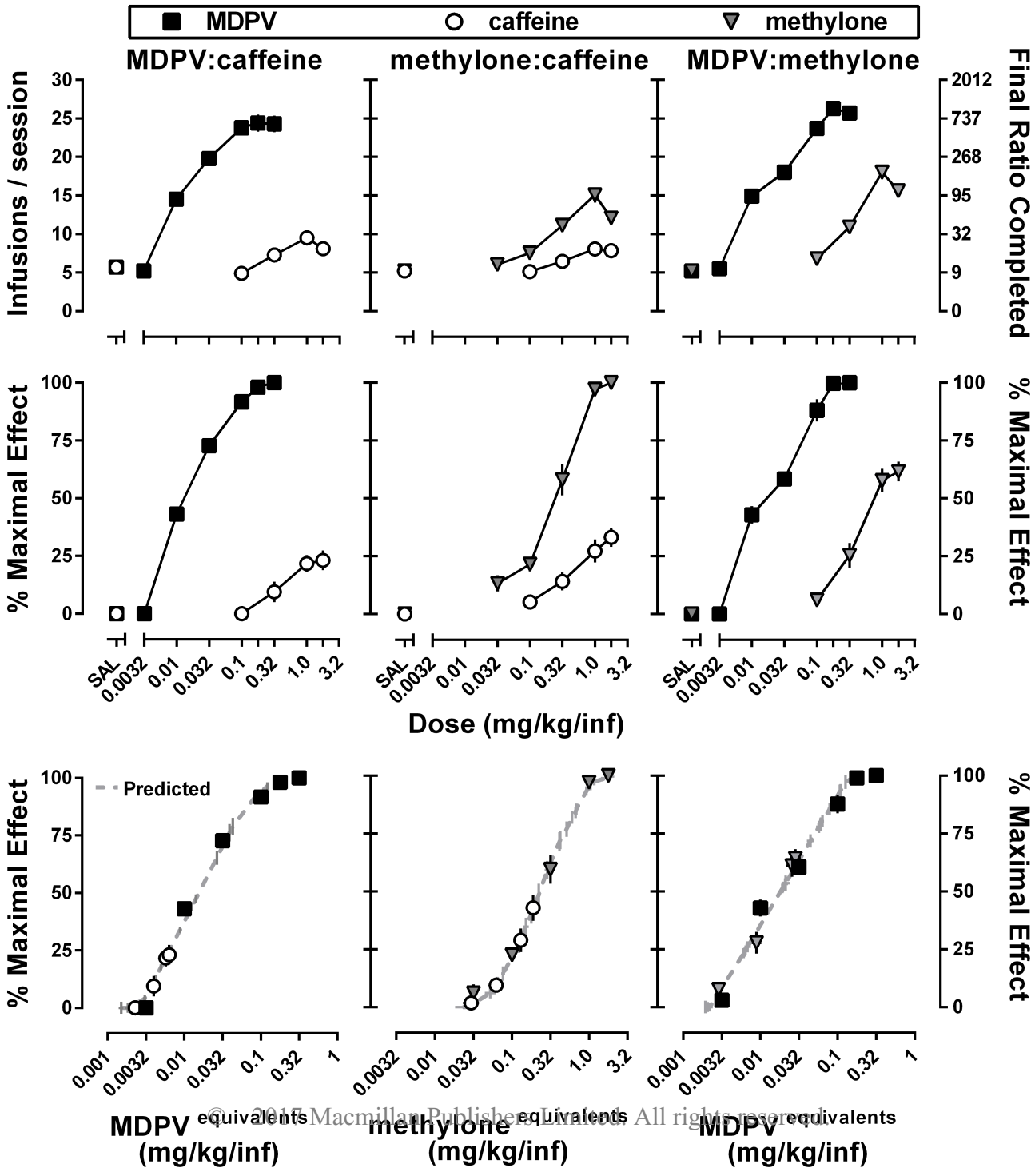
Table 1: Reinforcing effectiveness of *bath salts* constituents MDPV, methylone, and caffeine alone and in binary mixtures

	MDPV:caffeine		methylone:caffeine		MDPV:methylone	
	Emax (± SEM)	**Final Ratio (± SEM)**	**Emax (± SEM)**	**Final Ratio (± SEM)**	**Emax (± SEM)**	**Final Ratio (± SEM)**
Constituent 1	26.4 (1.0)	1168 (168)	14.8 (0.8)	118 (18)	26.3 (1.0)	1207 (177)
Constituent 2	8.7 (0.4)	27 (3)	8.3 (0.5)	24 (3)	18.1 (0.9)	271 (70)
10:1 mixture	25.5 (0.8)	947 (156)	16.2 (0.7	138 (20)	24.1 (1.5)	1007 (350)
3:1 mixture	24.0 (0.7)	672 (76)	17.7 (1.0)	208 (38)	19.1 (0.6)	246 (34)
1:1 mixture	21.6 (0.9)	445 (75)	16.6 (0.8)	157 (26)	18.6 (1.1)	266 (64)
1:3 mixture	19.3 (0.6)	256 (30)	12.8 (0.4)	62 (6)	16.7 (0.9)	169 (36)

Accepted manuscript

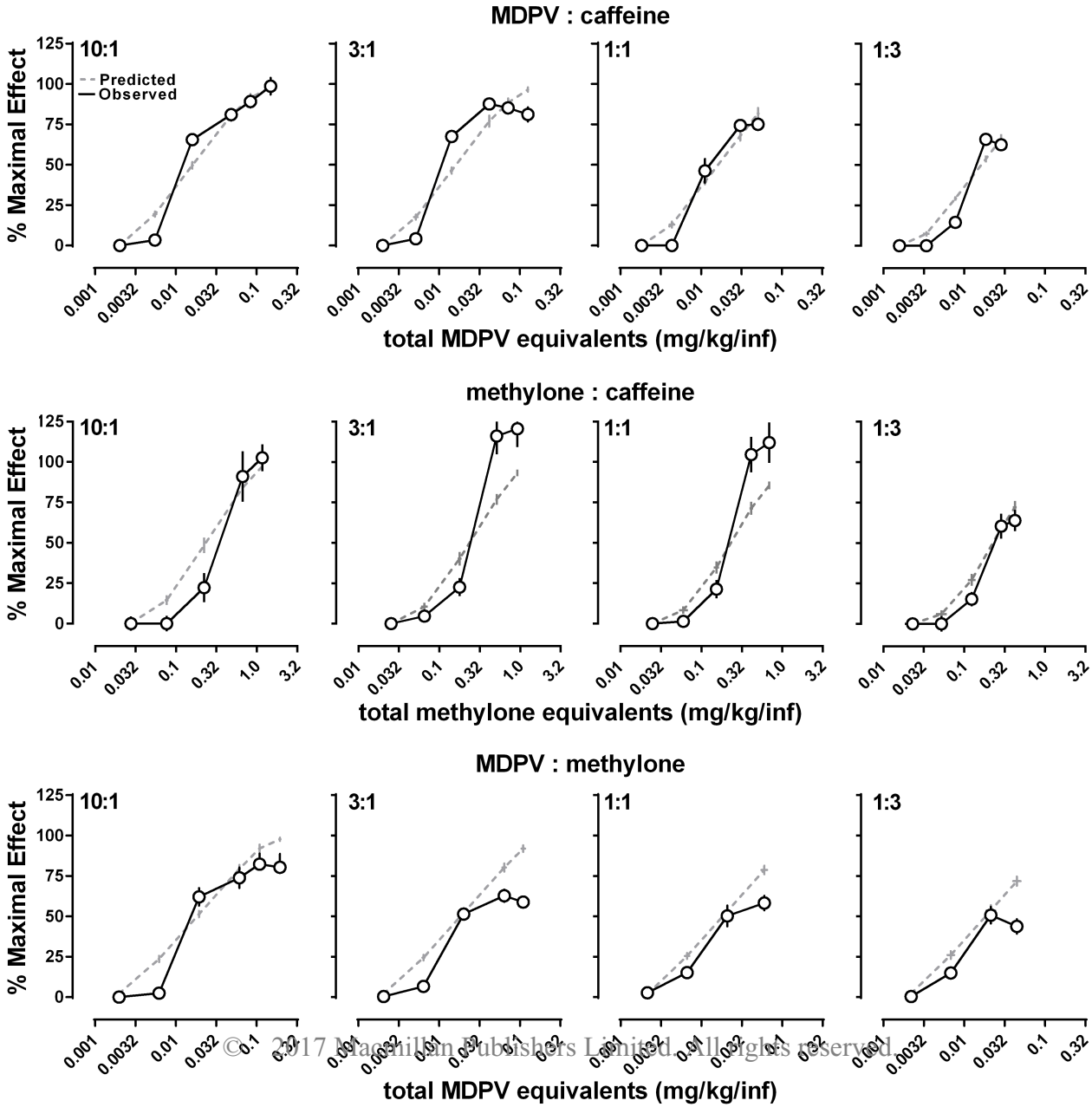
© 2017 Macmillan Publishers Limited. All rights reserved.

Figure 1:



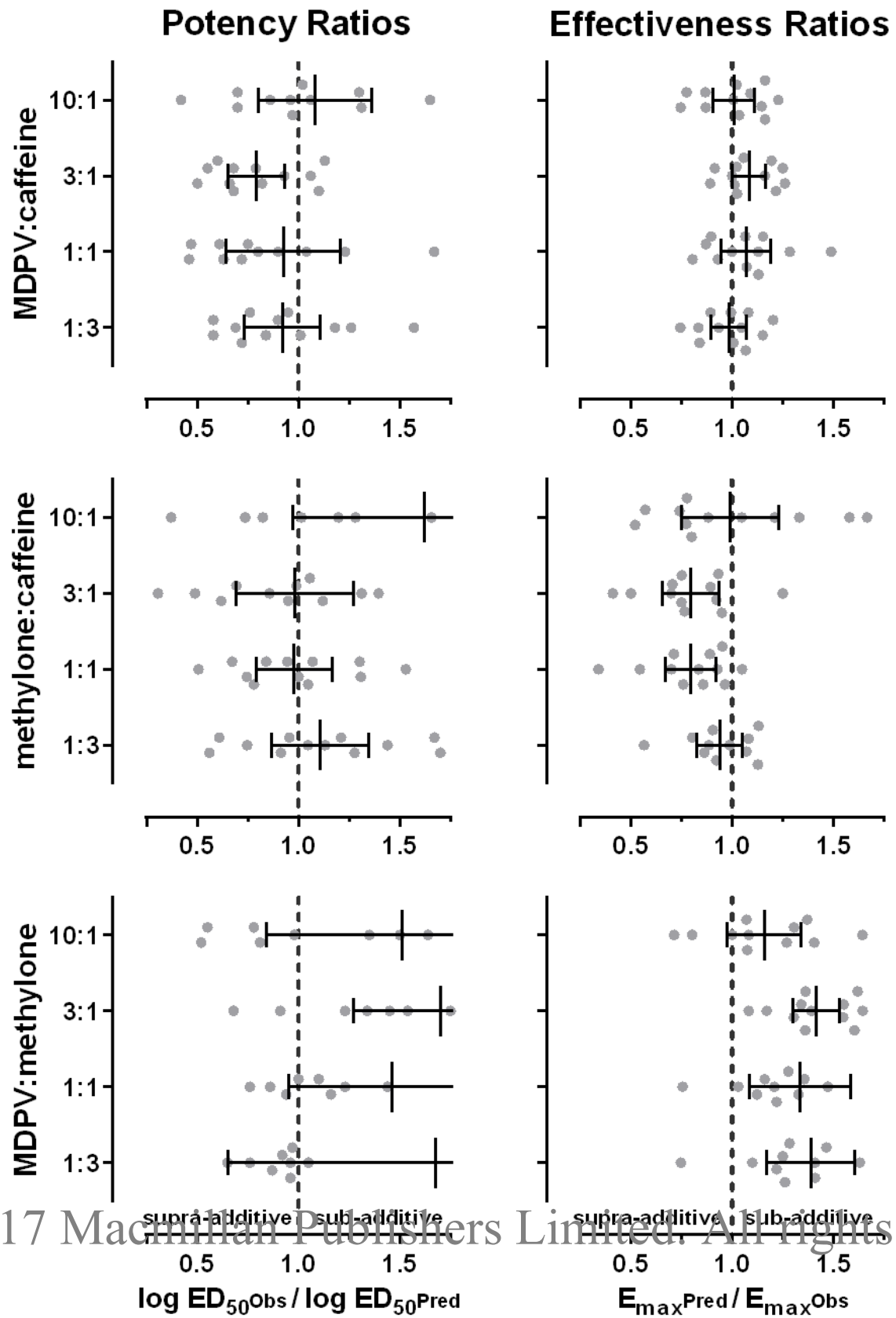
©2017 Macmillan Publishers Limited. All rights reserved.

Figure 2:



© 2017 Macmillan Publishers Limited. All rights reserved.

Figure 3:



2017 Macmillan Publishers Limited. All rights rese