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Effects of buprenorphine and methadone, two analgesics used for suppressing humans' addiction to morphine; a study using ants as biological models.

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ABSTRACT: *Using ants as biological models, we examined the effects of buprenorphine and methadone, two analgesics used for treating addicted persons, in order to precise their adverse effects and to see if they might replace morphine in some medical situations. Buprenorphine and methadone decreased the ants' speed of locomotion, increased their sinuosity, reduced their precision of reaction, response to pheromone, food consumption, 'audacity', brood caring and cognition, largely reduced the ants' tactile ('pain') perception and induced some aggressiveness between nestmates. Habituation to buprenorphine occurred for its effect on locomotion and precision of reaction, but not for its impact on tactile perception. Ants did not develop dependence on buprenorphine. This drug did not impact the ants' visual and olfactory conditioning and memory. The analgesic effect of buprenorphine persisted intact during four hours after its consumption ended, and vanished after ca 15 hours. Habituation to methadone occurred for the ants' locomotion, but not for their precision of reaction and their tactile perception. Ants developed no dependence on methadone. Under this drug consumption, the ants never acquire visual or olfactory conditioning. The analgesic effect of methadone quickly decreased during three to five hours, and then stayed at level for ca four hours, this level allowing absence of dependence. After that, its analgesic affect again decreased during two to three hours. The effect of methadone lasted so 10 – 13 hours. We conclude that, even if methadone could be used as an analgesic, buprenorphine should be preferred for such a use, and should be preferred to morphine, while methadone may be more efficient as a substitute of morphine or heroine as it induces effects similar to those of these drugs and suppress the exhilarating effect of heroine.*

KEY WORDS - *cognition, dependence, habituation, memory, tactile perception.*

I. INTRODUCTION

Analgesics are among the most important and the most drugs used in medicine. Morphine is one of them, probably the most commonly used, being often essential. On the other hand, persons dependent on morphine or heroine consumption can no longer efficiently live; they must be 'saved' by medicinal treatments. Two substances are used both as analgesics and to suppress humans' dependence on morphine: buprenorphine and methadone. According to practitioners, one or the other of these substances is used after some essays, at different amounts and with a variable efficiency. Some of their properties and characteristics are known and some of their ethological and physiological effects have been observed [1, 2]. However, not all of these effects are precisely known and the use of these substances as an analgesic and/or as a substitute to morphine or heroine remains not well documented.

Having previously studied the effects of morphine, we intended to do so for buprenorphine and methadone, using once more ants as biological models [3].

Since most biological processes are quite similar for all animals, including humans (i.e. genetics, metabolism, nervous cells functioning), a lot of invertebrates and vertebrates could be used as models for studying biological subjects [4, 5, 6]. Invertebrates are more and more used for this goal because they offer scientists many advantages, among others a short life cycle, a simple anatomy, and being available in large numbers [7, 8]. Some species are largely used as biological models, for instance, the flatworm *Dendrocelium lacteum*, the nematode worm *Caenorhabdotes elegans*, the mollusk *Aplysia californica*, the beetle *Tribolium castaneum*, the fruit fly *Drosophila melanogaster*, and the domestic bee *Apis mellifera*. Among the invertebrates, insects, especially social hymenoptera and among them, bees, are advantageously used as biological models (9, 10], but ants too can be used. Indeed, colonies containing thousands of ants can easily be maintained in laboratories, at low cost and very conveniently, throughout the entire year. Ants are among the most complex and social invertebrate animals as for their morphology, physiology, social organization and

behavior. They are among the most morphologically evolved hymenoptera, having indeed a unique resting position of their labium, mandibles and maxilla [11], as well as a lot of glands emitting numerous efficient pheromones [12]. Their societies are highly organized with a strong division of labor, an age-based polyethism and a social regulation [12]. Their behavior is well developed: they care for their brood, build sophisticated nests, chemically mark the inside of their nest, and, differently, their nest entrances, nest surroundings and foraging area [14]. They generally use an alarm signal, a trail pheromone, and a recruitment signal [14]; they are able to navigate using memorized visual and olfactory cues [15 and references therein]; they efficiently recruit nestmates where, when and as long as it is necessary [16], and, finally, they clean their nest and provide their area with cemeteries [17]. According to the complexity of their society and their behavior, it looks reasonable to use ants as biological models for studying physiological and ethological effects of neuronal active substances.

During many years, we worked on ant's species belonging to the genus *Myrmica*, and above all *Myrmica sabuleti* Meinert 1861. We know some of its ecological traits, eye morphology, visual perception, navigation system, visual and olfactory conditioning capabilities, and recruitment strategy [18, 19, 20, 21]. The ontogenesis of cognitive abilities of *Myrmica* species, including *M. sabuleti*, has also been approached [22, 23, 24, 25, 26]. Studies on the impact of age, activity and diet on the conditioning capability of the related species *M. ruginodis* [27] led to presume that ants could be good biological models. This was confirmed by the study of the effects of caffeine, theophylline, cocaine, and atropine [28], of nicotine [29], of morphine and quinine [3], of fluoxetine, an 'ISRS' antidepressant [30], as well as of anafranil, an 'ACT' antidepressant and of efexor, an 'IRSNa' antidepressant [31], and finally of carbamazepine [32], all of them on *M. sabuleti* as a model. Each time, we observed effects related to those observed on humans, and brought information and precision on them, of course on *M. sabuleti*, but clearly leading to presume similar effects for humans and other living organisms.

We thus here made a similar study as for morphine (same ant species, similar experimental protocols) for examining the physiological and the ethological effects of buprenorphine and methadone, aiming 1) to precise what would occur in ants and suggest which precautions should be taken when treating humans with these substances, 2) to precise possible differences between the effects of these two substances, 3) to compare these effects with those of morphine (in the case one or the other of these two substances may advantageously replace morphine in some treatments), 4) and finally, to help practitioners, pharmacists and veterinaries in choosing analgesics and drugs for treating pain and addicted persons as best as possible.

II. EXPERIMENTAL PLANNING

Eighteen behavioral and physiological traits were assessed on two colonies of *M. sabuleti* before and after they consumed buprenorphine, as well as on two other colonies of *M. sabuleti* before and after they consumed methadone. A fourth untreated colony was used to provide 'alien workers'. These traits had previously similarly been examined while studying effects of morphine on the same ant species [3].

1 – the speed of locomotion (and thus the general activity) through the ants' linear speed,
2 – the sinuosity of movement through the ants' angular speed,
3 – the precision of reaction through the orientation towards a source of their alarm pheromone,
4 – the response to pheromones through the trail following behavior,
5 – food consumption through the numbers of ants coming onto meat food,
6. – the "audacity" through the numbers of ants coming onto a test apparatus,
7 – the caring behavior through the behavior in front of larvae removed from the nest,
8 – cognition through the ability in performing a task requiring cognition (moving through twists and turns),
9 – the tactile sensation (or "pain" perception) through the ants' behavior on an uncomfortable substrate,
10 – the potential aggressiveness against nestmates through ants' behavior in the course of dyadic encountering,
11 – the expected aggressiveness against alien ants through ants' behavior in the course of dyadic encountering,
12 – the habituation to drug consumption through the speed and the sinuosity of movement, the orientation to an alarm signal and the behavior on an uncomfortable substrate, five or eight days after continuous drug consumption,
13 – the dependence on drug consumption through the numbers of ants choosing food containing the drug,
14 – the visual learning ability through the acquisition of visual conditioning,
15 – the visual memory through the duration of the remembering a learned visual cue,
16 – the olfactory learning ability through the acquisition of olfactory conditioning,
17 – the olfactory memory through the duration of the remembering a learned olfactory cue,
18 – the decrease of the effect of drug after its consumption ended (= after removing the drug from the ants' food), through the ants' behavior in an uncomfortable situation, in the course of time.

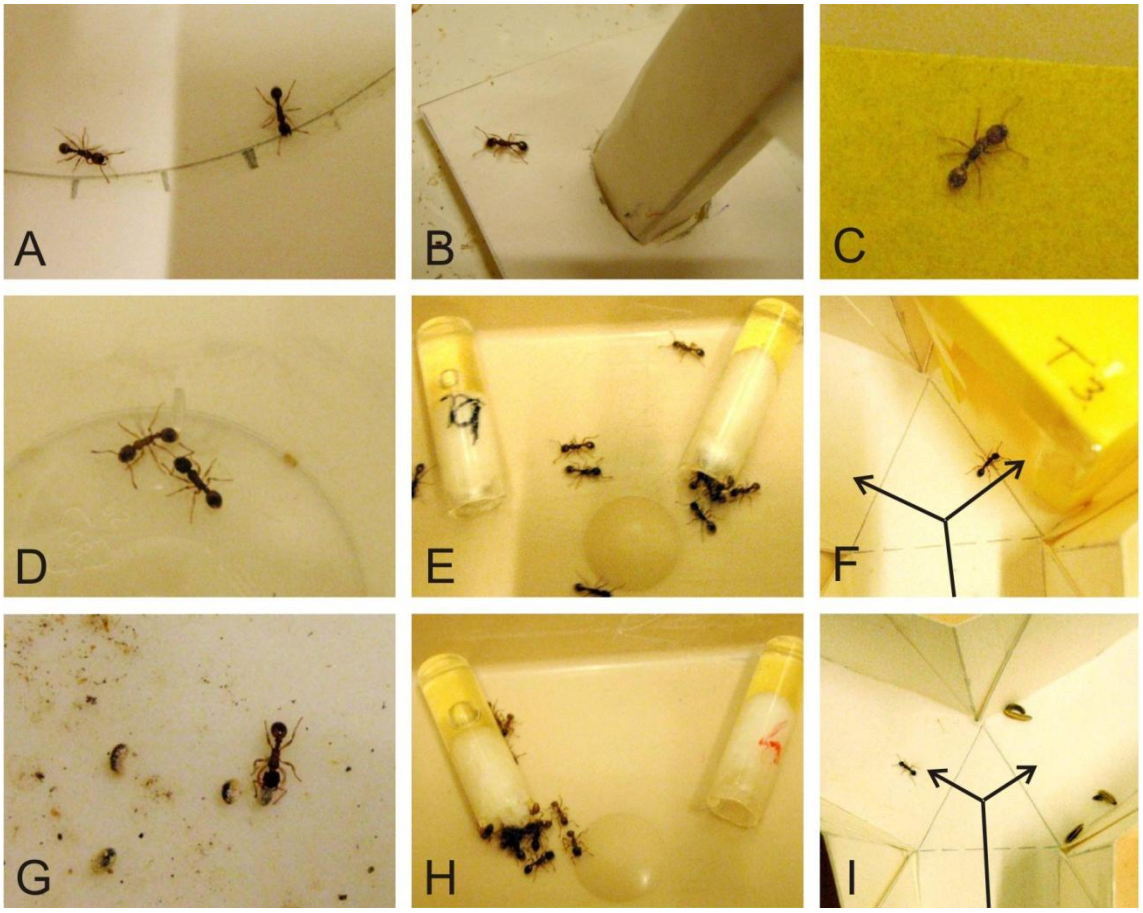

Figure 1. Some views of the experiments made with buprenorphine (A – B, D – F), methadone (H – I) or without these drugs (control: C, G). **A**: ants failing in correctly following a trail; **B**: an ant moving away from a risky apparatus; **C**: an ant under normal diet being reluctant to move on rough substrate; **D**: an ant under buprenorphine consumption presenting aggressive behavior against a nestmate; **E**: ants under buprenorphine consumption preferentially choosing liquid food without that drug (right tube); **F**: an ant under buprenorphine consumption giving, in a Y maze, the correct response i.e. choosing the way under a yellow hollow cube; **G**: an ant under normal diet taking care of a larva; **H**: ants under methadone consumption preferentially choosing liquid food free of that drug; **I**: an ant under methadone consumption and olfactory conditioning choosing, in a Y maze, the way without pieces of fennel. The arrows superposed on the Y apparatus indicate the entry branch and the choice.

III. MATERIAL AND METHODS

3.1, Collection and maintenance of the ants

The effects of buprenorphine were studied using two colonies of *M. sabuleti* collected in an abandoned slate quarry located in the Aise valley (Ardenne, Belgium). The study of the effects of methadone was made using two colonies of *M. sabuleti* collected at Audregnies, on an abandoned coal-mining heap (Terril de Ferrand, Hainaut, Belgium). A fifth colony, collected in an abandoned sandstone quarry at Treignes (Ardenne, Belgium), furnished the 'alien workers' required in one experiment. All the colonies were maintained in the laboratory in artificial nests made of one to three glass tubes half-filled with water, a cotton-plug separating the ants from the water. The glass tubes were deposited in trays (34 cm x 23 cm x 4 cm), which internal sides were slightly covered with talc to prevent the ants from escaping. These trays served as foraging areas, food being delivered in them. The ants were fed with sugar-water provided *ad libitum* in a small glass tube plugged with cotton, and with *Tenebrio molitor* larvae (Linnaeus 1758) provided twice a week on a glass slide. Temperature was maintained between 18°C and 22°C with a relative humidity of circa 80% all over the course of the study. Lighting had a constant intensity of 330 lux while caring for the ants, training and testing them. During other time periods, lighting was dimmed to 110 lux. The ambient electromagnetic field had an intensity of 2-3 $\mu W/m^2$. All the members of a colony are here named nestmates, as commonly done by researchers on social hymenoptera.

3.2, Acquisition of buprenorphine and methadone, realization of aqueous solutions for ants

Tablets of Temgesic containing 0.2 mg of buprenorphine hydrochloride, the active substance, produced by the manufacturer Reckitt Benckiser Healthcare Ltd (Dansom Lane, Hul HU8 7DS, UK), were provided by the pharmacist J. Cardon (1050 Brussels). The usual daily amount of that drug given to humans suffering from strong pain or morphine addiction varies between one tablet each six or eight hours, so at least 0.8 mg per day. The quantity of water humans ingest could be estimated at one liter per day. So, humans consuming buprenorphine commonly ingest about 0.8 mg of drug for one liter (1,000 ml) of ingested water. Insects consume about ten less water than mammals. It could be estimated that the most appropriate solution of buprenorphine to be given to ants for being in agreement with that commonly consumed by humans would be 0.8 mg of buprenorphine into 100 ml of water. Therefore, 0.2 mg of buprenorphine (one Temgesic tablet) were scratched and dissolved in 25 ml of a saturated solution of brown sugar, the ants' usual liquid food. The concentration in drug in the final solution was thus 0.2 mg in 25,000 mg of water, so 0.0008%. This solution was given to the ants, like their usual liquid food, in a small glass tube plugged with cotton. One hundred milligrams of methadone, produced by the manufacturer CERTA, were provided by the pharmacist J. Cardon (1050 Brussels). The product was provided as a white bright powder, at the highest level of purity possible. According to the amount of that drug given to humans suffering from pain perception or morphine addiction (= 60 mg per 24 hours), and to the quantity of water these humans drink, it could be established that the most appropriate solution of methadone to be given to the ants would be 60 mg into 100 ml of water. Therefore, 15 mg of methadone were weighted using a precision balance, and dissolved in 25 ml (= 25,000 mg of water) of a saturated solution of brown sugar, the ants' usual liquid food. The concentration in drug of the final solution was thus 15 mg in 25,000 mg of water, so 0.06%. Again, this solution was given to the ants, like their usual liquid food, in a small glass tube plugged with cotton.

The cotton of the liquid food tubes was refreshed each two days and the entire solution renewed each seven days. It was checked each day if ants actually consumed the given liquid food containing the drug.

3.3, Linear and angular speed (*1, 2*), orientation (*3*)

Ants' linear and angular speed was assessed for detecting excitement or sleepiness in the animals. Ants' orientation towards an isolated congener's head was assessed for examining the ants' precision of reaction. An isolated worker's head, with widely opened mandibles, is a source of alarm pheromone identical to that of an alarmed worker, in terms of the dimensions of the emitting source (the mandibular glands' opening) and of the quantity of pheromone emitted [33]. All the assessment was made on ants freely moving on their foraging area, each time on ants of two nests having never consumed buprenorphine or methadone, then on ants of these two nests having consumed the drug during one day. For each assessment, the movement of ten ants of each nest (n = 20 ants) was analyzed.

Trajectories were manually recorded using a water-proof marker pen, on a glass slide horizontally placed 3 cm above the experimental tray area, where the tested individuals were moving. A metronome set at 1 second was used as a timer for assessing the total time of each trajectory. Each trajectory was recorded until the ant reached the stimulus or walked for about 6 cm. All the trajectories were then copied with a water-proof marker pen onto transparent polyvinyl sheets. These sheets could then be affixed to a PC monitor screen and remained in place due to their own static electricity charge. The trajectories were then analyzed using specifically designed software [34], each trajectory being entered in the software by clicking as many points as wanted with the mouse (for instance, 20 points in a trajectory length of 5 cm) and by entering then the location of the presented worker's head. After that, the total time of the trajectory was entered, and the software was asked to calculate three variables defined as follows:

The linear speed (V) of an animal, which is the length of its trajectory divided by the time spent moving along this trajectory. It was here measured in mm/s.

The angular speed (S) (i.e. the sinuosity) of an animal's trajectory, which is the sum of the angles, measured at each successive point of the trajectory, made by each segment 'point i to point i – 1' and the following segment 'point i to point i + 1', divided by the length of the trajectory. This variable was here measured in angular degrees/cm.

The orientation (O) of an animal towards a given point (here a small blank piece of paper used as a control or an ant's head), which is the sum of the angles, measured at each successive point of the recorded trajectory, made by each segment 'point i of the trajectory - given point' and each segment 'point i - point i + 1', divided by the number of measured angles. This variable was here measured in angular degrees. When such a variable (O) equals 0°, the observed animal perfectly orients itself towards the given (source) point; when it equals 180°, the animal fully avoids the source point; when O is lower than 90°, the animal has a tendency to orient itself towards the source point and when it is larger than 90°, the animal has a tendency to avoid the source.

Each distribution of 20 variables was characterized by its median and quartiles (since being not Gaussian) and the distributions could be compared to one another using the non-parametric χ^2 test [35: 111-116]. Two distributions were considered as statistically different when $P < 0.05$.

3.4, Trail following behavior (*4*)

This behavior was assessed for examining the ants' response to pheromones. The trail pheromone of *Myrmica* ants is produced by the workers' poison gland. Ten of these glands were isolated in 0.5 ml (500µl) hexane and stored for 15 min at -25 °C. To perform one experiment, 0.05 ml (50µl) of the solution was deposited, using a metallic normograph pen, on a circle (R = 5 cm) pencil drawn on a piece of white paper and divided into 10 angular degrees arcs (= ang. deg.). One minute after being prepared, the piece of paper with the artificial trail was placed in the ants' foraging area. When an ant came into contact with the trail, its movement was observed (Fig. 1A). Its response was assessed by the number of arcs of 10 angular degrees it walked without departing from the trail, even if it turned back while walking on the trail. If an ant turned back when coming in front of the trail, its response was assessed as "zero arc walked"; when an ant crossed the trail without following it, its response equaled "one walked arc". Before testing the ants on a trail, they were observed on a "blank" circumference imbibed with 50µl of pure hexane, and the control numbers of walked arcs were so obtained (Table 1, C = control, T = test). On such experimental trails, *Myrmica* workers do not deposit their trail pheromone because they do so only after having found food or a new nest site. Each time, these handlings were made firstly on ants of two nests having never consumed buprenorphine or methadone, then on the same colonies having consumed one of this drug for four days. For each control and test experiment, 20 individuals of each two used colonies were observed (n = 40). Each distribution of values was characterized by its median and quartiles (since being not Gaussian). The distribution of values obtained for ants having consumed a drug were compared to the corresponding one obtained for ants having never consumed this drug, using the non parametric χ^2 test [35: 111-116].

3.5, Ants' food consumption (*5*)

Before the ants consumed buprenorphine or methadone, and after they had consumed one of these drugs for two days, the workers present on the meat food (pieces of *T. molitor* larva) at a time ants were fed were counted 10 times in the course of 10 min. The numbers obtained for the two kinds of food intake (with no drug, then with drug) were statistically compared using the Mann-Whitney U test [35: 128-137], and the mean as well as the extreme values of the recorded numbers were established.

3.6, Ants' "audacity" (*6*)

Before the ants consumed buprenorphine or methadone, and two days after they had consumed one of these drugs, a cylindrical tower built in strong white paper (Steinbach ®, height = 4 cm; diameter = 1.5 cm) was set on the ants' foraging area (Fig. 1B), and the ants present on it, at any place, were counted 10 times, in the course of 10 min. The mean and the extreme values of the obtained values were established each time and the two series of values were compared using the non parametric Mann-Whitney U test [35: 128-137].

3.7, Ants' brood caring behavior (*7*)

This trait was examined, for two nests, before the ants consumed buprenorphine or methadone, then two days after they had consumed one of these drugs. Each time, a few larvae were removed from the inside of the nest and deposited in front of the nest tube entrance. Five of them were carefully observed, as well as the ants' behavior in front of a larva (Fig. 1G). The numbers of the five observed larva still remaining out of the nest were counted after 0, 2, 4, 6, 8, 10 minutes, and the numbers recorded for each two colonies were added. The results obtained for ants consuming a drug were compared to those similarly obtained for the same ants having never consumed that drug using the non parametric Wilcoxon test [35: 87-95], the values of N, T and P being given in the results section.

3.8, An ants' cognitive ability requiring no memory (*8*)

This ability was assessed on ants of two nests first while these nests did not receive any drug, then five days after they had continuously either buprenorphine or methadone in their liquid food. The assessment was made using an adequate experimental apparatus schematically presented in the figure 3 of [29]. This apparatus consisted in a small tray (15 cm x 7 cm x 4.5 cm) inside of which pieces of white extra strong paper (Steinbach ®, 12 cm x 4.5 cm) were inserted in order to create a way with twists and turns between a loggia too narrow for 15 ants at a time (the initial loggia) and a larger one (the free loggia). Two such experimental apparatus were built and used, each one, for one of the two nests. Each time, for each nest and each feeding situation, 15 ants were collected from their colony and set all together, at the same time, in the initial loggia of the apparatus, and those located in this loggia as well as in the free loggia were counted after 0, 4, 8, 12, 16 and 20 min (Table 2,

cognition). The numbers obtained for ants consuming a drug were statistically compared to those previously obtained for these ants having never received that drug, using the non parametric Wilcoxon test [35: 87-95], the values of N, T, and P being given in the results section.

3.9, Ants' tactile perception (presumed 'pain sensation') (*9*)

This trait was assessed on ants of two nests first before they consumed buprenorphine or methadone, then five days after they had continuously received one of these drugs with their sugar food. The assessment was made by setting ants in an experimental apparatus made of a small tray (15 cm x 7 cm x 4.5 cm) into which a piece (3 cm x 11 cm) of rough emery paper (number 280) was duly folded (11 cm: 2 cm + 7 cm + 2 cm) and tied to the bottom and the borders of the tray, so dividing the tray in three zones: a small initial smooth zone (3 cm long, a too small space for 12 ants at a time), a 3 cm long zone on which ants' walking should be uncomfortable, and a large smooth zone (9 cm long). Two such apparatus were used, one for each of the two nests used. Each time, 12 ants were set, all together, at the same time, in the small initial zone. The ants present in each of the three zones of the apparatus were counted after 0, 2, 4, 6, 8, 10 min, and the linear as well as the angular speed of 12 ants' trajectories (so n = 24) made on the rough paper were assessed using the method briefly explained above (Fig. 1C; Table 2, pain perception). The numbers of ants obtained for ants having consumed a given drug were statistically compared to those previously obtained for these ants having never ingested that drug, using the non parametric Wilcoxon test [35: 87-95], the values of N, T and P being given in the results section, while the linear and angular speed of such ants were statistically compared using the non parametric χ^2 test [35: 111-116].

3.10, Ants' aggressiveness towards congeners or alien workers (*10, 11*)

This trait was quantified before the ants of two nests consumed buprenorphine or methadone, then six days after they had continuously consumed one of these drugs. Ants' aggressiveness towards nestmates as well as towards alien workers (i.e. belonging to another colony) was assessed in the course of dyadic encounters of five ants of each of the two used colonies, the encountering being conducted in a small glass (base diameter = 3 cm, top diameter = 4 cm, height = 5 cm), the borders of which had been slightly covered with talc to prevent escape. In total ten encounters with nestmates and ten encounters with alien workers were observed, each time before, and under a given drug consumption. Each time, one ant of a tested colony was observed for 3 minutes and its encounters with another ant were characterized by the numbers of times it did nothing (level 0 of aggressiveness), touched the other ant with its antennae (level 1), opened its mandibles in front of the other ant (level 2; Fig. 1D), gripped and/or pulled the other ant (level 3), and tried to sting or stung the other ant (level 4). The numbers recorded for each of the two used colonies were added (Table 2, aggressiveness), and the results obtained for ants consuming a drug were compared to those obtained for these ants having never consumed that drug, using the non parametric χ^2 test [35].

3.11, Ants' habituation to the drug consumption (*12*)

Five days after the ants of two nests had continuously consumed buprenorphine or methadone, their linear and angular speeds, as well as their orientation to an isolated worker's head, were assessed. Three days later, the ants' orientation to an alarm signal for buprenorphine only, as well as their linear and angular speed on a rough substrate were quantified (Table 3, habituation). The results were compared to those obtained before giving the drug to the ants, and after the ants had consumed the drug for one day (concerning their locomotion on the foraging area) and five days (concerning their locomotion on a rough substrate), using the non parametric χ^2 test [35].

3.12, Ants' dependence on drug consumption (*13*)

After the ants of two nests had continuously consumed buprenorphine or methadone for seven days, an experiment was performed for examining if they acquired some dependence on the consumed drug. Fifteen ants of each two used colonies were transferred into a small tray (15 cm × 7 cm × 5 cm), the borders of which had been covered with talc to prevent escape, and in which two tubes (h = 2.5cm, diam. = 0.5 cm) were laid, one containing sugar water, the other a solution of sugar water and either buprenorphine or methadone (the same solutions as those used in the course of the whole experimental work), each tube being plugged with cotton. In one of the trays, the tube containing the drug was located on the right; in the other tray, it was located on the left (Fig. 1E, H). The ants drinking each liquid food were counted 12 times in 15 min, the mean values being then established for each kind of food (Table 3, dependence). They were statistically compared to the values expected if ants randomly went drinking each kind of food, using the non parametric goodness of fit χ^2 test [35].

3.13, Ants' visual and olfactory conditioning and memory (*14, 15, 16, 17*)

Briefly, at a given time, either a yellow hollow cube (after 11 days of buprenorphine or methadone consumption) or pieces of fennel (after 18 days of one of these drug consumption) were set above pieces of *T. molitor* larvae given as food, and tied to the supporting piece of glass. The ants of two nests so underwent each time either visual or olfactory operant conditioning. Tests were then performed in the course of time, while the ants were expected acquiring conditioning then, after having removed the yellow cube or the pieces of fennel, while the ants were expected to partly lose their conditioning.

In detail, ants were collectively visually trained to a hollow yellow cube constructed of strong paper (Canson ®) according to the instructions given in [36] and set over the meat food which served as a reward. The color has been analyzed to determine its wavelengths reflection [37]. Only the ceiling of the cube was filled, this allowing ants entering the cube. Choosing the yellow cube was considered as giving the 'correct' choice when ants were tested as explained below. The ants were olfactory conditioned by setting pieces of fennel aside the tied pieces of *T. molitor* larva. Choosing the pieces of fennel was considered as giving the 'correct' choice when ants were tested as explained below.

Ants were individually tested in a Y-shaped apparatus (Fig. 1F, I) constructed of strong white paper according to the instructions given in [21], and set in a small tray (30 cm x 15 cm x 4 cm), apart from the experimental colony's tray. Each colony had its own testing device. The apparatus had its own bottom and its sides were slightly covered with talc to prevent the ants from escaping. In the Y-apparatus, the ants deposited no trail since they were not rewarded. However, they could utilize other chemical secretions as traces. As a precaution, the floor of each Y-apparatus was changed between tests. The Y-apparatus was provided with either a yellow cube, or pieces of fennel, in one or the other branch. Half of the tests were conducted with the cube or the odorous plant in the left branch and the other half with the cube or the odorous plant in the right branch of the Y maze, and this was randomly chosen. Control experiments had previously been made on never conditioned ants and on trained ants of colonies having never received the examined drugs (Table 4: * from [21]; ** from [29]). This had to be done because, once an animal is conditioned to a given stimulus, it becomes no longer naïve for such an experiment. It was thus impossible to perform, on the same ants, conditioning first without, and later on, with a drug in the ants' food. The only solution was to use previous results obtained in the course of identical experiments made on very similar colonies never fed with any drug.

To conduct a test on a colony, 10 workers - randomly chosen from the workers of that colony - were transferred one by one onto the area at the entrance of the Y-apparatus. Each transferred ant was observed until it turned either to the left or to the right in the Y-tube, and its choice was recorded. Only the first choice of the ant was recorded and this only when the ant was entirely under the cube, i.e. beyond a pencil drawn thin line indicating the entrance of a branch (Figure 1F, I). Afterwards, the ant was removed and transferred into a polyacetate cup, in which the border was covered with talc, until 10 ants were so tested, this avoiding testing twice the same ant. All the tested ants were then placed back on their foraging area. For each experiment, the numbers of ants belonging to the two used nests (so among n = 10 + 10 = 20 ants) which turned towards the "correct" yellow cube or pieces of fennel, or went to the "wrong" empty branch of the Y were recorded. The percentage of correct responses for the tested ant population was so established (Table 4). The results obtained for ants consuming a drug were compared to previous results obtained for ants that had never consumed that substance, using the non parametric Wilcoxon test [35]. They were also similarly compared to results previously obtained on ants consuming morphine [30]. The values of N, T, and P, according to the nomenclature given in the here above reference, are given in the results section.

3.14, Decrease of the effects of the drugs, after the end of their consumption (*18*)

Eighteen days after the ants had continuously consumed either buprenorphine or methadone, the liquid food containing the drug was removed from the ants' tray and replaced by sugar water free of any drug. This change was made at a given recorded time. After that, the ants' linear and angular speeds on a rough substrate were assessed after successive given time periods (Fig. 2). The results revealed the decrease of the effects of the drug on the ants' tactile perception. Their statistical significance could be estimated via the non parametric χ^2 test [35]. The variables 'linear and angular speeds on the foraging area, and the variable orientation to an alarm signal' were not used contrary to what was made in previous studies because, in the present case, a quick habituation appeared for these three or two variables to buprenorphine or methadone respectively. Curvilinear fitting of the decrease was done following a stepwise polynomial regression forward selection procedure described in Zar [38: 452-457].

IV. RESULTS

4.1, Effects of buprenorphine on eighteen physiological and ethological ants' traits

4.1.1, Locomotion (*1, 2*)

This trait was affected by buprenorphine consumption (values after one day: Table 1, buprenorphine, linear speed and sinuosity). Compared to usual behavior, the ants clearly moved more slowly ($\chi^2 = 19.67$, df = 2, $P < 0.001$), and more sinuously ($\chi^2 = 23.01$, df = 1, $P < 0.001$). Such an impact appeared as soon as the ants had drunk some sugar water containing the drug, and tried to return to their nest entrance. They moved with difficulty, slowly, going to their left and to their right, stopping, and then moving again very slowly. However, after a few days, such an unusual locomotion was less exhibited (see below, habituation to the drug consumption).

4.1.2, Precision of reaction (*3*)

Buprenorphine affected this trait as soon as after one day of consumption (Table 1, buprenorphine, orientation). In the presence of a worker's isolated head (= a source of alarm pheromone), the ants having consumed the drug since one day presented some deficiency in correctly orienting themselves towards the source of pheromone, this result being statistically significant ($\chi^2 = 9.17$, df = 3, $P \approx 0.01$). It was observed that the ants obviously perceived the alarm pheromone, but walked rather sinuously towards it, or failed coming onto the source, or even moved beyond it.

4.1.3, Response to pheromone (*4*)

The ants' trail following behavior was largely affected by buprenorphine consumption (Table 1, buprenorphine, trail following, T; Fig. 1A). Ants under normal diet correctly followed a circular trail (one poison gland extract per circumference R = 5 cm) while those consuming buprenorphine since four days presented difficulties in doing so, simply crossing the trail or following it only along a few arcs. Such an impact of the drug was obvious to the observer, and highly significant ($\chi^2 = 42.16$, df = 3, $P < 0.001$).

4.1.4, Food consumption (*5*)

This trait appeared to be affected by buprenorphine consumption. By comparison with what occurred under normal diet, after two days of this drug consumption the ants meanly counted on the meat food site were seven times less numerous (Table 1, buprenorphine, food consumption), a difference highly significant ($U = 20$, $Z = 5.16$, $P < 10^{-6}$).

4.1.5, "Audacity" (*6*)

Buprenorphine somewhat affected the ants' tendency to come onto a risky apparatus (Fig. 1B). Compared to the numbers of ants counted for colonies consuming no buprenorphine, for those having consumed the drug for two days, only half of the ants were counted on the apparatus (Table 1, buprenorphine, 'audacity'). Such an impact of the drug was statistically significant ($U = 116$, $Z = 2.43$, $P = 0.015$).

4.1.6, Brood caring (*7*)

This trait was only slightly affected by buprenorphine consumption. Under normal diet, all the ten larvae removed from their nest were re-entered within 10 min, while after having consumed the drug for two days, two of the larvae were not re-entered within the same time period (Table 2, buprenorphine, brood caring). Such a slight difference was however significant ($N = 5$, $T = 15$, $P = 0.031$) and might be due to the impact of buprenorphine on the ants' locomotion and precision of reaction.

4.1.7, A cognitive ability requiring no memory (*8*)

Such ability appeared to be affected by buprenorphine consumption. Under normal diet, two ants among 30 succeeded in crossing the twists and turns of the experimental apparatus, and only nine ants were still present in the initial loggia. After having consumed buprenorphine for five days, no any ant reached the free loggia beyond the twists and turns, and 23 ones were still present in the initial loggia (Table 2, buprenorphine, cognition). This is in agreement with the decrease of the ants' audacity under buprenorphine consumption. The obtained results were significant (initial loggia: $N = 6$, $T = 21$, $P = 0.016$; free loggia: $N = 4$, $T = 10$, $P = 0.063$).

4.1.8, Tactile ("pain") perception (*9*)

To the observer, this trait was obviously and largely affected by buprenorphine consumption (Table 2, buprenorphine, pain perception). Under normal diet, ants were reluctant in moving on the rough bottom separating the initial small zone and the large free one, moving very cautiously, with difficulty, and slowly progressing on the rough substrate (Fig. 1C). Their linear speed was lower ($\chi^2 = 44$, df = 3, $P < 0.001$) and their sinuosity larger ($\chi^2 = 39.54$, df = 1, $P < 0.001$) than on a normal substrate. After five days of buprenorphine

consumption, the ants were obviously less reluctant in moving on the rough substrate: they nearly immediately moved on it towards the large zone. However, due to their reduced audacity and speed of locomotion, they were not very numerous in reaching the large zone within the experimental time period. Therefore, the numerical results did not statistically differ from the control ones (initial zone: N = 6, T = 15, P = 0.219; rough zone: N = 5, T = 10, P = 0.313; large zone: N = 3, NS). But the ants' locomotion on the rough substrate clearly revealed the impact of buprenorphine on the ants' tactile perception. Indeed, the ants moved more quickly ($\chi^2 = 34$, df = 2, $P < 0.001$) and less sinuosity ($\chi^2 = 28.65$, df = 2, $P < 0.001$) than before consuming the drug. Also, by comparison with their locomotion on a normal substrate, the ants moved on a rough bottom at a similar speed ($\chi^2 = 0.79$, df = 2, NS) and less sinuously ($\chi^2 = 14.28$, df = 3, $P < 0.001$). This proved that, under buprenorphine, the ants less perceived the uncomfortable character of the rough substrate.

4.1.9, Aggressiveness towards congeners (*10*) and aliens (*11*)

Buprenorphine slightly affected these traits (Table 2, buprenorphine, aggressiveness). Under normal diet, the ants presented no aggressive behavior towards nestmates, but a strong one towards aliens (for instance: 71 *vs* zero encounters showing no sign of aggressiveness towards nestmates, and zero *vs* 83 + 38 encounters showing strong aggressiveness towards aliens). After having consumed buprenorphine for six days, the ants exhibited some aggressiveness towards congeners (Fig. 1D). For instance, by comparison with what occurred under normal diet, 29 *vs* 71 encounters occurred with no aggressive behavior, and 46 + 2 *vs* 13 ones occurred with aggressive behavior ($\chi^2 = 38.15$, df = 1, $P < 0.001$). On the other hand, the ants consuming buprenorphine appeared to be a little less aggressive in front of aliens: they presented more encounters with no aggressive behavior (5 + 44 *vs* 23) and less ones with strong aggressiveness (59 + 20 *vs* 83 + 38). These differences were statistically significant ($\chi^2 = 16.99$, df = 1, $P < 0.001$).

4.1.10, Habituation (*12*)

Ants' habituation to buprenorphine consumption was assessed five as well as eight days after the ants had continuously the drug in their liquid food. The results depended on the examined physiological trait (Table 3, buprenorphine, habituation).

After five days of buprenorphine consumption, the ants' linear speed was no longer low like after one day of that drug consumption ($\chi^2 = 19.6$, df = 1, $P < 0.001$) but again very similar to the control one ($\chi^2 = 4.5$, df = 2, NS). In the same way, the ants' angular speed after five days of buprenorphine consumption was no longer high like after one day of such a drug consumption ($\chi^2 = 16.42$, df = 2, $P < 0.001$), but again similar to the control one ($\chi^2 = 2.07$, df = 2, NS). Such a habituation to the drug as for the individuals' locomotion was obvious while looking to their moving on the foraging area. It was not exactly the same for the ants' precision of reaction. After five days of buprenorphine consumption, the ants' orientation towards an alarm signal was still similar to that observed after one day ($\chi^2 = 4.44$, df = 2, NS) and not different from the control value ($\chi^2 = 1.10$, df = 2, NS). The ants were thus in the process of habituation. Indeed, after eight days of buprenorphine consumption, the ants' orientation towards an alarm signal was far better than after one day ($\chi^2 = 9.5$, df = 2, $P < 0.01$) and statistically similar to the control one ($\chi^2 = 2.65$, df = 2, NS).

More important was to assess the ants' potential habituation to buprenorphine consumption as for their tactile perception (presumed to be "pain" perception) (Table 3, buprenorphine, habituation). After eight days of drug consumption, the number of ants counted in the course of time in the three zones of the appropriate apparatus (small, rough and large zone) still somewhat differed from the control ones (N = 5, T = 15, P = 0.031; N = 5, T = 10, P = 0.0313; N = 4, T = 10, P = 0.063 respectively) and were similar to those obtained after five days of buprenorphine consumption (N = 5, T = 15, P = 0.031; N = 6, T = 13, P = 0.344; N = 4, T = 10, P = 0.063 respectively). Also, after eight days of drug consumption, the ants' linear speed on the rough substrate was still as large as, and even a little larger than after five days ($\chi^2 = 8.48$, df = 3, $P < 0.02$) while the ants' sinuosity was as low as that observed after five days ($\chi^2 = 0.89$, df = 2, NS). In other words, after eight days of buprenorphine consumption, the ants went on moving frankly on an uncomfortable bottom, and not with reluctance as it was the case before consuming that drug, having so their tactile ("pain") perception still reduced by the drug. Such a result is in favor of a long lasting analgesic effect of the drug contrary to what was observed with morphine [3].

4.1.11, Dependence (*13*)

This physiological trait was assessed after the ants had continuously consumed buprenorphine for seven days (Table 3, buprenorphine, dependence; Fig. 1E). Very few dependence was observed. Indeed, in total (for the two used colonies), 71 ants were counted on the food containing buprenorphine while 53 ones were counted on the food free of that drug. This corresponded to 57.3% of the ants choosing food containing buprenorphine and 42.7% of the ants choosing food free of the drug. This result (71 *vs* 53) was not statistically different from

that expected if ants made their choice randomly (62 *vs* 62) ($\chi^2 = 2.60$, df = 1, NS). The individuals' addiction on buprenorphine was thus found to be very low and not significant.

4.1.12, Visual and olfactory conditioning ability and memory (*14, 15, 16, 17*)

These traits, assessed 11 days (visual conditioning and memory) and 18 days (olfactory conditioning and memory) after the ants continuously consumed buprenorphine, were unexpectedly not affected by that drug consumption (Table 4, columns 'control' and '+ buprenorphine').

The ants consuming buprenorphine progressively reached a visual conditioning score of 70% just like as under normal diet (= control), presenting only some longer latency in doing so. The difference between the ants' successive conditioning score's under the two different diets was not significant (Table 4, visual learning, N = 3, T = 4.5, NS). After removal of the visual cue (a hollow yellow cube), the ants went on giving the correct response with a score of at least 70% even after 72 hrs. This behavior was similar to that presented under normal diet. It even revealed a stronger visual memory (in agreement with the slightly longer latency period), but this difference was not significant (Table 4, visual memory, N = 3, T = 6, NS).

The ants consuming buprenorphine progressively reached an olfactory score of 90% to 75%, just like as under normal diet (80%), the difference between the successive conditioning scores they presented under the two different diets being not significant (Table 4, olfactory learning, N = 6, T = 13.5, $P \approx 0.30$). Note that all the conditioning scores obtained during the evenings (65%, 75% and 90%) were higher than all those obtained during the days (60%, 70% and 75%). After removal of the olfactory cue (pieces of fennel), the ants went on correctly responding to the cue, with lower and lower scores, as they did when being under normal diet, but they presented higher scores during four of the six performed tests. The difference between the successive conditioning scores the ants presented in the course of time under the two kinds of diet (normal and with buprenorphine) was at the limit of significance (Table 4, olfactory memory, N =: 4, T = 10, P = 0.063). Once more, during their loss of conditioning, the ants presented higher scores during the evenings (80%, 65% and 65%) and lower ones during the days (70%, 55% and 55%). Considering the six assessments made during the ants' acquisition of conditioning and the six ones made during their loss of conditioning, we obtained a significant difference between the ants' responses during the evening *vs* during the day (N = 6, T = 21, P = 0.016). This is in agreement with a larger use of olfaction during the evening by the ant *M. sabuleti* [21].

We should recall that under morphine consumption, the ants could never acquire any visual and olfactory conditioning, presenting thus no visual and olfactory memory [3], a fact related again in the discussion section.

4.1.13, Decrease of the effects after end of consumption (*18*)

The ants' linear and angular speeds on a rough bottom were assessed just before replacing their food containing buprenorphine by food free of drug, and after that, in the course of hours following this replacement. During the first four hours, the ants went on presenting, on a rough bottom, a rather high linear speed and a low angular speed, just as when under the impact of buprenorphine. There was thus a latency period before the decrease of the drug effect, which is best shown when drawing straight lines between the plotted points (Fig. 2, the latency period being encircled). After this four hours lasting latency period, the ants' movement on a rough bottom began to change, the linear speed decreasing and the angular speed increasing, each two according to a sigmoid curve (Fig. 2). Although expressing not such details, due to the fact that only 8 points were plotted in the course of time, the linear speed decrease and angular speed increase can be fitted by polynomial curves with a maximum power corresponding to a statistical significance at the $\alpha = 0.05$ level. These curves are $y = 10.52 - 0.14x - 0.034x^2 - 0.0012x^3$ (y measured in $mm.sec^{-1}$ and x in hours) for linear speed decrease and $y = 108.35 + 11.42x - 0.19x^2$ (y measured in angular $degrees.sec^{-1}$ and x in hours) for angular speed increase.

It lasted 13 hours for the ants' linear speed to be only slightly different from the control speed ($\chi^2 = 9.07$, df = 3, $0.02 < P < 0.05$), while at this time the angular speed still remained lower than the control one ($\chi^2 = 14.11$, df = 2, $P < 0.001$). After 16 hours of drug depriva, the linear and angular speed values no longer statistically differed from the control ones. So, on basis of the ants' linear and angular speeds on a rough bottom (= tactile perception), the effect of buprenorphine is expected to vanish about 15 hours after the end of this drug consumption. The effect of buprenorphine was thus longer lasting than that of morphine [3] and moreover presented an initial latency period before decreasing.

Table 1. Effects of buprenorphine and methadone on six ethological traits. Experimental details are given in the text. Briefly, buprenorphine, and methadone even more, decreased the ants' linear speed, increased their sinuosity, decreased their precision of reaction, trail following behavior (C: no pheromone; T: + pheromone), food consumption, and "audacity". P: level of probability; NS: no significant difference at P = 0.05.

Examined traits	No drug consumed	Under drug consumption	Statistics
buprenorphine			
Linear speed (mm/sec)	14.8 (13.6 - 16.3)	8.8 (8.2 - 11.4)	$P < 0.001$
Sinuosity (ang.deg./cm)	111 (106 - 116)	182 (157 - 195)	$P < 0.001$
Orientation to alarm pheromone (ang. deg.)	42.2 (31.9 - 58.0)	64.6 (48.6 - 85.5)	$P \approx 0.01$
Trail following (n^o of arcs walked)	C: 1.0 (1.0 - 2.0) T: 11.0 (7.8 - 18.0)	C: 1.0 (0.0 - 1.0) T: 3.0 (1.0 - 5.3)	NS $P < 0.001$
Food consumption	1.85 (0 - 3)	0.25 (0 - 1)	$P < 1\ 10^{-6}$
"Audacity"	2.15 (1 - 6)	1.10 (0 - 2)	$P = 0.015$
methadone			
Linear speed (mm/sec)	14.1 (13.2 – 15.7)	7.9 (7.1 – 8.9)	$P < 0.001$
Sinuosity (ang.deg./cm)	134 (125 – 155)	227 (207 – 245)	$P < 0.001$
Orientation to alarm pheromone (ang. deg.)	40.4 (35.8 – 51.2)	74.1 (63.9 – 89.3)	$P < 0.001$
Trail following (n^o of arcs walked)	C: 1.0 (1.0 – 2.0) T: 12.0 (8.0-16.0)	C: 1.0 (1.0 – 1.0) T: 1.0 (1.0 – 2.0)	NS $P < 0.001$
Food consumption	1.4 (1 – 2)	0.3 (0 – 1)	$P = 2.10^{-6}$
"Audacity"	1.7 (1 – 3)	0.45 (0 – 1)	$P = 1.10^{-6}$

Table 2. Effects of buprenorphine and methadone on four ethological and physiological traits. Experimental and statistical information are given in the text. Briefly, these two drugs somewhat affected the ants' brood caring, cognition and usual aggressive behavior, and largely reduced their tactile ("pain") perception.

Examined trait		Control (no drug)			**+ buprenorphine**		
Brood caring	time:	0 2 4 6 8 10 min			0 2 4 6 8 10 min		
	N° of larvae not re-entered:	10 9 6 4 2 0			10 10 9 6 4 2		
Cognition	N^o of ants in the:	initial loggia		free loggia	initial loggia		free loggia
	after 0 min	29		0	30		0
	4 min	23		0	30		0
The not counted ants are in the twists and turns	8 min	16		1	27		0
	12 min	14		1	24		0
	16 min	11		2	25		0
	20 min	9		2	23		0
Tactile perception	N°of ants in the:	small	rough	large zone	small	rough	large zone
after 5 days	after 0 min	24	0	0	22	2	0
	2 min	21	3	0	18	6	0
	4 min	22	2	0	18	4	2
	6 min	21	2	1	17	6	1
	8 min	16	6	2	17	3	4
	10 min	18	5	1	16	5	3
in the rough zone:	linear speed	5.17 (3.9 – 6.5)			9.2 (7.9 – 10.2)		
	angular speed	282 (248 – 312)			139 (116 – 151)		
Aggressiveness	levels:	*vs* congeners		*vs* aliens	*vs* congeners		*vs* aliens
	0	71		0	29		5
	1	66		23	68		44
	2	13		43	46		65
	3	0		83	2		59
	4	0		38	0		20

Table 2 continues on following page

Examined trait		Control (no drug)			**+ methadone**		
Brood caring	time:	0 2 4 6 8 10 min			0 2 4 6 8 10 min		
N° of larvae not re-entered:		10 8 5 5 3 2			10 8 7 7 7 6		
Cognition	N° of ants in the:	initial loggia		free loggia	initial loggia		free loggia
	after 0 min	29		0	29		0
The not counted ants are in the twists and turns	4 min	19		0	21		1
	8 min	15		2	19		1
	12 min	14		3	17		1
	16 min	12		6	14		1
	20 min	11		6	14		2
Tactile perception	N°of ants in the:	small	rough	large zone	small	rough	large zone
after 5 days	after 0 min	24	0	0	21	3	0
	2 min	22	2	0	17	5	2
	4 min	21	2	1	15	4	5
	6 min	20	3	1	13	4	7
	8 min	20	4	0	12	4	8
	10 min	20	3	1	11	4	9
in the rough zone:	linear speed	4.5 (3.7 – 4.9)			8.3 (7.7 – 8.8)		
	angular speed	284 (261 – 321)			170 (145 – 200)		
Aggressiveness	levels:	*vs* congeners		*vs* aliens	*vs* congeners		*vs* aliens
	0	47		5	18		7
	1	34		33	46		14
	2	13		59	33		41
	3	0		46	0		13
	4	0		33	0		5

Table 3. Habituation to, and dependence on, buprenorphine and methadone consumption. Information about the experimental protocols and the statistical analysis are given in the 'Material and Methods' and 'Results' sections. Briefly, ants presented quick habituation to buprenorphine as for their locomotion, a rather quick one as for their precision of reaction, and no habituation as for their tactile ("pain") perception. They developed no dependence on buprenorphine. They presented habituation to methadone as for their locomotion, but not as for their precision of reaction and tactile perception. They developed not dependence on methadone.

Examined traits	Variables assessed	Results		
buprenorphine				
Habituation				
locomotion after 5 days	linear speed (mm/sec)	14.5 (12.7 – 15.7)		
	angular speed (ang.deg./cm)	120 (95 – 135)		
precision of reaction after 5 days	orientation to an alarm signal	45.5 (37.2 – 65.1)		
after 8 days	(ang. deg. = °)	38.7 (29.8 – 64.6)		
tactile perception after 8 days	n°of ants in the three zones	small	rough	large zone
	after 0 min	24	0	0
	2 min	16	8	0
	4 min	15	7	2
	6 min	13	8	3
	8 min	13	5	6
	10 min	12	7	5
in the rough zone:	linear speed (mm/sec)	10.4 (9.5 – 12.1)		
	angular speed (ang.deg./cm)	134 (121 – 152)		
Dependence nest 1	n° and % of ants choosing food +	17 *vs* 39 = 30.4% *vs* 69.6%		
nest 2	buprenorphine *vs* food free of the	54 *vs* 14 = 79.4% *vs* 20.6%		
pool	drug.	71 *vs* 53 = 57.3% *vs* 42.7%		

Table 3 continued		**methadone**			
Habituation					
locomotion after 5 days		linear speed (mm/sec)	13.4 (10.7 – 15.2)		
		angular speed (ang.deg./cm)	158 (122 – 170)		
precision of reaction after 5 days		orientation to an alarm signal (°)	83.6 (74.5 – 99.6)		
tactile perception after 8 days		n°of ants in the three zones	small	rough	large zone
		after 0 min	18	3	3
		2 min	14	5	5
		4 min	15	4	5
		6 min	14	3	7
		8 min	14	3	7
		10 min	13	4	7
in the rough zone:		linear speed (mm/sec)	9.8 (8.1 – 12.5)		
		angular speed (ang.deg./cm)	146 (132 – 168)		
Dependence	nest 1	n° and % of ants choosing food +	18 *vs* 6 = 75.0% *vs* 25.0%		
	nest 2	methadone *vs* food free of the	1 *vs* 62 = 1.6% *vs* 98.4%		
	pool	drug.	19 *vs* 68 = 21.8% *vs* 78.2%		

Table 4. Visual and olfactory conditioning abilities and memory under normal diet, buprenorphine consumption and methadone consumption. Information concerning the methods and the statistical analysis are given in the 'Material and Methods' and 'Results' sections. In short, buprenorphine did not impact these ants' abilities and even slightly increased their olfactory memory, while methadone inhibed these abilities. *: results from [21], **: results from [29].

Trait assessed	*hours*	Control (no drug)	**+ buprenorphine**	**+ methadone**
Visual learning		*C 50%		
	7	**55%	50%	55%
	24	60%	50%	45%
	31	60%	60%	50%
	48	65%	65%	50%
	55	65%	70%	45%
	72	70%	70%	50%
Visual memory	*7*	**65%	75%	
	24	75%	75%	
	31	70%	70%	no memory
	48	70%	70%	
	55	70%	75%	
	72	70%	70%	
Olfactory learning		*C 50%		
	7	**55%	65%	40%
	24	65%	60%	40%
	31	70%	75%	50%
	48	75%	70%	40%
	55	80%	90%	50%
	72	80%	75%	50%
Olfactory memory	*7*	**65%	80%	
	24	60%	70%	
	31	60%	65%	no memory
	48	55%	55%	
	55	55%	65%	
	72	55%	55%	

4.2, Effects of methadone on eighteen physiological and ethological ants' traits

4.2.1, Locomotion (*1, 2*)

This trait was largely affected by methadone consumption (Table 1, methadone, linear speed and sinuosity). As soon as after one day of such consumption, the ants moved more slowly and more sinuously: they no longer moved straight along a few centimeters. The obtained numerical results significantly differed from the control ones (linear speed: $\chi^2 = 32.73$, df = 1, $P < 0.001$; angular speed: $\chi^2 = 24.00$, df = 1, $P < 0.001$).

4.2.2, Precision of reaction (*3*)

This trait was obviously affected by methadone consumption, as soon as after only one day of such consumption (Table 1, methadone, orientation: $\chi^2 = 19.60$, df = 1, $P < 0.001$). Most of the ants seemed unable to detect the presence of the alarm signal, and moved either in a wrong direction or beyond the signal.

4.2.3, Response to pheromone (*4*)

Under normal diet, ants moved rather well along a circumference marked with the species' trail pheromone, but under methadone consumption, they failed in following the presented trail (Table 1, methadone, trail following, T). They crossed the trail or followed it along only one or two arcs. The numerical results assessing this observation were highly significant ($\chi^2 = 67.76$, df = 2, $P < 0.001$).

4.2.4, Food consumption (*5*)

Ants consuming methadone were less numerous on the meat food site and each ant coming on that site rapidly left it obviously eating less. This observation led to significant results (Table 1, methadone, food consumption; U = 36, Z = 4.77, $P = 2.10^{-6}$). After the entire experimental work, when ants stopped consuming the drug, they again came onto the meat food and eat (observation not quantified).

4.2.5, "Audacity" (*6*)

Under methadone consumption, the ants were obviously reluctant in coming onto the presented risky apparatus, avoiding it or reducing their presence on it. The obtained numerical results were highly significant (Table 1, methadone, 'audacity'; U = 31.5, Z = 4.82, $P = 1.10^{-6}$).

4.2.6, Brood caring (*7*)

This trait was slightly affected by methadone consumption. After 10 minutes, six of the ten larvae the experimenter had removed from the nests were still on the foraging area (Table 2, methadone, brood caring). Such a result was at the limit of significance: N = 4, T = 10, P = 0.063. It was also observed that the ants did not ill-treat or kill the larvae, but did not take care of them since the larvae stayed at the same stage during two months, while in control nests, they developed up to one or two further larval stages.

4.2.7, A cognitive ability requiring no memory (*8*)

This trait was somewhat affected by methadone consumption. The ants were slower than usual to left the initial loggia, to cross the twists and turns, and to reach the free zone. They often stopped in the course of such a displacement. The obtained numerical results were slightly significant (Table 2, methadone, cognition; initial loggia: N = 5, T = 15, P = 0.031; free loggia: N = 5, T = 13.5, P = 0. 077).

4.2.8, Tactile ('pain') perception (*9*)

Under normal diet, ants were reluctant in crossing the uncomfortable rough zone of the apparatus: after ten minutes, twenty ants were still in the small zone in front of the rough one, while only one ant had crossed it and stayed beyond it, in the large zone. After having consumed methadone for five days, the ants frankly crossed the zone with the rough bottom. In ten minutes, nine ants were beyond the uncomfortable zone, up to the large one, while eleven ants were still at the start. The result was significant (Table 2, methadone, 'pain' perception; small zone: N = 6, T = 21, P = 0.016; uncomfortable as well as large zones: N = 5, T = 15, P = 0.031).

In the same way, under normal diet, the ants moved slowly and sinuously on the uncomfortable bottom, obviously perceiving its rough character. After having consumed methadone for five days, they moved more quickly and less sinuously on it. This observation was highly significant (Table 2, methadone, 'pain' perception; linear speed: $\chi^2 = 44.16$, df = 1, $P < 0.001$; angular speed: $\chi^2 = 34.12$, df = 2, $P < 0.001$). Moreover, under methadone consumption, the ants' sinuosity on the rough bottom was similar to that presented on a normal bottom after five days of drug consumption: 170 (Table 2) *vs* 158 ang. deg (Table 3, methadone, habituation; $\chi^2 = 5.09$, df = 2, $0.05 < P < 0.10$). Since the locomotion is difficult on the rough substrate, the ants' linear speed

was not equaled to that presented after five days of the drug consumption but was lower: 8.3 (Table 2) *vs* 13.4 mm/sec (Table 3, methadone, habituation; $\chi^2 = 33.12$, df = 1, $P < 0.001$).

Thus, methadone reduced the ants' tactile ("pain") perception. A further experiment examined if habituation occurred as for this physiological impact of the drug (see below "habituation"). Comparison with what occurred with buprenorphine will be made in the Discussion section.

4.2.9, Aggressiveness towards congeners (*10*) and aliens (*11*)

Under normal diet, the ants were not at all aggressive towards their nestmates but very quickly attacked alien ants, gripping then stinging them. This observation and the obtained numerical results (Table 2, methadone, aggressiveness) were of course highly significant (behavior of ants in front of congeners *vs* aliens: $\chi^2 = 101.10$, df = 1, $P < 0.001$). However, after having consumed methadone for six days, the ants became somewhat aggressive towards their nestmates. They mainly opened their mandibles when approaching them. The difference of behavior before and after consuming methadone was statistically significant: $\chi^2 = 23.31$, df = 2, $P < 0.001$. On the other hand, in front of alien ants, workers consuming methadone delayed in attacking, and so, were very often themselves attacked by the alien. Only 13 + 5 instead of 46 + 33 gripping or stinging behaviors were recorded for ants under methadone consumption and normal diet respectively (Table 3, methadone, aggressiveness). The ants' entire behavior under these two diets statistically differed: $\chi^2 = 12.28$, df = 2, $P < 0.01$.

4.2.10, Habituation (*12*)

Habituation appeared to occur as for the ants' locomotion. After five days of methadone consumption, the ants no longer moved more slowly or more sinuously. They again presented their usual linear and angular speed (Table 3, methadone, habituation). This result was statistically significant as follows. The linear speed occurring after five days of drug consumption was similar to the control one (0.40, df = 1, NS) and different from that observed after one day of consumption ($\chi^2 = 32.40$, df = 1, $P < 0.001$); the angular speed occurring after five days of drug consumption was similar to the control one (1.60, df = 1, NS) and different from that observed after one day of consumption ($\chi^2 = 15.17$, df = 1, $P < 0.001$).

On the contrary, no habituation occurred as for the ants' precision of response. The insects' bad orientation towards an alarm signal went on being impacted by methadone consumption (Table 3, methadone, habituation), the ants still not using true positive taxis while moving in the vicinity of the signal. This result was statistically significant. The ants' orientation after five days of methadone consumption was different from the control one ($\chi^2 = 13.33$, df = 1, $P < 0.001$) and similar to that presented after one day of consumption ($\chi^2 = 1.66$, df = 1, NS). This result will be compared to what occurred with buprenorphine (see the Discussion section).

As for the impact on the ants' tactile perception, no habituation occurred. The numbers of ants present in the course of time in the three experimental zones (small, rough, large) were similar and statistically not different after five (Table 2, methadone, tactile perception) as well as after eight days of methadone consumption (Table 3, methadone, habituation; small zone: N = 5, T = 8.5, P = 0.45; rough zone: N = 2, NS; large zone: N = 4, T = 7, P = 0.313). In the same way, the ants went on moving rather frankly on the rough bottom. They even walked a little more quickly after eight than after five days of drug consumption. The numerical results (Table 2, methadone, tactile perception; Table 3, methadone, habituation) clearly assessed this observation: linear speed on a rough bottom after five (8.3 mm/sec) *vs* eight days (9.8 mm/sec) of consumption: $\chi^2 = 8.08$, df = 1, $P < 0.01$; angular speed on the rough bottom after five (170 ang.deg./cm) *vs* after eight days (146 ang. deg./cm) of consumption: $\chi^2 = 3.98$, df = 2, NS.

Thus, for methadone, there is no habituation as for its analgesic effect, contrary to what occurred for morphine [3]. However, all the adverse effects observed for buprenorphine and methadone should be taken into account for selecting the best analgesic (see the Discussion section).

4.2.11, Dependence (*13*)

No dependence occurred on methadone consumption. Concerning the ants of nest 1, 18 ones were counted on food containing methadone, while six ones were counted on food free of the drug. But the 18 ones were counted essentially at the beginning of the experiment while the six ones were counted essentially during the last experimental minutes. As for the ants of nest 2, only one ant was counted on food containing methadone as compared to 62 ones on food free of it (Fig. 1H). So, in total, 19 ants were observed on food containing methadone and 68 ants on food free of the drug. This corresponded to 21.8% of the individuals choosing the food with methadone and 78.2% of the individuals choosing the food free of drug (Table 3, methadone, dependence). Thus, ants did not developed dependence on the drug and even exhibited some aversion for it, a result statistically significant ($\chi^2 = 27.59$, df = 1, $P < 0.001$). This should be taken in consideration when proposing methadone as an analgesic and/or substitute of drug (see the Discussion section).

4.2.12, Visual and olfactory conditioning ability and memory (*14, 15, 16, 17*)

These traits were impacted by methadone consumption (Table 4, control, + methadone).

Under such consumption, the ants never acquired visual conditioning, and presented thus no visual memory. The numerical results statistically differed from the control ones (N = 5, T = 15, P = 0.031), and were statistically similar to those obtained for morphine [3] (N = 2, NS). In the same way, under methadone consumption, the ants never acquired olfactory conditioning. All the obtained conditioning scores were lower than those obtained under normal diet (N = 6, T = 21, P = 0.016). They were either similar to, lower than or larger than those obtained under morphine consumption [3], and thus statistically similar to these scores under morphine (N = 4, T = 5, NS).

4.2.13, Decrease of the effects after end of consumption (*18*)

After being deprived of methadone, the ants' linear speed on a rough bottom firstly rapidly decreased while their angular speed rapidly increased. Then, between 3½ and 5 hours for the angular speed and between 5 and 7½ hours for the linear speed, a level appeared, after which the speeds again continued to decrease or increase. This level was best shown when drawing straight lines between the experimental points (Fig. 2, where the level is encircled). After a total of ten hours of drug deprival, the ants' linear speed on a rough bottom (4.6 mm/sec) was similar to the control one (that exhibited before methadone consumption) ($\chi^2 = 0.48$, df = 2, NS). Angular speed was statistically similar to control 13 hours after drug deprival. Of course, values obtained for 25 h of drug deprival were also statistically similar to control ones. The plotted points can be best fitted by polynomial curves with a maximum power corresponding to a statistical significance at $\alpha = 0.05$ level. These curves are $y = 9.36 – 0.72x + 0.02x^2$ (y measured in $mm.sec^{-1}$ and x in hours) for linear speed decrease and $y = 161.99 + 16.75x - 0.50x^2$ (y measured in angular $degrees.sec^{-1}$ and x in hours) for angular speed.

On basis of the ants' linear and angular speeds on a rough bottom, it can thus be deducted that the effect of methadone first rapidly decreased during about 3½ to 5 hours, then stayed at level for about four hours, and then continued to vanish during about 6 to 8 hours. Such a vanishing 'in three steps' was obvious for the experimenter while testing the ants. The effect of methadone on the ants' tactile perception was thus less long lasting than that of buprenorphine (see above), but longer lasting than that of morphine [3]. The fact that the effect of methadone did not decrease during ca five hours should help to avoid addiction on this drug [28, 29].

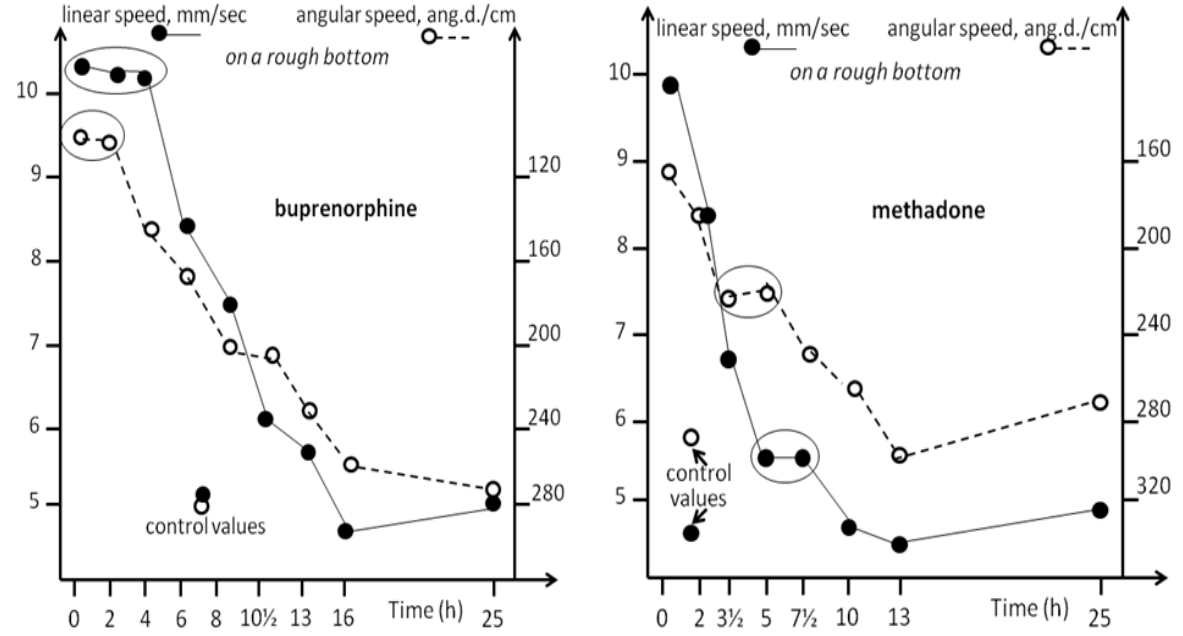

Figure 2. Decrease of the effect of buprenorphine and methadone after the end of consumption. The effect of buprenorphine decreased after a latency period (encircled) in ca 15 hours, what is advantageous for an analgesic use. That of methadone decreased immediately but stopped decreasing between ca 3½ to 7½ hours (encircled), what is advantageous as a substitute of morphine or heroine.

V. CONCLUSION – DISCUSSION

We made comparative studies on the effects of buprenorphine and methadone, using ants as biological models, what may help practitioners, pharmacists and veterinaries to adequately use these substances as analgesic and/or substitute to morphine or heroine.

We found that buprenorphine and methadone decreased the ants' speed of movement, increased their sinuosity, and decreased their precision of reaction, response to pheromones, food consumption, 'audacity', brood caring, and cognitive ability. The two drugs largely decreased the ants' tactile perception and induced some aggressiveness between nestmates. Habituation occurred to buprenorphine consumption as for its impact on ants' locomotion and precision of reaction, but not as for their tactile perception. Habituation occurred to methadone consumption as for its impact on ants' locomotion, but not for their precision of reaction (methadone differing in this matter from buprenorphine) and also not for their tactile perception. Nearly no dependence occurred on buprenorphine consumption (57% of ants preferred food containing the drug) while no dependence at all and even aversion occurred on methadone consumption (only 22% of ants preferred food containing the drug). Buprenorphine did not impact the ants' conditioning ability and memory, while under methadone consumption the ants never acquired such conditioning. This is another important difference between buprenorphine and methadone. After deprival of buprenorphine, its impact on tactile perception started with a latency period and vanished in about 15 hours. After deprival of methadone, its impact on tactile perception first quickly decreased during ca 4 to 5 hours, then presented a level for ca 4 hours, and after that again decreased, ending in a total of 10 to 13 hours. The intermediate level allows some absence of dependence on the drug.

A previous work [3] showed that morphine has all the adverse effects of buprenorphine and methadone, and has four and three respectively more ones. Morphine drastically impacted the ants' conditioning ability and memory; habituation occurred as for its effect on tactile perception; strong dependence occurred on morphine consumption; the effects of morphine quickly vanished in about eight hrs.

On basis of our results, we propose to use buprenorphine instead of morphine as a strong analgesic, and to keep methadone as a substitute of morphine or heroine to care addicted persons. Are such deductions in agreement with what is actually known about the two examined drugs? Most information was found on web sites [1, 2, 39, 40, 41, 42, 43, 44]; other ones were provided to us by practitioners.

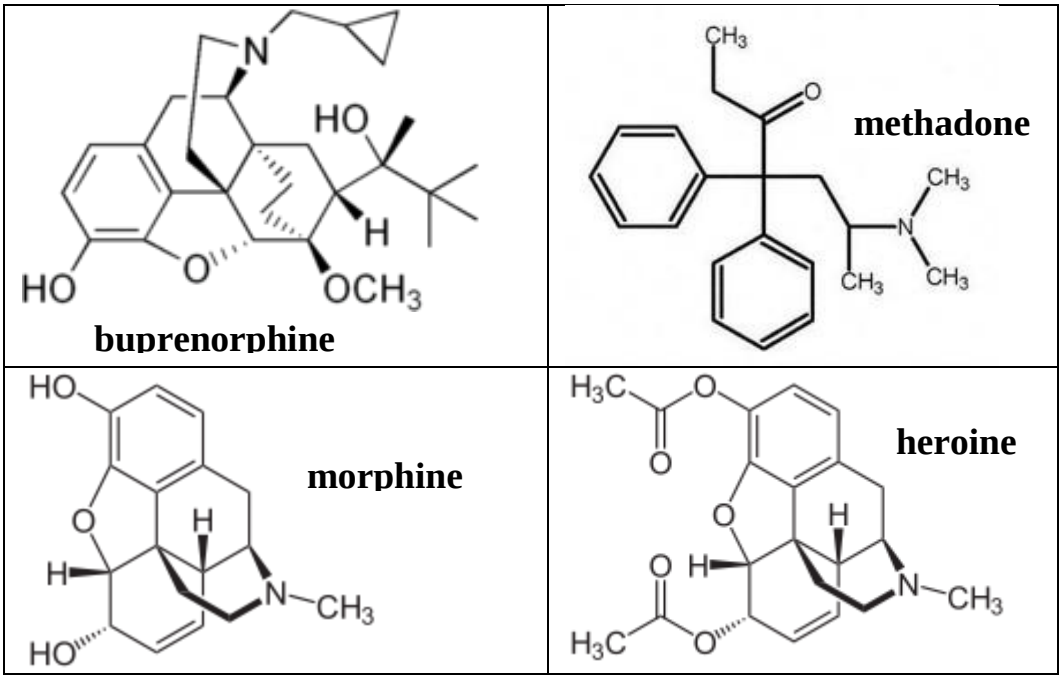

Figure 3. Chemical structure of the two drugs here studied (buprenorphine, methadone) and of the two ones to which they are compared (morphine, heroine) [reproduced from 1, 2, 41].

Morphine is extracted from opiacea. Heroine is obtained by di-acetylating morphine. Buprenorphine and methadone are non-natural chemicals.

Among others, buprenorphine and methadone are used as analgesic for humans and animals. Buprenorphine is very quickly absorbed by the organisms, may present pharmaceutical effects in humans up to 24 hours, and has some adverse effects such as sleepiness and nausea. Methadone is quickly absorbed by

organisms and is active for six to eight hours. It was firstly used as a strong analgesic in case of severe pain. Buprenorphine seems to be more adequate as analgesic while methadone would be more efficient as a substitute of opiacea [43]. Buprenorphine and methadone might also act as antidepressants, doing so even better than usual antidepressants (i.e. fluoxetine) [43]. Both substances are also used for caring persons dependent on morphine or heroine consumption. In other words, they are used to reduce problems and dangers caused by the consumption of opiacea extracts, and to try stopping addiction. Under methadone consumption, heroine has no longer an euphoric effect. Indeed, methadone and heroine have some chemical similarities: they have both a cetonic function, an amine function and two benzenic cycles (Fig. 3). Used as a substitute to opiacea, methadone appeared to have rather long lasting effects. It presents few adverse effects for addicted persons, being however susceptible to kill non addicted ones. Its use for treating addicted persons is not easy, though efficient (addicted persons accept methadone) because some dependence on any drug (morphine, heroine, methadone) may persist.

On basis of our results and on what is actually known about buprenorphine and methadone, we conclude that:

- Buprenorphine should advantageously be used as an analgesic instead of morphine, having no impact on memory, no addiction, and longer lasting effects.
- Methadone should be used preferentially as a substitute to morphine or heroine to save addicted persons: it has similar effects, no dependence at all, longer lasting effects, and under methadone consumption, heroine has no longer effects.
- Methadone should not be used as an analgesic, for dogs and cats among others, since it severely impacts memory.
- Buprenorphine and methadone should not be used as antidepressants, even if acting better than usual antidepressants such as fluoxetine, which induces aggressiveness between congeners [30], what buprenorphine and methadone also somewhat do. Moreover, we have here shown that buprenorphine and methadone largely decrease tactile perception in ants. We think that this highly useful perception should always be detained by cared depressive persons.

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