Acta Scientific Pharmaceutical Sciences (ISSN: 2581-5423)

Volume 3 Issue 3 March 2019

Research Article

Adverse Effects of Diclofenac Observed on Ants as Models

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Received: January 28, 2019; **Published:** February 07, 2019

Abstract

Diclofenac is a very largely used anti-inflammatory drug. Ecological and medicinal observations showed that it could be not as safe as it was initially estimated. Therefore, we examined its potential adverse effects using the ant *Myrmica sabuleti* as a model. Diclofenac appeared to increase the ants' sinuosity of locomotion, to decrease their orientation ability, audacity, cognition, escaping capability, conditioning ability and short term memory. It did not affect the ants' sensory perception as well as their social relationship. No adaptation occurred to these adverse effects and ants developed dependence on diclofenac consumption. The effect of this drug vanished in about 6 hours after weaning, with a quick decrease between 2 and 3 hours, what explained the development of some dependence. Based on the ants' reactions, we conclude that diclofenac is not a safe drug, and our work could define some of its harmful effects. For human care, diclofenac should be used only when necessary, momentarily and in small amounts. We advise practitioners and pharmacists to research for safer novel anti-inflammatory drugs.

Keywords: Adaptation; Cognition; Dependence; Locomotion; Memory; *Myrmica sabuleti*

Abbreviations

ang.deg.: Angular Degrees; ang.deg/cm: Angular Degrees per cm; mm/s: Millimeter per second; χ^2: Chi-Square; vs: Versus, n°: Number; cm: Centimeter; mm: Millimeter; ml: Milliliter; mg = Milligram; s: Second; min: Minute: h: Hours; t: Time; %: Percentage.

Introduction

Among the most sold and used anti-inflammatory drugs are those the active substance of which is the 'diclofenac' molecule (Figure 1). Diclofenac is also very largely used for caring of animals. It is a very efficient substance and it has been considered as safe during many years. However, at least two events draw our attention to safety problems. First, a dramatic decline of vultures occurred in India, Pakistan and Nepal since the early 1990s. This ecological disaster appeared to be due to the vultures' consumption of carcasses of domestic livestock which had been treated with diclofenac [1-4]. These vultures died within days from kidney failure, resulting in extensive stock piling of uric acid in the tissues [4,5]. This event also occurred in Africa [6]. Therefore, since 2006, diclofenac is banned for veterinary use in the Indian subcontinent [4,7], however not for human care. Second, three Danish researchers recently made a comparative study of 1.3 10^6 patients treated with diclofenac, 4 10^6 patients treated with ibuprofen, 291 10^3 patients treated with naproxen, and 764 10^3 ones treated with paracetamol [8]. The conclusions of this study are that the persons treated with diclofenac had a probability of suffering from a serious cardiovascular problem increased by 20%, 30% and 50% as compared with those treated with paracetamol or ibuprofen, treated with naproxen, or having received no treatment respectively. Furthermore, the instructions for use joined to diclofenac package nowadays recommend paying attention for young and old persons, and for those suffering from kidney and heart problems.

Persons consume diclofenac for treating an inflammatory reaction which results from some health problem. This health problem and its consequent inflammatory reaction cause by themsel-

Figure 1: Chemical structure of diclofenac, an arycarboxylic acid largely used as an anti-inflammatory drug, the potential adverse effects of which are here examined on ants. This drug appeared to be not fully safe; it somewhat impacted the ants' health (their locomotion, cognition, and memory among others).

ves some physiological impairment. What are the harmful effects of diclofenac independently of those due to a health problem and the consequent inflammatory reaction? Does diclofenac impact, by itself, some physiological and/or ethological functions or traits? Answering this question requires making experiments on models presenting no inflammatory reaction and being blind to the situation. Being accustomed to use ants as models for studying the effects of products used by humans [9-13], we intended here to examine the potential adverse effects of diclofenac on several physiological and ethological ants' traits.

Here below, we explain why we used ants as models, which species we employed, what we know on it, and which traits we intended to examine.

Why using ants as models?

Most animals' and humans' physiological and ethological traits are fundamentally similar [14]. They are thus firstly examined on animals as models (e.g. fruit flies, cockroaches, bees, mice, monkeys), and then on humans [15]. Invertebrates and among them insects are often used as models due to their rapid development and easy maintenance in a laboratory [16,17]. Hymenoptera, among others, are used [17]. Ants could advantageously be so [18]. Indeed, these social insects present, among others, colonial regulation, labor division and information exchange thanks to tactile and chemical signals (pheromones) [19,20,21]. They construct sophisticated nests, take care of their brood, and chemically mark the different parts of their habitat [19]. They navigate, recruit congeners, relocate their nest, clean its inside and create cemeteries [20]. On the basis of such a complex biology, they could serve as biological models. The impact of stressors, environmental changes, and drug consumption could be examined on them, and hypothesis concerning the effects of these factors on other organisms including humans can be emitted.

Which species was used?

We have intensely studied the ants of the genus *Myrmica*. We have looked, among others, to their ecology, eyes morphology, angle of vision, visual perception, recruitment strategy, navigation system, learning [22], and to the ontogenesis of some of their abilities [23]. The study of the effect of electromagnetic fields (EMF) on their learning, memory capabilities and responses to pheromones showed that they could be used as biological models [24,25]. Effectively, *Myrmica* ants were good models while examining on them the harmful impact of products used by humans [9-13]. In the present work, we used again the ant *M. sabuleti* Meinert 1861 as a model for studying the impact of diclofenac consumption on several physiological and ethological traits.

Which traits were examined?

The 17 following traits were examined: meat consumption, sugar water consumption, general activity, speed of locomotion, sinuosity of movement, orientation ability, audacity, tactile perception, brood caring, aggressiveness towards nestmates, aggressiveness against aliens, cognition, escaping ability, conditioning capability, adaptation to the adverse effects of the drug, dependence on the drug consumption, and decrease of the effect of the drug after weaning.

Material and Methods

Collection and maintenance of ants

The experiments were conducted on two colonies of *M. sabuleti* collected, in September 2018, in an abandoned quarry located in the Aise valley (Ardenne, Belgium). Another colony, collected the same day in the same valley, was used for performing the control experiment of the conditioning study. A fourth colony, collected in June 2018 at Marchin (Condroz, Belgium), also in an abandoned quarry, furnished the alien ants used in the experiment on the ants' aggressiveness. All these colonies were nesting under stones and contained about 500 workers, brood and a queen. They were maintained in the laboratory in two to three glass tubes half filled with water, a cotton plug separating the ants from the water. The nest tubes of each colony were set in a tray (34 cm x 23 cm x 4 cm) which

served as foraging area, and the ants received, in that area, pieces of *Tenebrio molitor* larvae (Linnaeus, 1758) three times per week, as well as sugar water continuously present in cotton plugged tubes. The ambient temperature was ca 20°C, the humidity 80%, the lighting 330 lux while working on ants, and the electromagnetism 2 μWm^2. These environmental conditions are optimum for the species. The ants are here often named 'nestmates' as researchers on social insects commonly do.

Solution of diclofenac given to the ants

On the basis of the instructions for use joined to the drug package as well as those found on several internet sites, humans who must be treated with diclofenac are advised to consume 150 mg, 100 mg, or 75 mg of the drug each day, according to the level of their health problem [https://base-donnees-publique.medicaments.gouv.fr/]. We opted for the mean dose of 100 mg per day. Tablets containing 75 mg of diclofenac were furnished by the pharmacist Wera (1170 Bruxelles). In general, a human consume one liter of drink water per day. Consequently, when treated with a dose of 100 mg of diclofenac per day, a human daily consume this dose together with one liter of water. The need for water of insects, and thus of ants, is 10 times lesser than that of mammals, essentially due to the different efficiencies of their excretory apparatus [14]. Consequently, for setting ants under a diclofenac concentration corresponding to that of a human treated with 100 mg of the drug per day, they must be provided with a solution of 75 mg in 75 ml of a liquid. The ants were provided with such a concentrated solution using their usual sugar water as liquid, solution delivered in their usual tubes plugged with cotton from which they could drink *ad libitum* according to their size and to their request. We checked several times per day if the ants drunk the provided solution and they effectively did.

Assessment of the examined traits

The protocols of these assessments have already been many times used, what allows only briefly relating them here and referring the readers to previous studies [11-13].

Food consumption, general activity

While ants were under normal diet then while they consumed diclofenac, those eating mealworm (*T. molitor*) pieces, drinking sugar water and being active anywhere (on the foraging area, on the food sites, and in the nest) were counted six times per day during six days, at the same times o'clock each day (as in [11-13]). The mean of the daily counts was established for each case (Table 1), as well as, for information only, the average of these means for the 6-days period (Table 1, last line).

Experimental days	Normal diet			Diet with diclofenac		
	Meat	Sugar water	Activity	Meat	Sugar water	Activity
I	1.67	2.83	10.33	1.67	2.50	11.50
II	1.50	2.00	10.67	1.67	2.50	9.10
III	3.17	4.00	14.00	1.67	2.50	12.83
IV	3.00	3.00	14.00	1.83	2.00	10.33
V	2.00	2.17	12.50	1.33	1.83	12.00
VI	2.33	1.67	14.67	1.67	1.67	10.00
I – VI	2.28	2.61	12.70	1.64	2.17	10.96

Table 1: Impact of diclofenac on the ants' food consumption and general activity.

The table gives the mean of 6 daily counts (days I to VI) of the observed ants, and the mean of these 6 means (last line) for each examined physiological trait. The three traits were slightly affected by diclofenac consumption. Details and statistics are given in the text.

Linear and angular speeds, orientation

These traits were quantified on ants moving on their foraging area, the linear speed without stimulating the ants and the orientation while stimulating them with a nestmate tied to a piece of paper (Figure 2A). Such a nestmate emits its alarm attractive mandibular glands pheromone [8-10]. For the ants' speeds on one hand and for their orientation on the other hand, the trajectory of 40 workers was recorded. They were then analyzed using appropriate software [26] based on the following definitions. The linear speed (in mm/s) is the length of a trajectory divided by the time spent to travel it; the angular speed (in angular degree/cm = ang.deg./cm) is the sum of the angles made by successive adjacent segments, divided by the length of the trajectory; the orientation (in ang. deg.) towards a location is the sum of the successive angles made by the direction to the location and the direction of the trajectory, divided by the number of angles measured. When the mean angle value is lower than 90°, the animal tends to orient itself towards the location; when the value is larger than 90°, it tends to avoid the location [26]. The median and quartiles of each distribution of 40 values were established.

Audacity

As previously, a cylindrical tower (height = 4 cm; diameter = 1.5 cm) tied to a squared platform (9 cm^2), made of white Steinbach® paper, was deposited in the ants' foraging area, and those present at any place on this apparatus were counted 10 times over 10 min (Figure 2 B) [11-13]. The counts obtained for the two colonies were added, and the mean and the extremes of these added counts were established. The counts obtained for two successive minutes were added for statistical analysis.

Tactile perception

Ants perceiving the uncomfortable character of a rough substrate walk on it slowly, sinuously, and with difficulties (Figure 2C). Ants weakly perceiving such an uncomfortable character walk more quickly and less sinuously. Therefore, for assessing the ants' tactile perception, their locomotion on a rough substrate was analyzed (as above: 'Linear and angular speeds'). A folded piece (3 cm x 2 + 7 + 2 = 11 cm) of emery paper n° 280 paper was tied to the bottom and the borders of a tray (15 cm x 7 cm x 4.5 cm), and the tray was so divided in a first 3 cm long zone, a second 3 cm long zone containing the emery paper, and a last 9 cm long zone [11-13]. Such an apparatus was constructed for each colony. For making an experiment, 12 ants of each colony were deposited in the first zone of their apparatus, and the trajectory of 24 ants walking on the emery paper was recorded. The ants' linear and angular speeds were then assessed as usually, and the median and quartiles of the obtained distributions of values were established.

Brood caring behavior

For each colony, a few larvae were removed from the nest and deposited in front of the entrance. Five of these larvae, and the ants' behavior towards them, were observed (Figure 2 D). The larvae among these 5 + 5 = 10 not yet re-entered in the nest were counted after 5 seconds, 2, 4, 6, 8, and 10 minutes.

Aggressiveness against nestmates and against aliens

As previously, these traits were assessed, for each colony, during five dyadic encounters between an ant of a colony and either a nestmate or an alien ant (Figure 2 E, F) [11-13]. Each encounter took place in a cylindrical cup (diameter = 2.5 cm, height = 1.8 cm) and lasted 5 minutes. The number of times the observed ant did nothing (level 0 of aggressiveness), contacted the opponent with its antennae (level 1), opened its mandibles (level 2), gripped the other ant (level 3), and tried to sting or stung the opponent (level 4) was counted. The numbers obtained for the two colonies were added. The ants' aggressiveness was also characterized by "a" = number of aggressiveness levels 2 + 3 + 4/number of levels 0 + 1.

Cognition

The experimental protocol set up when studying the effects of nicotine was again utilized [27]. An experimental apparatus was build for each colony. Two pieces of white paper (Steinbach®, 12 cm x 4.5 cm) duly folded were inserted in a tray (15 cm x 7 cm x 4.5 cm), dividing the tray into a first small zone, a zone with twists and turns, and a large zone into which some wet cotton had been placed (Figure 2 G). For conducting an experiment, 15 ants of each colony were transferred into the first zone of their apparatus, and the ants present in this zone and in the large one were counted after 30 seconds, 2, 4, 6, 8, 10 and 12 minutes. The numbers obtained for the two colonies were added.

Escaping ability

The study of this trait allows examining the ants' cognition and state of stress. For each colony, 6 ants were enclosed under a reversed polyacetate glass (h = 8 cm, bottom diameter = 7 cm, ceiling diameter = 5 cm) set in the ants' tray [11-13]. A notch (3 mm height, 2 mm broad) had been made in the bottom rim of the glass for allowing the ants escaping (Figure H). Thirty seconds, 2, 4, 6, 8, 10 and 12 minutes after the ants had been enclosed, those escaped and those still enclosed were counted. The results obtained for the two colonies were added. The ants' ability in escaping was also assessed by the proportion 'n° of ants escaped after 12 min/12'.

Conditioning and memory

The control experiment was that made two months before on a similar colony (see the subsection 'Collection and maintenance of ants'). The test experiment was made during the present study, using exactly the same protocol. Each time, a green hollow cube was set above the entrance of the sugar water tube provided to the colonies; the ants went so through visual conditioning. Their acquisition of such a conditioning was checked by making tests over time until their conditioning score no longer increased. To make a test, 10 ants of each colony were individually tested in a Y-apparatus provided with a green hollow cube in one of its branch. This Y-apparatus was made of strong white paper and was deposited in a separate tray (30 cm x 15 cm x 4 cm). The green cube was randomly located in the right or the left branch of this Y-apparatus. Moving into the branch containing the cube was considered as giving the correct response (Figure 2 I). Each test provided the response of 20 ants, and consequently the proportion of their correct responses, that is the ants' conditioning score.

Adaptation to potential adverse effects of diclofenac

Adaptation occurs when an adverse effect of the drug gets weaker and weaker in the course of its consumption. In the present work, the ants' linear and angular speeds were again assessed after 13 days of consumption, as they had been assessed after one day, and the obtained results were compared to one another.

Habituation to potential beneficial effects of diclofenac

Habituation to a drug occurs when a beneficial effect of the drug weakens over its consumption. Here, no beneficial effect could be revealed, and this drug characteristic could not be examined on ants.

Figure 2: Some photos of the experiments. Each time: 1 = control experiments; 2 = experiments on ants under diclofenac diet. **A:** ants coming to a tied nestmate emitting its attractive alarm pheromone, through positive taxis in 1 and kinesis in 2. **B:** ants coming onto an unknown risky apparatus, more numerously in 1. **C:** ants walking with difficulties on a rough substrate, in 1 as well as in 2**. D:** ants taking care of larva, in 1 as well as in 2. **E:** two nestmates presenting no aggressiveness, in 1 as well as in 2. **F:** an ant meeting an alien, stinging it in 1 and being stung by it in 2. **G:** ants having to cross a twists and turns path, 6 in 1 (red circles) and zero in 2 having done so. **H:** enclosed ants escaping in 1 and not escaping in 2. **I:** an ant, trained to a hollow green cube, giving the correct response in 1, and the wrong one in 2 (red circles).

Dependence on diclofenac consumption

Dependence on a substance occurs when individuals consuming this substance prefer food containing it to food free of it. In the present work, dependence was examined after the ants consumed the drug since 14 days. As in previous works [12,13], for each colony, 15 ants were transferred into a tray (15 cm × 7 cm × 5 cm) containing two tubes (h = 2.5 cm, diam. = 0.5 cm), one filled with sugar water, and the other filled with the sugar solution of diclofenac used throughout the present work. The tube containing the drug was located on the right in one tray, and on the left in the other tray. The ants coming onto each tube were counted 15 times over 15 min. The counts corresponding to each kind of liquid were separately added.

Decrease of the effect of diclofenac on the ants' locomotion, after its consumption was stopped

This study was made after the ants consumed diclofenac during 16 days, using an experimental protocol already many times employed [12,13]. The ants received a fresh solution of the drug 12 hours before the weaning time. After these 12 hours, the ants' sinuosity was assessed, i.e. at t = 0h. Then, weaning started, i.e. the solution of diclofenac was replaced by usual sugar water. Since this time, the ants' sinuosity was assessed each hour, in the manner it had been for the control (i.e. before the ants consumed the drug), after they consumed it for one day, and after they consumed it for 13 days, except that only 20 instead of 40 ant's trajectories were analyzed in order to make the assessments over the experimentation. The results are numerically given in Table 5 and graphically presented in Figure 3. The experiment ended when the ants' sinuosity no longer differed from that observed during the control (Table 2, normal diet, line 2; Table 5, last line).

Traits	Normal diet	Diet with diclofenac
Linear speed in mm/s	12.6 (10.9 - 14.5)	11.9 (10.3 - 13.6)
Angular speed in ang. deg./cm	124 (112 - 142)	179 (158 - 208)
Orientation in ang. deg.	35.6 (28.9 - 47.9)	70.6 (53.1 - 82.5)
Audacity	3.50 [2 - 5]	2.45 [1 - 4]
Tactile perception assessed on a rough substrate:		
Linear speed in mm/s	6.5 (5.6 - 7.7)	6.3 (5.2 - 7.2)
Angular speed in ang. deg./cm	259 (222 - 283)	304 (269 - 351)

Table 2: Impact of diclofenac on five ants' physiological traits.

Diclofenac increased the ants' sinuosity of movement, decreased their orientation and audacity, but did not affect their tactile perception. Details and statistics are given in the text. ang.deg.: Angular Degrees, ang.deg./cm: Angular Degrees per cm; mm/s: Millimeter Per Second.

Figure 3: Ants' adaptation to the effect of diclofenac on their locomotion, and ants' dependence on this drug consumption. **A:** ants' trajectories after one day of diclofenac consumption. **B:** ants' trajectories after 13 days of diclofenac consumption. The trajectories went on being very sinuous, more than without diclofenac; there was thus no adaptation to the adverse effects of the drug. **C:** ants tested in front of a tube filled with pure sugar water (on the right) and a tube filled with sugar water containing diclofenac (on the left with a red point). The ants went onto the liquid containing the drug; some dependence on diclofenac consumption occurred thus.

Statistical analysis

The numerical results concerning the ants' linear speed, angular speed, orientation, tactile perception, aggressiveness against nestmates and against aliens were ranked. Those obtained on ants under diclofenac diet were statistically compared to those obtained on ants under normal diet using the non-parametric χ^2 test [28]. The ants' aggressiveness against aliens was complementarily analyzed using the non-parametric 2 x 2 contingency table χ^2 test. As for the ants' food consumption, general activity, audacity, brood caring, cognition and escaping behavior, same comparisons were made using the non-parametric test of Wilcoxon [28]. Ants' dependence on diclofenac consumption was statistically analyzed using the non-parametric goodness of fit χ^2 test [28].

The statistical analysis of the decrease of the effect of diclofenac after weaning was made as follows. The distributions of the 20 sinuosity values obtained after given time periods were compared to that obtained at t = 0h and to the control one using the non-parametric χ^2 test. Moreover, using Statistica V.10 software, the non-parametric Kruskal-Wallis two-tailed test (K-W test) for multiple comparisons was used to compare the values of sinuosity to either the values at the start of weaning (t = 0) or to the control values (the latter made of 20 values out of 40 in such a way that the median and quartiles were similar to those of the 40 values) [28]. A Bonferroni adjustment is incorporated. The mathematical function describing the regression of the median value of the sinuosity on time after weaning was established using Statistica® v.10 software, and the best choice between powers of polynomial regressions was established using the procedure described in Zar [29].

Results and Discussion

Food consumption, general activity

These physiological traits were somewhat affected by diclofenac consumption (Table 1). The ants consuming it eat a little less meat than ants living under normal diet (N = 5, T = -14, P = 0.063), and drunk less sugar water, but not significantly (N = 5, T = -13, P = 0.094). They were also less, but not significantly, active (N = 6, T = -17.5, P = 0.093). Nevertheless, an increase of counted ants occurred in the course of time for the ants living under normal diet, what was not the case for the ants consuming diclofenac (Table 1, days 3, 4). It could thus be concluded that consuming diclofenac somewhat reduced the ants' activity and food intake.

Linear and angular speed

Diclofenac appeared to impact the ants' locomotion (Table 2, lines 1 and 2). It was obvious to the observer that ants consuming this drug walked erratically. In fact, they walked a little more slowly, but not significantly (χ^2 = 4.04, df = 2, 0.10 < P < 0.20), and significantly more sinuously (χ^2 = 36.46, df = 2, P < 0.001). The ants consuming diclofenac obviously seemed perturbed, and had their locomotion affected by that drug.

Orientation to an alarm signal

This ability was affected by diclofenac consumption (Table 2, line 3; Figure 2A). While under normal diet, the ants oriented themselves rather well towards a tied nestmate (making a positive taxis), but when consuming diclofenac, they badly oriented themselves, walked erratically and reached the presented tied nestmates, probably more through positive klino kinesis than through a true positive taxis. The difference of *sensu stricto* orientation between ants under normal and diclofenac diets was statistically significant: χ^2 = 24.95, df = 2, P < 0.001. We checked this decrease of orientation ability under diclofenac diet by making again the experiment and found similar results, i.e. orientation value = 65.8 (55.2 - 73.0) angular degrees. Such a weaker orientation under diclofenac diet is probably due to the ants' larger sinuosity of movement, but it may also result from a decrease of the ants' sense of perception, a hypothesis checked by a following experiment (see below, the subsection 'Tactile perception').

Audacity

Diclofenac somewhat reduced the ants' inclination to come onto a risky unknown apparatus (Table 2, line 4; Figure 2B). The numbers of ants counted on such an apparatus were significantly lower for ants consuming the drug than for ants living under normal diet (N = 5, T = -15, P = 0.031). This may be due to a physiological perturbation and was in agreement with the ants' change in locomotion (see above).

Tactile perception

This physiological trait was not affected by diclofenac consumption (Table 2, last line; Figure 2C). On the rough substrate, the ants living under normal diet as well as those consuming diclofenac moved slowly, sinuously, with difficulties. Since the drug increased the ants' sinuosity of movement on any substrate, this locomotion characteristic was, on a rough substrate, larger for ants consuming diclofenac than for ants living under normal diet. Except this difference, the ants maintained under one or the other kinds of diet had their locomotion similarly (nearly proportionally) modified on a rough substrate (the linear speeds similarly decreased: $\chi^2 = 0.39$, df = 2, $0.80 < P < 0.90$; the angular speeds similarly increased, with that under diclofenac diet as expected still larger than that under normal diet: $\chi^2 = 11.53$, df = 2, $0.001 < P < 0.01$).

Brood caring behavior

This ethological trait was not impacted by diclofenac (Table 3, line 1; Figure 2 D). Indeed, ants consuming the drug took care of their larvae and re-entered those experimentally removed from the nest such as ants living under normal diet (N = 2, T = -3, NS). Consequently, diclofenac did not affect the ants' brood caring, and thus probably their different social tasks and relationships. The next experiment examined the impact of the drug on the ants' relationships with nestmates and aliens.

Aggressiveness against nestmates

Diclofenac did not impact this ethological trait (Table 3, line 2; Figure 2 E). Ants living under normal diet as well as those consuming the drug were never aggressive againts the encountered nestmate. There was no difference between the behavior of ants living under one and the other kinds of diet ($\chi^2 = 0.72$, df = 2, $0.50 < P < 0.70$), and the variable assessing their aggressive level had similar values (ants under normal diet: 0.08; ants under diclofenac diet: 0.10). Consequently, diclofenac did not affect the ants' social relationships.

Aggressiveness against aliens

This ethological trait was only slightly affected by diclofenac consumption (Table 3, line 3; Figure 2 F). While experimenting, we observed that, compared to ants living under normal diet, those

Traits	Normal diet	Diet with diclofenac
Brood caring: n° of larvae not re-entered over 10 min	t: 30'' 2' 4' 6' 8' 10' 10 8 6 5 3 0	t: 30'' 2' 4' 6' 8' 10' 10 8 6 4 2 0
Aggressiveness:		
against nestmates:	68 84 13 0 0 0.08	55 76 14 0 0 0.10
(n° of levels 0 to 4, value of 'a')	levels: 0 1 2 3 4 'a'	levels: 0 1 2 3 4 'a'
against aliens:	7 28 85 58 37 4.86	7 28 78 53 16 4.20
Cognition: n° of ants in front (*f*) and beyond (*b*) a difficult path over 12 min	t: 30'' 2' 4' 6' 8' 10' 12' *f:* 28 24 21 17 14 10 9 *b:* 0 0 0 2 5 6 7	t: 30'' 2' 4' 6 ' 8' 10' 12' *f:* 27 21 18 15 13 13 12 *b:* 0 0 0 0 0 0 1
Escaping behavior: n° of ants in and out of the enclosure over 12 min.	t: 30'' 2' 4' 6' 8' 10' 12' in: 12 11 9 7 5 3 2 out : 0 1 3 5 7 9 10	t: 30'' 2' 4' 6' 8' 10' 12' in: 12 12 11 10 9 9 9 out: 0 0 1 2 3 3 3
Conditioning ability: score (in %) over time (in hours)	t: 7 24 31 48 55 72 score: 60 70 75 80 85 90	t: 7 24 31 48 55 72 score: 45 50 50 50 40 45

Table 3: Impact of diclofenac on five ants' physiological and ethological traits.

The ants' brood caring and aggressiveness were not impacted by diclofenac; consequently, the drug did not affect the insects' social relationships. Diclofenac largely reduced the ants' cognition, escaping ability, conditioning and short term memory, acting thus on the cerebral functions. Statistics and more details are given in the text. Min: minute; t: time; ': minute; '': second; %: proportion, 'a': variable assessing the aggressiveness = n° levels 2 + 3 +4 / n° levels 0 + 1

consuming diclofenac behaved similarly except that they seldom stung the alien opponent, and were thus often stung themselves (Figure 2 F2). The numerical results actually showed that the only difference in the numbers of aggressive levels between ants under one and the other kinds of diet concerned the stinging behavior, more frequently exhibited by ants under normal diet. The variable assessing the ants' aggressiveness equaled 4.86 for ants under normal diet, and 4.20 for those consuming diclofenac. However, on the whole, there was no statistically significant difference between the overall aggressiveness of these two kinds of ants ($\chi^2 = 6.15$, df = 3, $0.10 \sim P < 0.20$). Note that this χ^2 value resulted from a lower number of stinging presented by ants consuming diclofenac (16 and 166 other acts vs 37 and 178 other acts), what was in itself statistically significant ($\chi^2 = 5.38$, df = 1, $0.02 \sim P < 0.05$). It could thus be concluded that diclofenac did not affect the ants' social relationship (an alien stayed considered as an alien) although it somewhat reduced the ants' tendency or rapidness to kill an intruder.

Cognition

This trait was somewhat affected by diclofenac consumption (Table 3, line 4; Figure 2 G). Ants consuming this drug moved away from the small area located in front of the twists and turns path, exactly as did the ants living under normal diet (N = 7, T = +11, -17, P = 0.344), but they walked erratically through the difficult path, often came back on their way, and finally only one of them reached the large area beyond the twists and turns at the end of the 12 experimental minutes (N = 4, T = -10, P = 0.063). After having done so, this sole ant came back into the difficult way. It could thus be concluded that diclofenac somewhat reduced the ants' ability or motivation in crossing a difficult path.

Escaping ability

Diclofenac impacted this trait (Table 3, line 5; Figure 2 H). Enclosed ants living under normal diet walked along the rim of the enclosure, and when they found the exit, they often succeeded in escaping. While consuming diclofenac, the ants walked rather erratically in the enclosure. Moreover, when they came just in front of the exit, unexpectedly, they seldom went out of the enclosure. A few ants even began to go out and returned inside the enclosure. The difference of behavior between the ants living under one and the other kinds of diet was significant (N = 6, T = +21 (ants still enclosed), -21 (ants escaped), P = 0.016). The variable assessing the ants' ability in escaping equaled 0.83 for ants under normal diet and 0.25 for those consuming diclofenac. On basis of these numerical results and on the observation of the experimented ants, it could be concluded that ants consuming diclofenac were somewhat stressed or perturbed. Their cognition was perhaps reduced compared to ants living under normal diet (see the previous experiment and the here below one).

Conditioning ability, short term memory

Diclofenac decreased the ants' ability in acquiring conditioning (Table 3, last line; Figure 2 I). Under normal diet, the ants progressively reached a conditioning score of 90%. While consuming the drug, they never acquired any conditioning: they presented a score of only 45% after 72 hours of training. The difference of conditioning capability between the ants maintained under one and the other kinds of diet was significant: N = 6, T = -21, P = 0.016. This result was in agreement with those on the ants' cognition and escaping ability (see above).

Adaptation to the adverse effect of diclofenac on the locomotion (and thus the physiology)

After the ants consumed diclofenac during 13 days, their linear and angular speeds were again assessed as they had been after one day of consumption. The values obtained for the two variables were very similar to and not statistically different from those obtained after one day of ants' drug consumption (linear speed: $\chi^2 = 1.87$, df = 3, $0.50 < P < 0.70$; angular speed: $\chi^2 = 0.97$, df = 3, $0.80 < P < 0.90$) (Table 4, upper part). Consequently, the ants did not adapt themselves to the impact of diclofenac on their locomotion, and therefore on their physiology. The drug kept its adverse effects over time (Figure 3 A, B).

Adaptation:	Control	after one day	after 13 days
Linear speed in mm/s	12.6 (10.9 - 14.5)	11.9 (10.3 - 13.6)	11.5 (10.4 - 12.6)
Angular speed in ang.deg./cm	124 (112 - 142)	179 (158 - 208)	178 (166 - 201)
Dependence:	colony A	colony B	colonies A + B
n° of ants on the liquid			
- Drug-free	35	2	37
- Containing the drug	49	52	101

Table 4: Adaptation to the impact of diclofenac on the locomotion, and dependence on the drug consumption.

No adaptation to the effect of diclofenac on the ants' locomotion occurred after 13 days of that drug consumption. After 14 days of diclofenac consumption, the ants appeared to have developed some dependence on this drug consumption. More details and statistics are given in the text. ang.deg./cm: angular degrees per cm; mm/s: millimeter per second

Dependence on diclofenac consumption

Some dependence on diclofenac consumption occurred (Table 4, lower part; Figure 3 C). Ants of colony A presented only a slight preference for that drug, with 58.3% of them having chosen the liquid with the drug, what was statistically not different from a random ants' choice between the two liquids ($\chi^2 = 0.86$, df = 1, $0.30 < P < 0.50$). On the contrary, ants of colony B presented a strong significant dependence on diclofenac, 96.3% of them having chosen the liquid with the drug, what significantly differed from a random choice ($\chi^2 = 27.24$, df = 1, $P < 0.001$). Pulling the counts gave 37 ants seen on the drug free liquid and 101 ones on the liquid containing diclofenac. This corresponded to 73.2% of ants choosing the liquid with the drug, what statistically differed from a random ants' choice between the two liquids ($\chi^2 = 14.72$, df = 1, $P < 0.001$). The difference between the responses of colonies A and B may result from physiological differences between them. For instance, ants of colony A may have consumed the drug just before the experiment while ants of colony B may have not done so. An addiction can depend on the individuals' physiological state as was shown in a study on the effects of nicotine. Hungry ants became dependant of that alkaloid while well fed ones never became so [27].

As a general rule, dependence on a product occurs when the effect of that product rapidly decreased over time after its consumption was stopped [30-32]. It was thus logical to look, in the next and last experiment, to the loss over time of the effects of diclofenac after its consumption was stopped.

Loss of the effects of diclofenac once its consumption was stopped

Numerical results are given in table 5 and graphically presented in Figure 4. The two kinds of statistical analysis are in accordance with one another. The comparison of the sinuosity values obtained over time with those recorded at t = 0 showed that during the two first hours after weaning, diclofenac was fully active. After that, a quick decrease occurred till 3 hours after weaning, but it was only at 5 hours after weaning that the sinuosity became significantly different from that recorded at t = 0h. Six hours till 7 hours after weaning, the sinuosity again decreased quickly, getting then values similar to the control ones.

The decrease was also pointed out by comparing the sinuosity values obtained over time to the control ones. During the two first hours after weaning, the sinuosity values were much higher than the control ones. After that, a first quick decrease of the effect of diclofenac occurred from 2 till 3 hours, although the values recorded at 3, 4 and 5 hours after weaning were still different from the control ones. Six hours after weaning, the values no more significantly differed from the control ones, although a second quick decrease was observed till the seventh hour.

In summary, after weaning, the effect of diclofenac subsisted intact during 2 hours, then quickly decreased during one hour, continued to slowly decrease for two more hours, and about 6 hours after weaning it again rapidly decreased and then fully vanished. The two quick decreases of the effect of the drug, but essentially

Time (hours)	Ants' sinuosity (ang.deg./cm)	Chi-square test		KW test	
		vs t = 0	*vs* control	*vs* t = 0	*vs* control
0	192 (178 - 204)				
1	203 (183 - 213)	NS	P < 0.001	P = 1	P = 0.0018
2	191 (153 - 205)	NS	P < 0.001	P = 1	P = 0.0018
3	162 (141 - 182)	0.10<P<0.05	P < 0.001	P = 0.176	P = 0.0162
4	152 (146 - 180)	0.10<P<0.05	0.001<P<0.01	P = 0.1104	P = 0.0252
5	144 (125 - 167)	0.001<P<0.01	0.02~P<0.05	P = 0.0032	P = 0.0579
6	140 (107 - 157)	P<0.001	0.10<P<0.20	P = 0.0016	P = 1
7	125 (116 - 142)	P<0.001	0.50<P<0.70	P = 0.0016	P = 1
8	125 (111 - 150)	P<0.001	NS	P = 0.0016	P = 1
Control	124 (112 - 142)				

Table 5: Decrease of the effect of diclofenac on the ants' locomotion after weaning.

The ants' sinuosity, affected by diclofenac, was assessed after weaning. A first very quick decrease of the effect of the drug occurred between 2 and 3 hours after weaning a second rather quick decrease occurred between 6 and 7 hours after weaning. Such quick decreases could lead to dependence on diclofenac consumption. More information is given in the text. The present results are graphically presented in Figure 4. ang.deg./cm: angular degrees per cm; t: time. KW: non-parametric Kruskal-Wallis two-tailed test.

Figure 4: Decrease of the ants' sinuosity of locomotion after diclofenac consumption was stopped. Left graph: observed values; right graph: third-power regression curve providing the optimum fit to the observed values. A first quick decrease occurred from 2 to 3 hours after weaning, and a second rather quick one occurred from 6 to 7 hours after weaning. Such quick decreases may lead to dependence on diclofenac consumption. Numerical results and statistics are given in Table 5; details can be found in the text.
ang.deg./cm: angular degrees per cm.

the first occurring one, may induce dependence on the drug consumption [30-32].

The curve fitting the best the variation of the sinuosity values over time after the consumption of diclofenac was stopped corresponded to a third-power polynomial function, the equation of which was:

$S = 197.67 + 1.58\ t - 4.45\ t^2 + 0.30\ t^3$ with S = sinuosity and t = time in hours.

The determination coefficient R^2 equaled 0.95.

Conclusion

Warned by the ecological disastrous decline of vultures and by the report of Danish researchers on the safety of diclofenac (see the introduction section), we examined the potential harmful effects of this drug on animals presenting no inflammatory reaction, i.e. on ants as models. We found that this drug increased the ants' sinuosity of movement, that it decreased their orientation ability, audacity, cognition, escaping ability, conditioning capability, and short term memory, and that it can lead to some dependence on its consumption. The most important adverse effects are the decrease of cognition and memory and a possible development of some dependence. Moreover, no adaptation occurred to the adverse effects of diclofenac on the ants' locomotion. After weaning, the effect on the sinuosity of locomotion rapidly decreased after about 2 hours, and again after about 6 hours. When a rapid decrease of the effect of a drug is perceived by a consumer, it generally leads to some dependence; we often showed this all along our studies of the effects on ants of products consumed by humans [30-32].

Diclofenac did not affect the ants' food consumption and general activity (or only slightly), tactile perception (thus, it did not act as an anti-pain), brood caring and relationship with nestmates.

We recorded the experimental values without looking to the control ones, i.e. being rather blind to the situation. We obviously saw that under diclofenac diet the ants' behavior and physiology were modified. In agreement with the conclusions about the danger of diclofenac drawn from veterinary observations made in India [1-3], from the Danish nationwide study [8], and from the negative consequences on health related in the Medications Lexicon [33], we warn patients and practitioners against the dangers of unconsidered use of this drug.

Any inflammatory reaction is painful, and not without secondary unsafe effects. It may be associated with blood coagulation problems [34], diseases in organs [35], thrombosis [36], and psychiatric perturbations [37]. Anti-inflammatory drugs are thus necessary for humans as well as for animals. Novel anti-inflammatory drugs, safer than diclofenac, should be researched. In India, meloxicam has been proved to be of low toxicity for the vultures; it is nowadays used for cattle care, what should substantially reduce the vultures' mortality [4, 38]. However, although apparently safer than diclofenac [e.g. 39], meloxicam appears not to be without risks for the humans' health [40]. This product should thus be submitted to further experimentation on other animals.

Conflict of Interest

We affirm having no conflict of interest concerning the use of diclofenac or any other anti-inflammatory drug. We work on the ethology and the physiology of ants and received no money for making our research.

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Volume 3 Issue 3 March 2019