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Research Article

Side Effects of Chloroquine and Hydroxychloroquine Examined on Ants as Models

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Abstract

Chloroquine and hydroxychloroquine are two drugs used for treating persons suffering from malaria and auto-immune diseases such as lupus erythematosus and rheumatoid arthritis. They are efficient but used at the limit of their overdose and present several adverse effects. We examined the side effects of these drugs on ants as models, and found that they impacted their food consumption, activity, audacity, tactile perception, social relationships, cognition, and that no adaptation occurred to these adverse effects. However, no dependence occurred. Quantitative differences appeared between the side effects of the two examined drugs, chloroquine affecting more the traits requiring cognition and hydroxychloroquine the traits requiring muscle functioning. One or the other of these drugs should thus be used according to the health and physiology of the patients. Also, after weaning, the effect of chloroquine on ants vanished in 30 - 33 hours and that of hydroxychloroquine in 30 hours, though patients are advised to daily consume these drugs which have a long residence time in humans, what should lead to overdose. Therefore, for safety and reason of ease, and taking account of side effects seen in humans, we advise that chloroquine should be consumed by patients not suffering from cerebral impairments every 36 hours during 6 days and not the seventh day, while hydroxychloroquine should be daily consumed by patients not suffering from neuromuscular impairments, during 6 days and not the seventh day.

Keywords: *Cognition; Dosage; Memory; Muscles; Myrmica sabuleti; Overdose*

Abbreviations

ang.deg.: Angular Degrees; ang.deg./cm: Angular Degrees Per cm; CQ: Chloroquine; HCQ: Hydroxychloroquine; mm/s: Millimeter Per Second; χ^2: Chi-Square; *vs:* Versus; n°: Number; cm: Centimeter; mm: Millimeter; mL: Milliliter; µL: Microliter; ng; Nanogram; mg: Milligram; kg: Kilogram; s: Second; min: Minute; h: Hour; t: Time; %: Percentage

Introduction

Chloroquine and to a lower extend, hydroxychloroquine, are two 4-aminoquinolone drugs largely used for treating persons suffering from malaria as well as to prevent its contraction. They are also used for caring of persons suffering from autoimmune diseases, such as rheumatoid arthritis [1-3], systemic lupus erythematosus [3,4], Sjögrens's syndrome and osteoarthritis [3]. Since 2020, the most popularized and controversial proposed use of these two drugs is that for treating patients suffering from the recent Covid-19 pandemic caused by the SARS-CoV-2 coronavirus as they were suspected to inhibit steps of the replication of enveloped RNA viruses [5]. However, even if being effective in their medical use, chloroquine essentially, and to a lower extend hydroxychloroquine, are used at a dose approximating their toxicity threshold, and present severe side effects. Early studies stated that moderately low therapeutic over dosage of chloroquine may cause psychosis, delirium, personality changes, depression [6] and chloroquine as well as hydroxychloroquine may cause retinopathy, anorexia, nausea, neuromyopathy and central nervous system disturbances [7]. The instructions for use joined to the package of Plaquenil®, a commercialized hydroxychloroquine, warns that this drug, even if used at a therapeutic dose, may, among others, frequently cause blurred vision, skin and gastrointestinal disorders, loss of appetite (leading to anorexia), headache, emotional instability, as well as neuromuscular and cardiac disorders. Chloroquine and hydroxychloroquine have a terminal elimination half-live lasting 1 - 2 months [3] owing to a large volume of distribution in blood as well as in aqueous cellular and intracellular compartments, and resulting in long mean residence times: ca 38 days for chloroquine and 54 days for hydroxychloroquine [8,9]. This can be the explanation for involuntary drug overdose over consumption time.

The quinolones accumulate in lysosomes, change local pH concentrations and can inhibit molecular pathways involved in immune activation, this inhibiting the production of pro-inflammatory cytokines [9]. Side effects are mainly gastrointestinal effects, but the worst is retinopathy due to the affinity of quinolones with melanin-containing tissues [9].

Chloroquine and hydroxychloroquine are commercialized as a racemic mixture of their R(+) and S(-) enantiomers, but the pharmacological properties of these stereoisomers remain to be thoroughly established. It is known that in mammals the S(+) isomer of these quinolones is more rapidly eliminated than the R(-) isomer, owing to hepatic metabolism and a faster renal clearance [10], so that a mean R:S ratio of 2.2 in blood can be found after 6 months of hydroxychloroquine multiple dose treatment against rheumatoid arthritis [11]. The R:S ratio in the retina and choroid tissues of pigmented rabbits injected with hydroxychloroquine approximates 50:50 while that in albinos rabbits is 65:35, showing that the S-HCQ enantiomer has more affinity for the melanin-containing ocular tissues [12]. Although the two enantiomers of chloroquine were found to differ in efficacy and toxicity in mice against *Plasmodium berghei* and *vinckei* malaria agents [13,14], *in vitro* experiments did not show a difference in potency against a sensitive *P. falciparum* strain, although the S(+) enantiomer was slightly more active against a resistant strain than was the R(-) isomer [15]. *In vitro* also, the S-chloroquine isomer was found to be a more potent inhibitor of induced ileum smooth muscle contractions than the R isomer [16]. Discrepancies between *in vitro* and *in vivo* experiments on the quinolone enantiomers should warn us against too rapidly established conclusions about their *in vivo* efficacy, but the *in vitro* studies are often a first indicative step that cannot be disregarded. As an example, the S forms of chloroquine and hydroxychloroquine have recently been found to be 27% and 60% more active than their R forms against SARS-CoV-2 [17].

Being accustomed to study on ants as models the adverse effects of situations, products and drugs used by humans (up to now 36 products were studied [18-21]), we intended to examine in the same way the potential adverse effects of chloroquine on two ant colonies and of hydroxychloroquine on two other ant colonies. Here below, we recall, in this introduction section, why using ants as biological models and which ethological and physiological traits we aimed to examine. Then, in the following sections, we successively summarize our methods, relate our results on ants, comment them and compare them to what is nowadays known on the two considered drugs.

Most biological processes are similar for all animals, including humans (i.e. genetics, metabolism, nervous cells functioning). Invertebrates and vertebrates can thus be used as models for studying biological traits [22-24]. The use of invertebrates increases because they have a short life cycle, a simple anatomy, and are small [25,26]. Some species are commonly used, e.g. the flatworm *Dendrobium lacteum*, the nematode worm *Caenorhabditis elegans*, the mollusk *Aplysia californica*, the beetle *Tribolium castaneum*, the fruit fly *Drosophila melanogaster*, the domestic bee *Apis mellifera*. Insects, especially social *Hymenoptera*, are advantageously used [27,28]. Ants can thus be used as models, the more so they present sophisticated biological traits. They are eu-social animals, navigate using learned cues, recruit nestmates, differently mark parts of their living area, take care of their brood, clean their nest, build cemeteries [29], can recognize themselves in a mirror, are imprinted to the appearance of their congeners, learn several behaviors in the presence of older congeners [30,31] and have numerical abilities [32-35]. Their responses are influenced by the distance and the size effects, and the physiological law of Weber can be applied to them [36,37].

Since we examined on ants the side effects of products used by humans (antidepressants, analgesics, sweeteners, drugs, etc. [18-21]), we observed impacts identical to those seen in humans, as well as some ones that were not yet divulgated, and we could define information on these products such as the decrease of their efficiency after weaning, and the occurrence of some dependence.

In the present work, we intend to examine on the ant *Myrmica sabuleti* Meinert, 1861 as a model, the impact of chloroquine and of hydroxychloroquine on its food consumption, general activity, locomotion, orientation ability, audacity, tactile (pain) perception, social relationships, cognition, stress, learning ability and memory, as well as the adaptation to the side effects of these drugs, the dependence on these drugs consumption, and the decrease of their effects after their consumption was stopped. The methods used are similar to those employed in previous works [38-41].

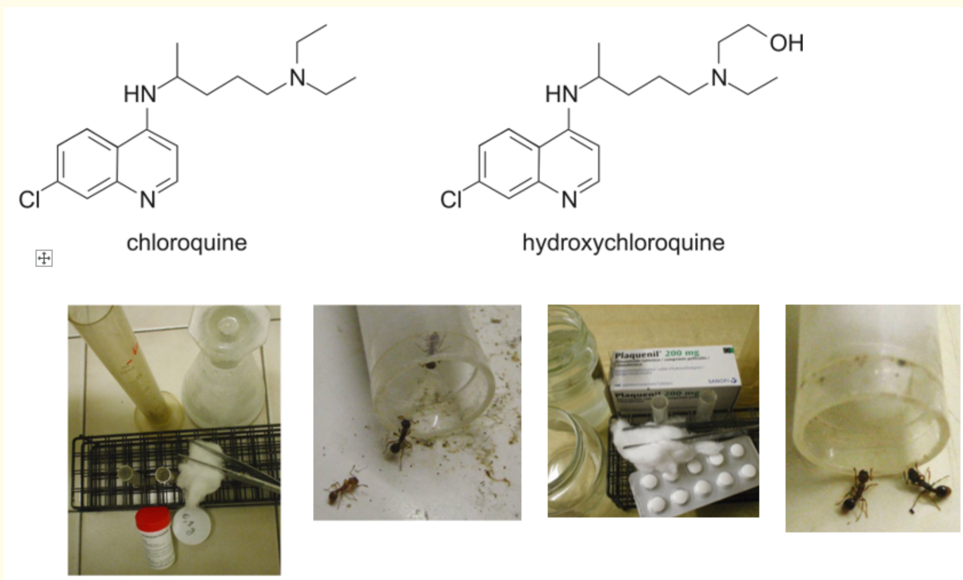

Figure 1: *Chemical structure of the two compounds the side adverse effects of which are here examined on ants as models. Making of an aqueous sugared solution of each of these substances, and consumption of these solutions by ants.*

Material and Methods

Collection and maintenance of ants

The experiments were performed on four colonies of *M. sabuleti* collected in September 2019 in an abandoned quarry located at Olloy/Viroin (Ardenne, Belgium). These colonies contained about 500 - 700 workers, a queen and brood and lived in grass as well as under stones. Each one was kept in the laboratory in one to three glass tubes half filled with water, with a cotton plug to separate the ants from the water. These nest tubes were set in a tray (34 cm x 23 cm x 4 cm) serving as a foraging area. Pieces of *Tenebrio molitor* larvae (Linnaeus, 1758) were deposited three times per week in the tray of each colony, which was moreover permanently provided with a 30% aqueous solution of sugar delivered in a tube plugged with cotton. While working on ants, the lighting of the laboratory equaled about 330

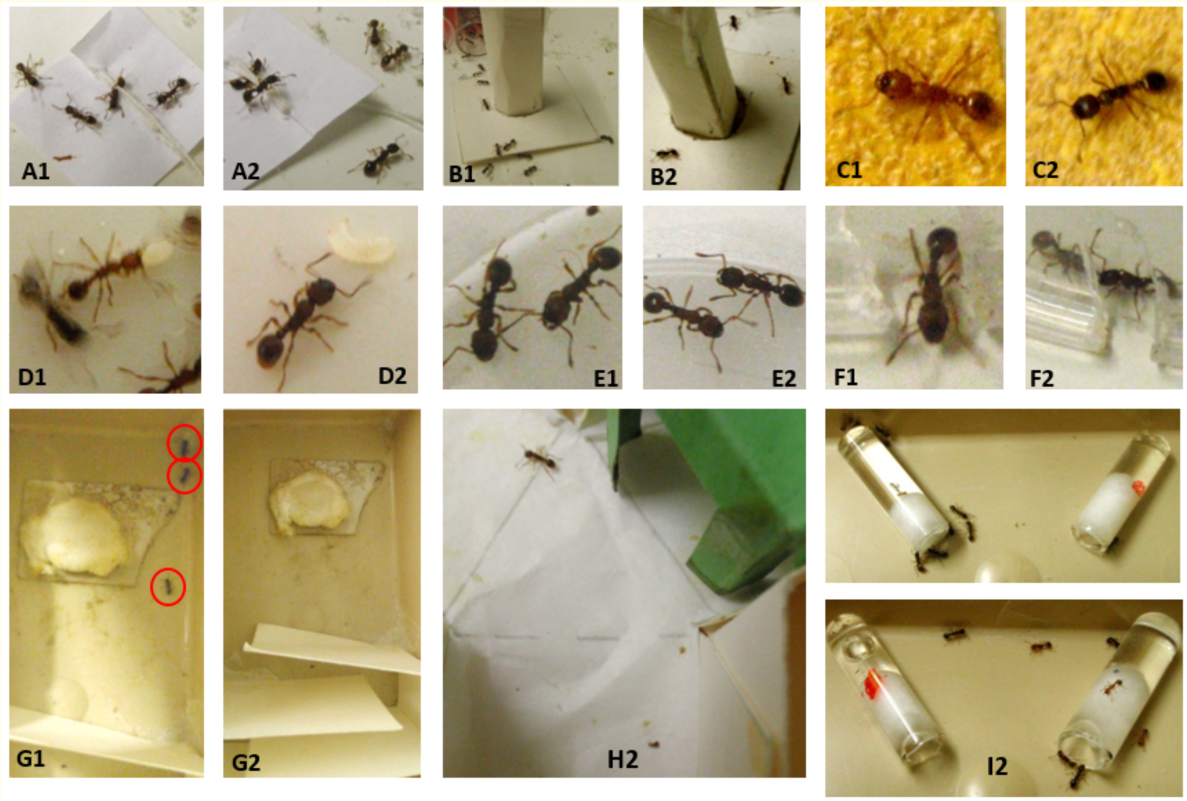

Figure 2: *Some views of the experiments made for examining effects of chloroquine on ants. Photos labeled '1'= normal diet, labeled '2'= chloroquine diet. A: ants' orientation to a tied nestmate, what was affected by the drug; B: ants coming onto an unknown apparatus, their number being reduced under the drug diet; C: ants walking on a rough substrate, with less difficulty when consuming the drug; D: an ant under normal diet transporting a larva; an ant consuming the drug only staying in front of it; E: nestmates encountering each other, and somewhat more spreading their mandibles apart while being under the drug diet; F: an ant under normal diet moving through the exit of an enclosure, and an ant under the drug diet not escaping; G: ants having to cross a path with twists and turns, and not succeeding in doing so when consuming the drug; H2: an ant under chloroquine diet giving the wrong response in the Y-apparatus; I2: ants under chloroquine diet preferring a drug-free sugar solution, and not a solution containing chloroquine (red dot). Details are given in the text and numerical results in table 2 to 5.*

lux. Permanently, the ambient temperature equaled *ca* 20°C, the humidity *ca* 80%, and the electromagnetism 2 μWm^2. Such conditions are adequate for *M. sabuleti*. In this paper, the ants are often named workers or nestmates as commonly done by researchers on social insects.

Solution of chloroquine and hydroxychloroquine given to the ants

A package of chloroquine phosphate produced by the firm Fagron (www.fagron.be, batch n° 19H07-B05-364215) and a package of hydroxychloroquine sulfate (as the medicine Plaquenil®, produced by Sanofi, Belgium) were furnished by the pharmacist Wera (Brussels, Belgium). Humans treated with these two drugs are advised to consume 100 mg of chloroquine as well as 200 mg of hydroxychloroquine per day. They absorb each of these amounts together with about one liter of water per day. Insects, and thus ants, due to their physiology and anatomy, consume about ten less water than mammals. Therefore, for maintaining the ants under a chloroquine or a hydroxychloroquine diet similar to that of humans, a solution of 100 mg chloroquine in 100 ml of the sugar water commonly given to the ants, as well as a solution of one tablet of 200 mg of hydroxychloroquine in 100 ml of this sugar water was made (Figure 1). Each solution was delivered in the ants' usual sugar water cotton plugged tubes. The cotton plug of these tubes was refreshed every 2 - 3 days and the entire solution was renewed every 7 days. It was checked each day if ants drunk the provided solutions, and effectively they did so. The control experiments were performed firstly, simultaneously on the ants of the four colonies maintained under normal diet. Then, experiments were performed on the ants of two of the colonies (A, B) living with a diet containing the solution of chloroquine, and thereafter, on the ants of the two other colonies (C, D) living with a diet containing the solution of hydroxychloroquine. Doing so allowed minimizing the time during which the ants consumed each drug.

Meat and sugar water consumption, general activity

For each kind of diet (normal, chloroquine and hydroxychloroquine), during six days, six times per day, the ants present on the meat food and those at the entrance of the sugar water tube were counted. The ants' general activity was quantified by the number of ants moving at any place (on their foraging area, near their nest entrance and inside their nest).

The ants were so counted four times during the day and twice during the night, each day at the same times o'clock and this for each of two colonies maintained under normal diet, chloroquine diet, or hydroxychloroquine diet. For each kind of diet, the mean of these 6 times X 2 colonies = 12 counts was established for each kind of behavior (eating meat, drinking, being active) (Table 1 and 7, the six first lines). The means of the six successive daily means were also calculated (Table 1 and 7, last line).

For each kind of behavior (eating, drinking, general activity), the six daily means obtained for the chloroquine diet (Table 1) and those obtained for the hydroxychloroquine diet (Table 7) were compared to the six daily means obtained under the corresponding normal diet (Table 1 and 7 respectively) using the non-parametric test of Wilcoxon [42], the level of probability being set at 0.05.

Linear and angular speeds; orientation to a tied nestmate

These three traits were quantified on foragers (i.e. ants moving in their foraging area). The speeds were measured without stimulating the ants, the orientation while presenting them with a nestmate tied to a piece of paper (Figure 2A and 3A). Such a tied nestmate emits its mandibular glands secretion which is the attractive alarm pheromone. For assessing the ants' speeds under no stimulation during one experiment and the ants' orientation under stimulation during another subsequent experiment, the trajectory of 40 foragers were recorded. These trajectories were analyzed using appropriate software [43] 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 degrees/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 successive angles made by the direction to the location and the direction of the trajectory, divided by the number of angles measured. If the obtained value of orientation is lower than 90°, the animal tends to orient itself towards the location; if the obtained value of orientation is larger than 90°, the animal tends to avoid the location. For each variable (linear speed, angular speed, and orientation), the median and the quartiles of the distribution of 40 values were established (Table 2, 8, lines 1, 2, 3). The distributions obtained for ants under chloroquine and hydroxychloroquine were compared to the corresponding distributions obtained under normal diet by using the non-parametric χ^2 test [42].

Audacity

A cylindrical tower (height = 4 cm; diameter = 1.5 cm) tied to a squared platform (9 cm^2), both in white Steinbach® paper, was deposited in the ants' foraging area [26-29]. The foragers present on this apparatus, at any place, were counted 10 times over 10 minutes (Figure 2B and 3B) and the numbers obtained for the two colonies experimented at the same time were added. The mean and extremes of the recorded numbers were established (Table 2 and 8, line 4). For statistically analyzing the results, the numbers of ants counted for the two colonies during two successive minutes were added, and the five summed values obtained for ants under chloroquine diet, as well as those obtained for ants under hydroxychloroquine diet were compared to the five summed values obtained under the corresponding normal diet by using the non-parametric test of Wilcoxon [42].

Tactile (pain) perception

Ants reaching a rough substrate and perceiving its uncomfortable character change their locomotion and walk on that substrate with difficulty, slowly, and sinuously, often touching the substrate with their antennae (Figure 2C and 3C). An ant poorly perceiving the uncomfortable character of a rough substrate walks on it more quickly and less sinuously. Therefore, to assess the ants' tactile perception, 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). The tray presented then a first 3 cm long zone, a second 3 cm long zone with the emery paper, and a last 9 cm long zone. To make an experiment on one colony, 12 ants of that colony were deposited in the first zone of the apparatus, and their trajectories on the emery paper were recorded. For each considered diet, two colonies were used, 24 trajectories being thus recorded. The ants' linear and angular speeds presented on a rough substrate could thus be quantified as described in the subsection relative to the ants' linear and angular speeds. The median and quartiles of the obtained distributions of values were established (Table 2 and 8, the two last lines), and the distributions obtained for ants consuming chloroquine or hydroxychloroquine were compared to those obtained under the corresponding normal diet by using the non-parametric χ^2 test [42].

Brood caring behavior

For each two experimented colonies at a time, a few larvae were removed from the inside of the nest and deposited outside near the entrance. Five of these larvae were observed during five minutes (Figure 2D and 3D). The larvae among these 5 ones not re-entered in the nest after 30 seconds, 1, 2, 3, 4 and 5 minutes were counted. The numbers obtained for the two colonies were added (Table 3 and 9, first line). The six summed values corresponding to ants consuming chloroquine or hydroxychloroquine were compared to the six summed values obtained for ants of the same colonies when they were under normal diet by using the non-parametric test of Wilcoxon [42].

Social relationship towards nestmates

Social insects of a same colony present no aggressiveness towards one another, but drugs may affect this native social relationship. To assess potential aggressiveness against nestmates, five dyadic encounters were performed on ants of each of the two colonies used at a time (a total of 10 encountering experiments). Each encountering occurred in a cylindrical cup (diameter = 2 cm, height = 1.6 cm), the borders of which had been slightly covered with talc to prevent escaping. During each encountering, one ant of the pair was observed during 5 min and its behavior towards the other ant was 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 (level 2), gripped and/or pulled the other ant (level 3), tried to sting or stung the other ant (level 4) (Figure 2E and 3E). The numbers of these different behaviors obtained for the two colonies were added (Table 3 and 9, line 2). The sums relative to ants under a chloroquine or a hydroxychloroquine diet were compared to the sums relative to ants under the corresponding normal diet by using the non-parametric χ^2 test [42]. The ants' level of aggressiveness was also quantified by the variable 'a', which equaled the number of recorded aggressiveness levels 2 + 3 + 4 divided by the number of recorded levels 0 + 1.

Cognition

For assessing this trait on a colony, 15 of its ants were placed in an own apparatus (Figure 2G and 3G) comprising a tray (15 cm x 7 cm x 4.5 cm) inside of which two duly folded pieces of white extra strong paper (Steinbach®, 12 cm x 4.5 cm) were inserted in order to create a twists and turns path with a small area 2 cm long in front of that path and a larger area 8 cm long beyond the path. The ants were set all together in the small area. Then, the ants still in this area and those having reached the large area beyond the difficult path were counted after 2, 4, 6, 8, 10 and 12 min. The numbers obtained for the two colonies used at a time were added (Table 3 and 9, line 3). The sums obtained for ants consuming chloroquine, as well as for those consuming hydroxychloroquine were statistically compared to those previously obtained for ants under the corresponding normal diet using the non-parametric Wilcoxon test [42].

Escaping from an enclosure

Six ants of a colony were enclosed under a reversed polyacetate cup (h = 8 cm, bottom diameter = 7 cm, ceiling diameter = 5 cm) placed on the foraging area. They were introduced into the enclosure through a hole (diameter = 3 mm) made in the ceiling of the glass. The lower part of the inner surface of the glass was slightly covered with talc to prevent the ants climbing. For allowing the ants escaping, and assessing their escaping ability, a notch (3 mm height, 2 mm broad) had been made in the rim of the bottom of the cup (Figure 2F and 3F). The ants' escaping ability was assessed by counting those escaped after 2, 4, 6, 8, 10 and 12 minutes. The results obtained for the two colonies used at a time were added (Table 3 and 9, last line). The six sums obtained for ants consuming chloroquine or hydroxychloroquine were compared to those obtained for ants living under the corresponding normal diet by using the non-parametric Wilcoxon test [42].

Visual conditioning and memory

At a given time, a green hollow cube was placed above the entrance of the sugar water tube of the two colonies that were experimented at the same time, and the pieces of *T. molitor* larvae as meat food were relocated near the cube and the sugar water tube. The foragers could see the visual cue (the cube) and the reward (the food) and associate the two stimuli (conditional and unconditional), undergoing so visual operant conditioning. The hollow cube under which ants could go was build in strong green paper (Canson®), the wavelengths reflection of which has been previously determined [44]. From the time of this set up, the ants were tested first over their conditioning acquisition, then after the cue removal, over their loss of conditioning. Ten ants of each two colonies experimented at the same time were individually tested in a Y-apparatus (Figure 2H and 3H). These Y-apparatus were made of strong white paper, had their sides slightly covered with talc to prevent escaping, had their floor covered with a thin paper changed between each test, and were set in a tray (30 cm x 15 cm x 4 cm). Also, each Y-apparatus was provided with a green hollow cube in one of its branch, half of the tests being made with the cube in its left branch and the other half with the cube in its right branch. The ants' choice of the branch containing the green cube was considered as giving the correct response. Control experiments had been previously made on ants of a distinct colony living under normal diet [45] because, if the controls had been done during the present work, the ants under drug diet would have been already conditioned, i.e. no longer naïve as for the green cube, and their conditioning ability under medication could not been examined. To make a test on a colony, 10 ants of that colony were one by one, transferred into a Y-apparatus, in the part lying before the two branches. Each ant was observed until it turned into the left or the right branch of the Y-apparatus, and its choice was recorded when it was beyond a pencil-drawn line indicating the entrance of the branch. After having been tested, the ant was kept into a polyacetate cup until the 10 ants were tested, this for not testing twice the same ant. After the test, the 10 ants were returned into their foraging area, near their nest entrance. For each test, the numbers of ants of the two colonies experimented at the same time (n = 10 ants x 2 colonies = 20 responses) and giving the correct response were recorded, and the proportion of correct responses was calculated (Table 4 and 10). The successive numbers of correct responses obtained for ants consuming chloroquine or hydroxychloroquine were statistically compared to those obtained for ants living under normal diet by using the non-parametric Wilcoxon test [42].

Adaptation (tolerance) to chloroquine and hydroxychloroquine side effects

Adaptation to a drug or a situation occurs when an individual less and less suffers from the unwanted effects of this drug or situation over time. For examining potential adaptation to a drug, a trait impacted by it must be again assessed after several days of use. In the present work, the ants' linear and angular speeds were impacted by chloroquine as well as hydroxychloroquine. They were thus again assessed after 6 days of these drugs consumption, exactly as they had been assessed after one day of consumption. The results obtained after 6 days of consumption were compared to those obtained after one day of consumption using the non-parametric χ^2 test [42].

Dependence on chloroquine or hydroxychloroquine consumption

Becoming dependent on a drug or a situation consists in wanting going on enjoying this drug or situation after having used it for some time. When an individual dependent on a drug has the choice between living with and living without the drug, it chooses living with it. In the present work, dependence on chloroquine or hydroxychloroquine was examined after the ants had consumed one or the other of these drugs during 8 days. To do so, for each two colonies under the drug diet, 15 ants were deposited in a tray (15 cm × 7 cm × 5 cm) which contained two cotton plugged tubes (h = 2.5 cm, diam. = 0.5 cm), one filled with sugar water, the other filled with the sugar solution containing the drug they consumed since 8 days. The tube containing the drug was located on the right in the tray of one colony, and on the left in the tray of the other colony (Figure 2I, 3I). Every minute during 15 minutes, the ants of each colony coming at the entrance of each tube were counted, and the 15 counts were added for each tube (Table 5 and 11). The sums obtained for the two colonies were compared to the numbers expected if the ants randomly went onto the two tube entrances, by using the non-parametric χ^2 goodness-of-fit test [42].

Decrease of the effects of chloroquine and of hydroxychloroquine after their consumption was stopped

This decrease was studied after the ants had consumed either chloroquine or hydroxychloroquine during 10 days. The experimental protocol was identical to that used in previous studies, e.g. in [38-41]. The ants were provided with a fresh solution of the drug 12 hours before weaning. After the end of these 12 hours, the ants' linear and angular speeds for chloroquine as well as for hydroxychloroquine were assessed as they had been in the course of this work except that 20 instead of 40 ants' trajectories were analyzed for being able to finalize all the assessments in the course of the experiment. Just after these assessments, i.e. at t = 0h, weaning started: the sugared solution of the drug was removed and replaced by a usual normal sugared solution. From this time, the ants' linear and angular speeds were assessed every three hours until these traits became similar to those of ants under normal diet (i.e. the control corresponding to each kind of drug). The numerical results of this study are given in table 6 and 12, and illustrated in figure 3 and 5. The distributions of the locomotion values obtained over time after weaning were compared to those obtained just before weaning (at t = 0) as well as to those obtained for ants under normal diet (i.e. controls) by using the non-parametric χ^2 test for independent samples [42]. The resulting P values (Table 6 and 12) were adjusted for multiple comparisons using the Benjamini-Hochberg procedure [46] after choosing a false discovery rate (FDR) of 0.05. For calculation of the adjusted P values, < 0.001 was used as =0.001 and intermediate P values such as $X_1 < P < X_2$ were used as $P = (X_1-X_2)/2$.

Comparison of the side effects of chloroquine and hydroxychloroquine

It is of interest to compare the two drugs by the strength of their side effects. For assessing this quantitative difference, we established for each drug and for each of the ants' traits a variable '|value obtained under normal diet - that obtained under the diet containing the drug| x 100/sum of these two experimental values' in order to compare traits continuously varying without a given limit (i.e. food consumption, general activity, locomotion speeds, orientation, audacity, social relationship), and a variable '|value obtained under normal diet - that obtained under the diet containing the drug| x 100/the maximum possible value of the examined trait' in order to compare traits varying up to a given limit (i.e. brood caring, cognition, escaping, conditioning). Moreover, dependence on the drug and duration of its effect after weaning were also compared. This is summarized in table 13.

Day	Normal diet			Diet with chloroquine		
	Meat	Sugar Water	Activity	Meat	Sugar Water	Activity
I	1.67	5.50	15.20	1.00	3.50	10.50
II	3.00	2.58	16.80	1.50	3.50	7.75
III	2.58	3.08	15.50	2.00	3.00	10.08
IV	2.50	4.50	10.50	0.50	2.92	7.50
V	2.92	7.50	10.00	0.50	1.83	7.75
VI	3.50	6.50	6.33	0.25	2.00	7.75
I - VI	3.20	4.94	12.39	0.96	2.79	8.56

Table 1: *Impact of chloroquine on ants' food consumption and general activity. For each trait, the table gives the mean of 12 daily counts, and the mean of the six obtained means. Chloroquine appeared to decrease each of the three considered traits.*

Traits	Normal diet	Diet with chloroquine
Linear speed (mm/s)	11.5 (10.0 - 13.3)	8.9 (7.9 - 9.9)
Angular speed (ang.deg./cm)	116 (102 - 126)	179 (163 - 189)
Orientation (ang.deg.)	34.8 (27.1 - 44.9)	61.1 (41.4 - 70.1)
Audacity (n°)	2.50 [1 - 4]	1.60 [1 - 2]
Tactile perception:		
Linear speed (mm/s)	4.6 (4.2 - 6.0)	8.1 (7.5 - 8.8)
Angular speed (ang.deg./cm)	287 (244 - 311)	162 (148 - 175)

Table 2: *Impact of chloroquine on five ethological and physiological ants' traits. The table gives the median (and quartiles) or the mean [and extremes] of the obtained values. Chloroquine impacted the five traits. Details are given in the text and photos are shown in figure 2.*

Results

Effects of chloroquine

Consumption of meat and sugar water, general activity

Numerical results are given in table 1. Compared to the ants' behavior under normal diet, the three traits were impacted by chloroquine consumption. Under that diet the ants eat less meat than usually, what was statistically significant (N = 6, T = -21, P = 0.016) and seemed to increase over time. The ants consuming chloroquine also drunk less sugar water (N = 6, T = -19, P = 0.047), an effect which had also some tendency to increase over time. Moreover, under chloroquine diet, the ants became less active (N = 6, T = -20, P = 0.031). These three impacts of chloroquine were very obvious for an observer watching the ants in their environment (foraging area, food sites, inside of the nest).

Traits	Normal diet	Diet with chloroquine
Brood caring: n° of not re-entered larvae over time	30s 1 2 3 4 5 min 9 7 5 3 1 0	30s 1 2 3 4 5 min 12 11 10 9 7 5
Aggressiveness towards nestmates: n° of each level	Levels 0 1 2 3 4 variable a 75 55 11 0 0 0.08	Levels 0 1 2 3 4 variable a 24 57 50 0 0 0.62
Cognition: n° of ants in front and beyond a difficult path	In front / Beyond	In front / Beyond
After 2min	19 / 0	22 / 0
4min	18 / 1	19 / 0
6min	17 / 1	18 / 0
8min	14 / 3	17 / 1
10min	11 / 5	14 / 1
12min	10 / 7	13 / 2
Escaping ability: n° of ants escaped among 12	Time: 2 4 6 8 10 12 min 2 4 4 6 8 10	Time: 2 4 6 8 10 12 min 0 1 2 3 3 4

Table 3: *Impact of chloroquine on four ethological and physiological ants' traits. The drug affected the four traits. Explanation is given in the text and photos are shown in figure 2.*

Time	Normal diet	Diet with chloroquine
Conditioning		
7 hours	55%	5 + 4 *vs* 5 + 6 45%
24 hours	60%	5 + 5 *vs* 5 + 5 50%
31 hours	60%	5 + 5 *vs* 5 + 5 50%
48 hours	65%	5 + 6 *vs* 5 + 4 55%
55 hours	65%	5 + 4 *vs* 5 + 6 45%
72 hours	70%	5 + 5 *vs* 5 + 5 50%
Cue Removal		
7 hours	65%	
24 hours	75%	
31 hours	70%	Cannot be examined
48 hours	70%	
55 hours	70%	
72 hours	70%	

Table 4: *Impact of chloroquine on the ants' conditioning. Ants consuming chloroquine could not acquire conditioning; their short and middle-term memory were thus affected. Details on the experimental protocol can be found in the text.*

Adaptation to CQ	Normal diet	1 day on CQ diet		6 days on CQ diet
Linear speed, mm/s	11.5 (10.0-3.3)	8.9 (7.9 - 9.9)		5.9 (5.3 - 6.6)
Angular speed, ang.deg./cm	116 (102-126)	179 (163 - 189)		197 (167 - 231)
Dependence on CQ after 8 days of consumption	N° of ants on the sugar water		N° of ants on the sugared CQ	
	Colony A: 29 Colony B: 21 = 75.8%		Colony A: 10 Colony B: 6 = 24.2%	

***Table 5:** Ants' adaptation to side effects of chloroquine (CQ) and dependence on its consumption. Ants did not adapt themselves to the impact of chloroquine on their locomotion, but developed no dependence on this drug consumption. Explanation can be found in the text, and photos of the experiment relative to dependence are shown in figure 2.*

Time (hours)	Linear speed (mm/s)			Angular speed (ang.deg./cm)		
	Values	*vs* t = 0	statistics *vs* control	Values	*vs* t = 0	statistics *vs* control
0 Weaning	6.0 (5.0-7.3)			222 (197-272)		
3	6.3 (5.6-6.9)	0.60	< 0.002	222 (203-247)	0.40	< 0.001
6	6.7 (6.2-7.2)	0.06	< 0.002	214 (189-237)	0.27	< 0.001
9	7.5 (6.7-8.2)	0.01	< 0.002	198 (184-217)	0.12	< 0.001
12	7.1 (6.1-7.3)	0.11	< 0.002	171 (152-220)	0.004	< 0.001
15	7.9 (7.3-8.5)	< 0.002	< 0.002	142 (122-158)	< 0.002	0.003
18	8.1 (6.9-9.1)	< 0.002	< 0.002	156 (141-177)	< 0.002	< 0.001
21	8.7 (7.7-9.6)	0.004	< 0.002	157 (129-170)	< 0.002	< 0.001
24	9.1 (8.1-10.1)	< 0.002	< 0.002	147 (141-161)	0.01	< 0.001
27	9.4 (9.0-10.6)	< 0.002	0.048	148 (138-162)	0.01	< 0.001
30	10.3 (9.0-10.8)	< 0.002	0.22	143 (132-160)	< 0.002	< 0.001
33	10.0 (9.1-10.9)	< 0.002	0.048	126 (104-144)	< 0.002	0.25
36	11.1 (9.7-11.8)	< 0.002	0.30	128 (116-149)	< 0.002	0.09
39	11.7 (9.4-12.7)	< 0.002	0.22	112 (87-120)	< 0.002	0.25
Control	11.5 (10.0-13.3)			116 (102-126)		

***Table 6:** Decrease of the effects of chloroquine after its consumption was stopped. The decrease concerned the effect of the drug on the ants' locomotion. The table gives the median (and quartiles) of the ants' linear and angular speeds obtained every three hours after weaning, as well as the results of non-parametric χ^2 tests versus the values obtained before weaning (at t = 0) and versus the corresponding control values. A graphical representation of the results is shown in figure 3. The effects of chloroquine slowly decreased with two time periods without decreasing, and totally vanished in about 39 hours. Benjamini-Hochberg adjusted P values.*

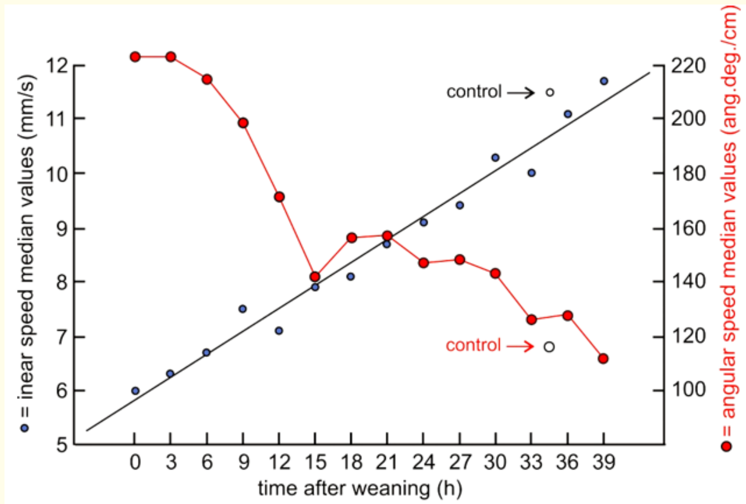

Figure 3: *Decrease of the effects of chloroquine after weaning. Numerical results are given in Table 6, and details in the text. The effect of the drug on linear speed slowly and linearly decreased after weaning while that on angular speed rapidly decreased until ca 12 hours, after what it remained rather stationary till 30 hours, and then again decreased until vanishing.*

Linear and angular speeds

The ants' locomotion was affected by chloroquine consumption: their linear speed was lower and their angular speed (i.e. the sinuosity of their walk) higher than those of ants living under normal diet (Table 2, lines 1, 2). This was statistically significant (linear speed: χ^2 = 25.08, df = 3, P < 0.001; angular speed: χ^2 = 68.55, df = 2, P < 0.001) and obvious while observing the foragers. In the subsection relative to the ants' adaptation, we examine if the ants' locomotion was still affected after 6 days of chloroquine consumption.

Orientation to a tied nestmate

Chloroquine impacted this trait (Table 2, line 3). While ants under normal diet easily oriented themselves towards a tied nestmate, those consuming the drug did so less well, with difficulty (i.e. they 'missed' the tied nestmate, walked beyond it or not exactly towards it) (Figure 2A). This statistically significant (χ^2 = 20.33, df = 2, P < 0.001) effect of the drug was also obvious to the observer. It may be due to the ants' increase of sinuosity, as well as to a decrease of their olfactory perception, the latter potential cause being considered in three following subsections (tactile perception, brood caring behavior, relationship towards nestmates).

Audacity

Numerical results are given in table 2, line 4, and show that this ethological trait was impacted by chloroquine consumption. Indeed, the ants under that drug diet were significantly (N = 5, T = 15, P = 0.031) less inclined to come onto the presented risky apparatus than those living under normal diet (Figure 2B), and this observation was also obvious while experimenting. This was in agreement with the ants' lower activity while consuming chloroquine (see the subsection relative to general activity).

Tactile (pain) perception

This physiological trait was impacted by chloroquine consumption (Figure 2C). Indeed, ants consuming that drug walked on a rough substrate at a higher linear speed and a lower angular speed than ants under normal diet (Table 2, the two last lines). The differences were significant: linear speed: $\chi^2 = 25.16$, df = 1, P < 0.001; angular speed: $\chi^2 = 34.28$, df = 1, P < 0.001. Such a decrease of tactile (pain) perception may reveal a decrease of perception, among others of the olfactory perception what could explain, at least partly, the ants' poor orientation towards a tied nestmate (see the subsection relative to orientation). A potential adverse effect of chloroquine on the individuals' perception would also affect the relation between the members of a colony, a possibility examined in the two following subsections.

Brood caring behavior

Chloroquine consumption affected this ethological trait (Table 3, line 1 and figure 2D). While consuming this drug, the ants somewhat poorly took care of their brood. They seemed to perceive the larvae less well than while living under normal diet, and 5 larvae instead of none among the 10 removed from the nest were not re-entered after 5 minutes. It also might be that the ants consuming chloroquine had some difficulty in holding the larvae and transporting them to the nest, what should imply an impact of the drug on the muscle function. Such a difference between the ants under one and the other diet was significant (N = 6, T = 21, P = 0.016). This was in agreement with the ants' poor orientation towards a tied nestmate as well as with their weaker tactile perception when they consumed chloroquine (see the two here above subsections).

Social relationship with nestmates

Chloroquine slightly affected the social relationship existing between the members of the colony. Under that drug consumption, the ants stayed less often motionless side by side, and more often spread their mandibles apart (Figure 2E). The numbers of times the ants presented the levels 0, 1, 2 and 3 of aggressiveness differed between ants living under the one and the other kind of diet (Table 3, line 2; $\chi^2 = 57.44$, df = 2, P < 0.001). Also, the variable 'a' assessing their aggressiveness level equaled 0.08 under normal diet and 0.62 under chloroquine diet. This result was in agreement with those relative to the ants' brood caring behavior, to the ants' orientation towards a tied nestmate and to the ants' tactile perception. Chloroquine may somewhat decrease the individuals' perception.

Cognition

This trait was affected by chloroquine consumption (Table 3, line 3 and figure 2G). Under normal diet, 7 ants among 30 could cross the twists and turns path within 12 minutes; under chloroquine diet, only 2 ants could do so. This difference was significant for the numbers of ants still in front of the difficult path over time (N = 6, T = 21, P = 0.016) and for the numbers of ants beyond that path over time (N = 5, P = 15, P = 0.031). It may be presumed that chloroquine somewhat affected the ants' brain functioning, what was checked in the two following subsections.

Escaping from an enclosure

This ethological and physiological trait was impacted by chloroquine consumption (Table 3, last line and figure 2F). Under normal diet, the enclosed ants erratically walked for a few seconds, then became calmer, moved essentially all along the rim of the enclosure, found the exit, and went out. Under chloroquine diet, the enclosed ants erratically walked for several minutes, what may be considered as reacting to a stressing situation, and even when moving along the rim of the enclosure, most failed in finding the exit. After the 12 experimental minutes, only 4 ants could go out while 10 ones could do so under normal diet. The difference of behavior between ants under one and the other kind of diet was significant (N = 6, T = -21, P = 0.016). Compared to those under normal diet, the ants consuming chloroquine appeared thus to be in a state of stress and maybe to have their cognitive ability somewhat reduced. This later presumption was investigated in the next experiment.

Visual conditioning and memory

These important physiological traits were affected by chloroquine consumption (Table 4 and figure 2H). Under normal diet, the ants easily acquired visual conditioning and kept it during several days, while the ants consuming the drug never could acquire any conditioning. When tested in the Y-apparatus, the latter obviously randomly went to the 'right' or the 'wrong' branch of the apparatus. On the basis of the ants' presented scores, the difference between ants under normal diet and under chloroquine diet was statistically significant (N = 6, T = -21, P = 0.016). As no conditioning occurred under chloroquine diet, it could be stated that the ants' short-term memory was affected. Due to this lack of conditioning, the ants' long-term and middle-term memory could not be assessed. However, the ants consuming the drug went on re-entering their nest, going towards their food sites, and recognizing their nestmates. It could thus be presumed that, at least to some extent, the ants' long-term memory was not affected by chloroquine consumption. As for the ants' middle-term memory, it might be somewhat impacted by the drug since even after 72 training hours, the ants still failed in acquiring conditioning to the green hollow cube.

Adaptation (tolerance) to side effects of chloroquine

No adaptation to the side effects of chloroquine occurred after 6 days of consumption (Table 5, first line). At that time, the ants' linear and angular speeds were still different from those of ants living under normal diet, and they were even more different than those presented by ants having consumed chloroquine for 1 day. The later observation was statistically significant: linear speed: $\chi^2 = 43.35$, df = 2, P < 0.001; angular speed: $\chi^2 = 13.13$, df = 2, 0.001 ~ P < 0.01. These results as well as the observation of ants moving in their foraging area showed that chloroquine essentially impacted the ants' speed of locomotion, and that this effect increased over the drug consumption, a fact that will be considered in the section relative to discussion.

Dependence on chloroquine consumption

After 8 days of chloroquine consumption, the ants developed no dependence on that drug, on the contrary (Table 5, last line and figure 2I). Indeed, only 10 ants of colony A and 6 of colony B were sighted on the sugared solution of chloroquine while 29 ants of colony A and 21 of colony B were sighted on the drug-free sugar water. This corresponded to 75.8% of ants having chosen the solution without chloroquine and 24.2% of them having chosen the solution containing the drug, a difference statistically significant ($\chi^2 = 8.31$, df = 1, 0.001 < P < 0.01). Consequently, the ants preferred a diet without chloroquine instead of a diet with it, developing thus no dependence on that drug consumption.

Decrease of the effects of chloroquine after its consumption was stopped

This was studied after the ants consumed the drug during 10 days, using the impact of the drug on the ants' locomotion. Measured on the ants' linear speed, the impact regularly and slowly decreased over time after weaning, according to a linear regression (linear speed = 5.82 + 0.14 h; $R^2 = 0.98$). It became no longer significant 30 hours after weaning and totally vanished in 39 hours (Table 6 and figure 3). Measured on the ants' angular speed, the impact of the drug rapidly decreased until 12 hours, after what it became more or less stationary until 28-30 hours, and then vanished in a total of 39 hours (Table 6 and figure 3). In the whole, about 15 hours after weaning, the effect of chloroquine was no longer equal to its initial one, the side effects of the drug significantly vanishing in 30-33 hours, and totally in 39 hours.

Effects of hydroxychloroquine

Meat and sugar water consumption, general activity

Hydroxychloroquine affected these three physiological traits (Table 7). Under this drug diet, the ants consumed less meat than ants living under normal diet, a difference statistically significant (N = 6, T = -21, P = 0.016). They also consumed less sugar water, but this difference was only near the limit of significance (N = 6, T = +2.5, -18.5, P = 0.063). The ants under hydroxychloroquine diet were somewhat less active than those maintained under normal diet (N = 6, T = +1, -20, P = 0.031). These impacts of hydroxychloroquine on the ants' physiology were obvious to the observer who also saw great differences between the individuals all along the experimental work.

Linear and angular speeds

Hydroxychloroquine impacted this physiological trait (Table 8, lines 1, 2). Ants consuming this medicine walked at a lower linear speed and at a higher angular speed than ants living under normal diet, with great variability among the individuals. These differences were significant: linear speed: $\chi^2 = 61.15$, df = 2, P < 0.001; angular speed: $\chi^2 = 57.39$, df = 2, P < 0.001. This was also obvious while observing the experimented ants, some of them walking with great difficulty. Six days later, it was examined if the ants could adapt themselves to this strong adverse effect of hydroxychloroquine after they had consumed that drug during these 6 days (see below, subsection on adaptation).

Orientation to a tied nestmate

This ethological trait was impacted by hydroxychloroquine consumption (Table 8, line 3 and figure 4A). While ants under normal diet easily reached a tied nestmate, those consuming hydroxychloroquine often failed to do so (their median angle of orientation often overtook 45 angular degrees). This difference was significant: $\chi^2 = 48.36$, df = 2, P < 0.001. This may be due to a high sinuosity of movement, as well as to some impact of the drug on the olfactory perception, what was checked by the following experiments (see the subsections relative to brood caring and social relationship).

Audacity

Hydroxychloroquine affected this ethological trait (Table 8, line 4 and figure 4B). The ants consuming this drug were significantly (N = 5, T = 15, P = 0.031) less inclined to come onto the presented unknown apparatus than those living under normal diet. Hydroxychloroquine affected thus the ants' behavior, what was in agreement with the ants' lower general activity previously observed (see above the subsection relative to such activity).

Tactile (pain) perception

This physiological trait was impacted by hydroxychloroquine consumption (Table 8, the two last lines and figure 4C). On a rough substrate, with a large variability between the individuals, the ants consuming the drug moved more quickly and less sinuously than ants living under normal diet, perceiving thus less well the uncomfortable character of the substrate. This difference of perception was significant: linear speed: $\chi^2 = 24.00$, df = 1, P < 0.001; angular speed: $\chi^2 = 33.56$, df = 1, P < 0.001). Hydroxychloroquine affected thus the ants' sensitive perception, what agreed with their poor perception of the alarm pheromone emitted by a tied nestmate (see the above subsection relative to the ants' orientation to such a nestmate). Such a decrease of perception may also explain the results of the two following experiments.

Brood caring behavior

Hydroxychloroquine affected this behavior (Table 9, line 1 and figure 4D). The ants consuming this drug presented broad difficulties in taking, holding and transporting a larva. They took a long time before reaching the nest entrance with a not correctly held larva. Consequently, the numbers of not re-entered larvae over the 5 experimental minutes was higher than those counted for ants under normal diet, and this difference was significant (N = 6, T = + 21, P = 0.016). On the basis of our observations, this may be due to some muscular disfunctioning, a presumption in agreement with the lower linear speed of ants consuming hydroxychloroquine.

Social relationship towards nestmates

Hydroxychloroquine consumption somewhat impacted this ethological trait. While ants living under normal diet were not at all aggressive towards one another and often stayed aside doing nothing, those consuming the drug avoided nestmates and presented some aggressive reaction in front of them (i.e. spreading the mandibles apart) (Table 9, line 2 and figure 4E). This difference of behavior between ants under the two kinds of diet was significant ($\chi^2 = 28.25$, df = 2, P < 0.001), and the variable 'a' assessing this behavior also showed such a difference. It may be due to a poorer perception of the colony odor by ants consuming hydroxychloroquine, what would be in agreement with these ants' poorer perception of their specific alarm pheromone (see the subsection relative to orientation), and maybe to their decrease of tactile (pain) perception (see the subsection relative to this perception).

Day	Normal diet			Diet with hydroxychloroquine		
	Meat	Sugar Water	Activity	Meat	Sugar Water	Activity
I	1.25	1.50	9.00	1.15	2.00	7.75
II	2.50	1.75	9.75	0.40	0.40	7.08
III	1.25	1.42	10.00	0.50	0.50	7.25
IV	1.50	1.50	9.00	0.48	0.50	7.50
V	1.00	2.50	9.50	0.50	2.00	10.50
VI	1.50	2.00	12.25	0.75	1.58	10.00
I - VI	1.50	1.78	9.92	0.65	1.16	8.35

Table 7: *Impact of hydroxychloroquine on the ants' food consumption and general activity. For each trait, the table gives the mean of 12 daily counts (I to VI) and the mean of the six obtained means (I – VI). Hydroxychloroquine consumption decreased the three considered traits.*

	Normal diet	Diet with hydroxychloroquine
Linear speed (mm/s)	11.8 (11.0 - 12.9)	7.2 (6.3 - 8.0)
Angular speed (ang.deg./cm)	114 (95 - 126)	199 (181 - 217)
Orientation (ang.deg.)	25.1 (19.8 - 34.9)	60.3 (50.4 - 71.6)
Audacity (n°)	2.25 [1 - 3]	0.95 [0 - 2]
Tactile perception:		
Linear speed (mm/s)	5.0 (4.2 - 6.3)	8.6 (7.9 - 9.3)
Angular speed (ang.deg./cm)	284 (238 - 312)	168 (149 - 186)

Table 8: *Impact of hydroxychloroquine on five ethological and physiological ants' traits. The table gives the median (and quartiles) or the mean [and extremes] on the obtained values. Hydroxychloroquine impacted all the traits. Details are given in the text, and photos are shown in figure 4.*

Traits	Normal diet	Diet with hydroxychloroquine
Brood caring: n° of not re-entered larvae over time	30s 1 2 3 4 5 min 9 6 4 3 1 0	30s 1 2 3 4 5 min 10 10 10 8 6 4
Aggressiveness towards nestmates: n° of each level	Levels 0 1 2 3 4 variable a 78 50 13 0 0 0.10	Levels 0 1 2 3 4 variable a 42 67 42 0 0 0.39
Cognition: n° of ants in front and beyond a difficult path	In front beyond	In front beyond
After 2min	24 0	20 0
4min	22 0	19 0
6min	18 2	19 0
8min	14 4	17 1
10min	11 6	16 1
12min	11 7	15 1
Escaping ability: n° of ants among 12 escaped over time	Time: 2 4 6 8 10 12 min 2 4 6 8 9 10	Time: 2 4 6 8 10 12 min 2 4 4 5 5 6

Table 9: *Impact of hydroxychloroquine on four ethological and physiological ants' traits. Hydroxychloroquine affected the ants' social relationship (lines 1, 2) as well as their cognition and state of stress (lines 3, 4), though somewhat less than did chloroquine (see table 3). Explanation can be found in the text, and photos in figure 4.*

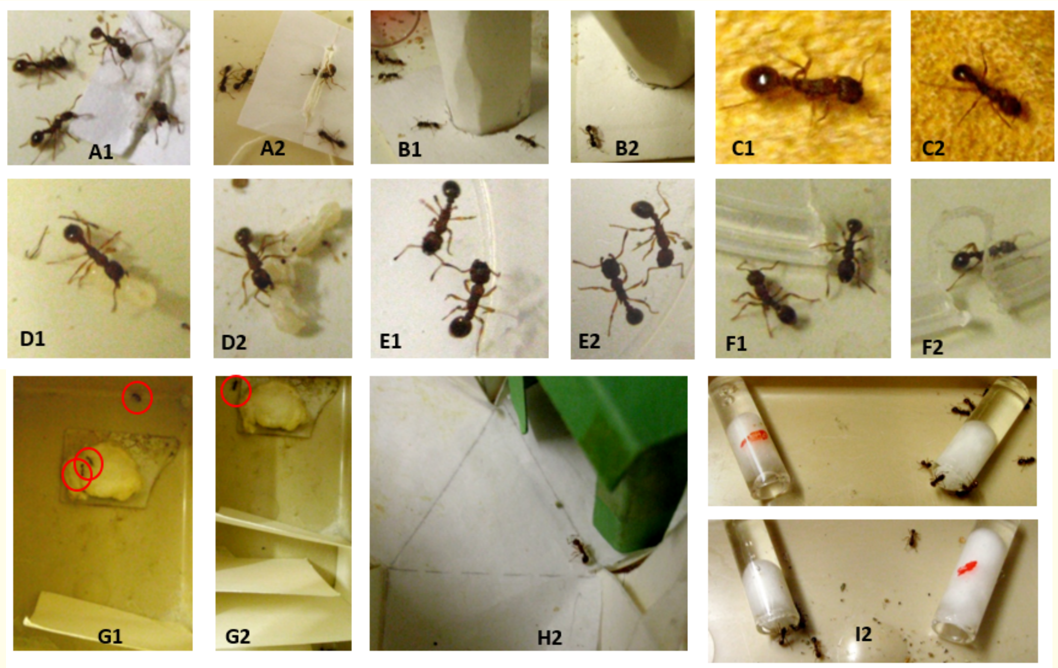

Figure 4: *Some views of the experiments made for examining effects of hydroxychloroquine on ants. Photos labeled '1'= normal diet, labeled '2'= hydroxychloroquine diet. A: ants' orientation towards a tied nestmate; B: ants' coming onto an unknown apparatus; C: ants walking on a rough substrate, with difficulty under normal diet. D: an ant in front of a larva removed from the nest, unable to hold it under hydroxychloroquine diet. E: two nestmates, presenting aggressiveness under hydroxychloroquine diet; F: ants in an enclosure near the exit of it; G: ants having to cross a path with twists and turns; H: an ant under hydroxychloroquine diet hesitating in giving the right response in the Y-apparatus; I: ants under hydroxychloroquine diet in front of a solution containing that drug (red dot) and a solution free of drug, and preferring the later one. Details are given in the text, and numerical results in table 8 to 11.*

Time	Normal diet	Diet with hydroxychloroquine
Conditioning		
7 hours	55%	4 + 6 *vs* 6 + 4 50%
24 hours	60%	6 + 4 *vs* 4 + 6 50%
31 hours	60%	6 + 6 *vs* 4 + 4 60%
48 hours	65%	6 + 6 *vs* 4 + 4 60%
55 hours	65%	6 + 8 *vs* 4 + 2 70%
72 hours	70%	6 + 8 *vs* 4 + 2 70%
Cue Removal		
7 hours	65%	8 + 8 *vs* 2 + 2 80%
24 hours	75%	7 + 6 *vs* 3 + 4 65%
31 hours	70%	6 + 7 *vs* 4 + 3 65%
48 hours	70%	7 + 7 *vs* 3 + 3 70%
55 hours	70%	7 + 7 *vs* 3 + 3 70%
72 hours	70%	8 + 8 *vs* 2 + 2 80%

Table 10: *Impact of hydroxychloroquine on the ants' conditioning. Hydroxychloroquine did not affect the ants' conditioning ability, and thus their short and middle-term memory. It did not impact the ants' long-term memory. Explanation about the method and the results is given in the text and a photo is shown in figure 4.*

Adaptation to HCQ	Normal diet	1 day on HCQ diet	6 days on HCQ diet
Linear speed, mm/s	11.8 (11.0 - 12.9)	7.2 (6.3 - 8.0)	6.8 (6.2 - 7.8)
Angular speed, ang.deg./cm	114 (95 - 126)	199 (181 - 217)	198 (176 - 223)
Dependence on HCQ after 8 days of consumption	N° of ants on the sugar water	N° of ants on the sugared HCQ	
	Colony C: 48 Colony D: 38 = 87.6%	Colony C: 7 Colony D: 5 = 12.4%	

Table 11: *Ants' adaptation to side effects of hydroxychloroquine (HCQ) and dependence on its consumption. Ants did not adapt themselves to the side effect of hydroxychloroquine on their locomotion, but this effect did not increase much over time. They developed no dependence on that drug consumption; on the contrary, they appeared to avoid it. Details are given in the text, and two photos are shown in figure 4.*

Time (hours)	Linear speed (mm/s)			Angular speed (ang. deg./cm)		
	Values	*vs* t = 0	statistics *vs* control	Values	*vs* t = 0	statistics *vs* control
0 Weaning	6.0 (5.7-7.1)			202 (183-226)		
3	6.6 (6.1-7.3)	0.50	<0.001	200 (167-212)	0.75	<0.001
6	6.8 (6.1-7.5)	0.50	<0.001	203 (178-215)	0.75	<0.001
9	7.0 (6.4-7.8)	0.50	<0.001	203 (178-223)	0.75	<0.001
12	8.1 (7.0-8.9)	0.11	<0.001	175 (165-194)	0.11	<0.001
15	8.5 (7.2-9.4)	<0.002	<0.001	161 (151-190)	0.042	<0.001
18	8.9 (8.3-10.2)	<0.002	<0.001	163 (154-185)	0.030	<0.001
21	8.9 (8.3-10.6)	<0.002	<0.001	147 (128-176)	<0.003	<0.001
24	9.5 (8.8-10.3)	<0.002	<0.001	133 (112-161)	<0.003	<0.001
27	10.0 (9.3-11.6)	<0.002	<0.001	127 (114-153)	<0.003	0.003
30	11.8 (10.0-12.7)	<0.002	0.40	119 (103-129)	<0.003	0.40
Control	11.8 (11.0-12.9)			114 (95–126)		

Table 12: *Decrease of the effect of hydroxychloroquine after its consumption was stopped. The decrease was studied using the impact of the drug on the ants' locomotion. The table gives the median (and quartiles) of the ants' linear and angular speeds assessed every three hours after weaning, and the results of non-parametric χ^2 tests versus the values obtained before weaning (at t = 0) and versus the control values. Details can be found in the text and a graph of the results is shown in figure 5. The effects of hydroxychloroquine totally vanished in 30 hours. Benjamini-Hochberg adjusted P values.*

Cognition

This trait was only slightly affected by hydroxychloroquine consumption (Table 9, line 3 and figure 4G). While 19 among 30 ants under normal diet went across the twists and turns path, 15 among 30 ants consuming the medicine did so, the difference being not significant (N = 6, T = -7, +14, P = 0.281). The ants consuming hydroxychloroquine were thus nearly as prompt as those not consuming this medicine to leave the narrow space lying in front of the twists and turns. However, while 7 ants under normal diet succeeded in reaching the zone located beyond the difficult path, only 1 ant consuming hydroxychloroquine could do so, the difference of the numbers of ants beyond the difficult path being near the limit of significance, due to the sample smallness (N = 4, T = -10, P = 0.063). Consequently, though a large variability existed between individuals, ants consuming the examined medicine were less able to find their way through a twists and turns path. This may be due to their large sinuosity of movement (see the subsection relative to the ants' linear and angular speeds) as well as to their difficulty in walking along a difficult path, maybe due to impact of the drug on muscle functioning. This might also be due to some impact of the drug on the ants' cognitive abilities, a presumption investigated thanks to the two following experiments.

Escaping from an enclosure

This physiological and ethological trait, which varies according to the state of stress and the cognitive abilities of the experimented individuals, was slightly impacted by hydroxychloroquine consumption (Table 9, last line and figure 4F). Though during the first four minutes of the experiment 4 ants living under normal diet as well as 4 ants consuming the drug could escape from the enclosure, at the end of the 12 experimental minutes, only 6 ants consuming the drug escaped while 10 ants under normal diet did so. The difference between the

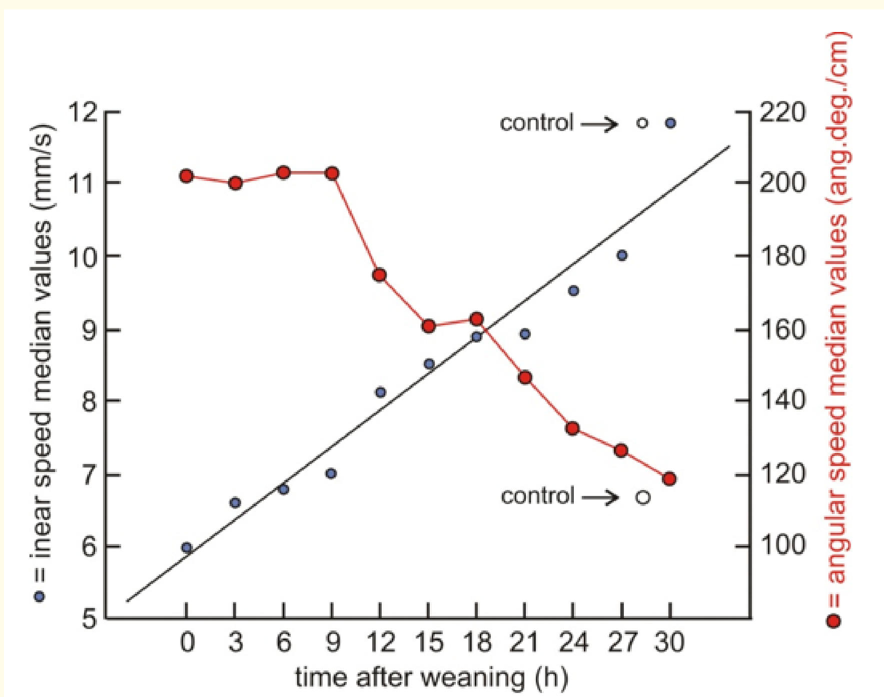

Figure 5: *Decrease of the effects of hydroxychloroquine after weaning. Numerical results are given in table 12, and details in the text. The effects of the drug slowly decreased in 28 -30 hours after weaning, with two time periods without decrease (0 - 9 hours and ca 15 - 18 hours after weaning).*

ants maintained under one and the other kinds of diet was at the limit of significance (N = 4, T = -10, P = 0.063). It could thus be concluded that ants consuming hydroxychloroquine were a little more excited than those living under normal diet and may have their cognitive abilities impacted. The latter presumption was checked thanks to the following experiment.

Visual conditioning and memory

The ants' acquisition of conditioning was not significantly impacted by hydroxychloroquine consumption (Table 10 and figure 4H). During the first 24 hours, the ants consuming this drug hesitated during a rather long time before choosing one or the other branch of the Y-apparatus. Then, they became more rapid in making their choice and finally often gave the correct response. This revealed a slight impact of the drug on the ants' cognition and on their short and middle-term memory, though, in fine, over the 72 experimental hours, their conditioning scores did not statistically differ from those presented by ants living under normal diet (N = 4, T = -8, +2, P = 0.188). After removal of the cue, the ants living under normal diet kept their conditioning during at least 72 hours, with an overall mean score of 70%, and ants consuming hydroxychloroquine did so also with an overall mean score of 71.7% (Table 10, means of 6 successive scores). Also, there was no statistical difference between the successive conditioning scores obtained over time by the ants living under one or

Traits	Chloroquine			Hydroxychloroquine		
	Normal Diet	Drug Diet	% Impact	Normal Diet	Drug Diet	% Impact
Meat consumption	3.20	0.96	53.8%	1.50	0.65	39.5%
Sugar water consumption	4.94	2.79	27.8%	1.78	1.16	21.1%
General activity	12.39	8.56	18.4%	9.92	8.35	8.6%
Linear speed	11.5	8.9	12.7%	11.8	7.2	24.2%
Angular speed	116	179	21.4%	114	199	27.2%
Orientation	34.8	61.1	27.4%	25.1	60.3	41.2%
Audacity	2.50	1.60	22.0%	2.25	0.95	40.6%
Tactile perception						
Linear speed	4.6	8.1	27.6%	5.0	8.6	26.5%
Angular speed	287	162	27.8%	284	168	25.7%
Brood caring	10	5	50.0%	10	6	40.0%
Social relationship	0.08	0.62	77.1%	0.10	0.39	29.0%
Crossing a difficult path	7	2	16.6%	7	1	20.0%
Escaping behavior	10	4	50.0%	10	6	33.3%
Conditioning: final score	70%	50	20.0%	70%	70%	0%
Memory: mean of 6 scores	70%	Irrelevant	Irrelevant	70%	71%	~ 0%
Adaptation						
Linear speed	8.9	5.9	20.3%	7.2	6.8	2.9%
Angular speed	179	197	4.8%	199	198	0.0%
Dependence		24.0%			12.4%	
Time for loss of effect		~30-33 h			30 h	

Table 13: *Side effects of chloroquine and hydroxychloroquine: a comparison. For each trait, the table gives the experimental values obtained for ants under normal and drug diets (values retrieved from tables 1-12), and the percentage of the impact of the drug calculated as explained in 'Materials and Methods', in the subsection relative to the present comparison. The side effects of the two drugs were qualitatively similar but quantitatively different, chloroquine impacting more the traits requiring cognition and hydroxychloroquine more those requiring muscle functioning.*

the other kind of diet (N = 4, T = +6.5, -3.5, P = 0.375). Consequently, hydroxychloroquine consumption did not affect the ants' long-term memory. Effectively, ants under hydroxychloroquine consumption continued to find their way to the food sites and the nest entrance.

Adaptation (tolerance) to side effects of hydroxychloroquine

No adaptation occurred to the effect of hydroxychloroquine on the ants' locomotion (Table 11, upper part). After 6 days of that drug consumption, the ants' linear and angular speeds were nearly identical to those they presented after one day of that drug consumption. There was no statistical difference between the values obtained for these two variables for ants living under one or the other kind of diet: linear speed: $\chi^2 = 0.31$, df = 1, $0.80 < P < 0.90$; angular speed: $\chi^2 = 0.14$, df = 1, $0.90 < P < 0.95$. However, the impact of the drug seemed to not worsen much over this drug consumption; it seemed to stay at its initial level, a presumption checked just before weaning, when aiming to study the decrease of the effect of hydroxychloroquine after its consumption was stopped (see the subsection relative to this decrease).

Dependence on hydroxychloroquine consumption

The ants developed no dependence at all on hydroxychloroquine consumption (Table 11, lower part and figure 4I2). Indeed, 48 ants of colony C and 37 of colony D were counted at the entrance of the tube containing the sugar water free of the drug, while only 7 ants of colony C and 5 of colony D were counted at the entrance of the tube containing the sugared solution of the drug. This corresponded to 87.6% of the ants' choices of the solution free of hydroxychloroquine and 12.4% of the ants' choices of the solution containing the drug. The difference between these two proportions was significant in favor of the drug-free liquid ($\chi^2 = 30.99$, df = 1, $P < 0.001$). Consequently, the ants did not become dependent on hydroxychloroquine consumption, and when having the choice, they contrarily avoided consuming the drug.

Decrease of the effects of hydroxychloroquine after its consumption was stopped

Numerical results are given in table 12 and are graphically presented in figure 5.

The values obtained at t = 0, just before weaning, showed that the ants, having been under hydroxychloroquine for ca 10 days, did not adapt themselves to the side effects of hydroxychloroquine which very slightly increased over time. The impact on the ants' linear speed appeared to regularly and linearly decrease (linear speed = 5.86 + 0.17 h; R2 = 0.94) and became statistically different from the initial one (that at t = 0) since 15 hours after weaning. The ants' linear speed remained different from that presented under normal diet until 27 hours after weaning and became similar to the control value only 30 hours after weaning. Consequently, on basis of the ants' linear speed, hydroxychloroquine was still efficient during 28 - 29 hours after its consumption was stopped. As for the values of the ants' angular speed, they stayed similar to the initial one (that at t = 0) until about 15 hours after weaning. They stayed different from that presented under normal diet until 27 hours after weaning and became similar to the control values only 30 hours after weaning. Consequently, on basis of the ants' angular speed, hydroxychloroquine remained efficient during 28 - 29 hours after the end of its consumption, what was also deduced from the results relative to linear speed.

The decrease of the effect of hydroxychloroquine was very slow or null during the first nine hours and perhaps somewhat again between about 15 and 18 hours after weaning. These stationary periods allowed to slow the loss of the hydroxychloroquine efficiency, what was in agreement with the absence of dependence on this drug consumption (the relation between slow decrease and absence of dependence has often been observed [47]).

Comparison of the side effects of chloroquine and hydroxychloroquine

This comparison is numerically presented in table 13. It appeared that the adverse effects of chloroquine and hydroxychloroquine were qualitatively identical but not quantitatively. Chloroquine impacted more than hydroxychloroquine the ants' food consumption, activity, social relationship, escaping behavior (which requires cognition and absence of stress), and conditioning (i.e. learning and memorization). Hydroxychloroquine affected more than chloroquine the ants' locomotion, orientation, audacity (coming on a risky apparatus), and crossing a difficult path. Brood caring was impacted by each of the two drugs but in different ways: ants consuming chloroquine had their social relationship largely impacted while ants consuming hydroxychloroquine had muscular weakness revealed by difficulties in holding larvae. The two drugs similarly affected the ants' tactile perception. In fact, besides the latter trait similarly affected by the two drugs, chloroquine impacted essentially the traits requiring cognitive abilities while hydroxychloroquine affected essentially the traits which required muscle functioning. No adaptation occurred to the adverse effects of the two drugs, but over time the side effects increased more for chloroquine than for hydroxychloroquine. No dependence on each of the two drugs appeared, the ants even avoiding them and this avoidance was stronger for hydroxychloroquine than for chloroquine. As far as can be deduced from their effects on ants, on the whole, chloroquine appears to be less safe than hydroxychloroquine.

Discussion and Conclusion

Chloroquine and hydroxychloroquine are two drugs the action and adverse effects of which are still largely debated, the more so since they are used for treating persons suffering from the coronavirus responsible for the 2020 pandemic. Working on ants as models, we tried to somewhat clarify the situation as for the side effects of these drugs. Table 13 summarizes a comparison of the effects of the two examined medicines on ants used as a model. Chloroquine appeared to decrease the ants' food consumption, general activity, locomotion, orientation ability, audacity, tactile perception, social relationships, cognition, learning and memory, and to increase their state of stress. It thus essentially impacted the traits requiring cognition. No adaptation occurred to the side effects which, on the contrary, as measured by the ants' locomotion, appeared to increase over the drug consumption. Chloroquine leaded to no dependence at all and when having the choice, the ants even preferred food without that drug. After the consumption of chloroquine was stopped, its effect vanished slowly in about 30 - 33 hours, with an absence of decrease or a very slow one from about 15 till 30 hours after weaning. Hydroxychloroquine impacted nearly all the same traits as those impacted by chloroquine, but otherwise or quantitatively differently. It did not affect the ants' memory, poorly affected the traits requiring cognition, but largely impacted those requiring muscle functioning. For instance, it impacted brood caring because the ants had difficulties to hold the larvae. No adaptation occurred to these side effects, which did not increase over the drug consumption. Hydroxychloroquine leaded to no dependence at all; the ants even avoided consuming it. After weaning, the effect of hydroxychloroquine stayed stationary for about 9 hours, then slowly decreased and vanished in 30 hours. The somewhat slow decrease of the effect of the examined drugs after weaning is in agreement with the lack of dependence on their consumption, a causal connection many times observed during drugs use [47].

Our results call for some other remarks. The ants' decrease of food consumption, activity and locomotion was very obvious when comparing the appearance of the colonies maintained under the two drugs diet with other colonies maintained under normal diet. The impact of the examined drugs on the ants' orientation, tactile perception and social relationship may partly result from a decrease of the ants' sensitive (olfactory, tactile, visual) perception. On the other hand, the decrease of linear speed and of larvae transport (two traits requiring good muscle function) may be caused by an impact of the drug on the muscular and/or the nervous systems. The decrease of the ants' cognition and escaping ability under chloroquine consumption may be at least partly explained by an increase of the ants' stress under such a diet. The decrease of the ants' learning and memory caused by chloroquine consumption may reveal some impact of the drug on the ants' brain. Overall, taking account of all the examined traits, hydroxychloroquine seemed to be somewhat less toxic than chloroquine (see again the comparison summarized in table 13).

Our observations have to be compared to what is known on the clinical effects of the two examined drugs. A comparison of these two medicines has been made from a medical point of view by Ruiz-Irastorza., *et al.* [48]. As we, these authors concluded that hydroxychloroquine should be preferred to chloroquine when considering their adverse effects. The muscular weakness we observed in ants under hydroxychloroquine diet may correspond to cases of neuromyotoxicity and myopathy observed in humans, although they are rare events [49], most of these cases having been found to be associated with cardiac myotoxicity, including muscle fiber atrophy with myocyte vacuolization and sarcoplasmic bodies [50-52]. Central nervous system (CNS) side effects (among others, headaches, nightmares [4,53]) may occur due to cerebral cortex stimulation. It may be that some of the side effects of chloroquine and hydroxychloroquine can be explained by the same mechanisms as those leading to beneficial effects. Bhattacharyya., *et al*. [54], experimenting on rats, found that chloroquine impacts the functioning of important enzyme complexes such as the lysosomal enzyme system, antioxidant enzymes, NADPH-induced lipid peroxidation, and the cellular glutathione content.

The CQ/HCQ retinopathy is influenced not only by the daily dose, the maximum amount of which has to be established in function of the body weight and of the kind of quinolone compound used [7], but also by the duration of use and by the cumulative dose [55]. Dose-response assessment remains thus a priority for a safe and efficient treatment [9]. As far as our results on ants can be applied to humans, we can suggest two rules for treating patients with chloroquine or hydroxychloroquine while minimizing the adverse effects of these drugs. First, cerebrally fragile patients should be cared thanks to hydroxychloroquine, while those with muscular problems should be cared thanks to chloroquine. Secondly, according to the instructions for use joined to the drugs, patients consume one or the other of these drugs every 24 hours. Doing so finally leads to side effects similar to those caused by overdose. For avoiding such an occurrence, thus for security reasons and for the ease of use, chloroquine should be consumed every 36 hours during 6 days and no longer the 7th day, while hydroxychloroquine should be daily consumed during 6 days and no longer the 7th day, all this at the usual dose of 100mg of chloroquine and 200mg of hydroxychloroquine per day.

In fact, research remains to be conducted in order to use these drugs the most efficiently and safely as possible; among others, it should be looked to a possible differential potency and toxicity of their stereoeisomers.

Chloroquine is a chemical derivative of quinine, a drug used since a long time for treating persons suffering from malaria and for preventing contracting that illness. We have previously studied on ants as models the physiological and ethological side effects of quinine [56]. Though chloroquine and quinine are rather similar chemical compounds, their physiological and ethological effects on living organisms largely differ. The effects of chloroquine here examined are summarized in the first alinea of the Discussion section. As for quinine, we found that it increased the ants' activity and maybe their food consumption; it did not affect their orientation, audacity and social relationship; but increased their cognition, learning and memorizing abilities. No habituation to and no dependence on quinine occurred. The effects of quinine on ants vanished in about 10 hours after weaning, what is followed by a recovery period (half-life in humans is 11h: [57]). Humans are advised to consume quinine every 24 hours, thus far after its effects vanished, what unfortunately allows the development of the sexual cells of the parasite causing malaria (*Plasmodium spp.*) and therefore its sexual reproduction. Being active during more than 24 hours after weaning, chloroquine allows preventing this sexual reproduction. However, due to adverse effects, we go on suggesting consuming chloroquine only every 36 hours during 6 days and no longer the seventh day.

Along the present experimental work, we observed a large individual variability concerning the state of health of the ants while they consumed the drugs, as inferred from their locomotion, food consumption, and general activity (such as foraging, transporting corpses and refuse, getting involved in social relationships). The young ants appeared to be the most affected by the drugs (personal observation). An important individual variability was also found in humans. After 43 patients had received at least during 6 months a racemate-HCQ therapy to treat rheumatoid arthritis, the R:S ratio of the HCQ enantiomers in the blood displayed a two to three fold inter-subject variability [11] while a 11-fold range of concentration was observed [58]: patients should thus be treated on a case-by-case basis.

In conclusion, although much remains to be elucidated about the action of chloroquine and hydroxychloroquine, these two drugs are efficient and should be used while trying to reduce their adverse effects. The present works allows suggesting two ways to do so: the use of one or the other drug according to the state of health and the physiology of the patients, and the use of a particular dosage for each drug. Using chloroquine or hydroxychloroquine by taking account of new insights into the differential activity and toxicity of their enantiomers, of the patients' idiosyncrasy, and of the drug dosage, while simultaneously increasing the patients' immunity, should be until now the best way to help patients recovering from their illness.

Conflict of Interest

We affirm having no conflict of interest concerning the use of chloroquine and hydroxychloroquine. We are ethologists and work on ants' behavior and cognition.

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