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Research Article

Glyphosate Impact on *Apis mellifera* Navigation: A Combined Behavioral and Cheminformatics Study

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Abstract

Future global nutrition may require adjusting pesticide strategies to ensure the vitality of pollinator insects. Motivated by recent reports that the herbicide glyphosate (GPH) may negatively impact the health of the western honey bee, *Apis mellifera*, this combined behavioral biology/chemical informatics study examines how GPH exposure affects bee navigation, and proposes possible neurophysiological processes underlying these effects.

The behavioral apparatus in this study included a controlled environment honey bee colony equipped with simple and complex entrance mazes. Bee cohorts received either a full sublethal dose (ED_{50}) of GPH, a half sublethal dose (ED_{25}), or non-toxic sucrose water (control group). GPH effect was quantified through maze completion time and path regressions, while insect behavior was monitored for qualitative mannerisms. Transit time correlated strongly with GPH dose, while regressions data offered more nuanced indications. Retesting 24 hours following intoxication revealed measurable, but incomplete, insect recovery.

Toxic mechanism was inferred from reverse molecular screening. PubChem was mined for bioactivity data for GPH and close chemical analogs, illuminating 84 candidate biochemical targets (72 proteins from *A. mellifera;* 12 reference targets from *H. sapiens*) including phosphoglycerate mutase, and metabotropic, NMDA, AMPA and kainate glutamate receptors. Molecular simulations (docking GPH analogs into targets, followed by dynamics validation of interesting cases) predict that GPH will outcompete well known agonists of *A. mellifera* AMPA and NMDA receptors, implying that GPH is significantly more neurotoxic to bees than to humans.

Informatics and behavioral studies concur that neuromuscular coordination and sensory input may be impaired at moderate doses, as cognition may at higher doses.

Keywords: *Apis mellifera; Glutamate Receptors; NMDA Receptor; AMPA Receptor; Neuromodulation; Glyphosate; Cognitive Assessment; Colony Collapse Disorder; Structural Biology; Cheminformatics; Molecular Simulations*

Introduction

Modern commercial agriculture is a complex process in which crop yield is contingent upon careful control of numerous factors that influence plant health and productivity. Two of the most critical resources in pursuing such balance are pollinators to ensure that crops are properly germinated, and effective pesticides to minimize damage from predation and competition. It is not difficult to see a potential conflict between these factors, given that many pesticides have toxicity to off-target species, and pollinators are living creatures whose biology has not necessarily evolved tolerance for synthetic agrochemicals.

Among the many pollinators, it has been the welfare of honey bees that has most captivated global attention. *Apis mellifera (A. mellifera*), the western honey bee, guarantees vital food sources for humans, animals, and themselves, through plant cross pollination. These insects foster herbal biodiversity, regenerate forests, and aid in agricultural production [1], delivering services that are valued at roughly $20 billion per year to the American agricultural industry [2].

Since 2006, an alarming loss of honey bee colonies, termed Colony Collapse Disorder (CCD), has emerged as a major environmental threat [3]. CCD entails worker bees disappearing en masse from a colony, leaving only queen, honey and brood. The current decline in *A. mellifera* population is attributed to a multi-faceted combination of agrochemicals, parasites, viruses, nutritional deficiency and changes in habitat [4].

Clearly one of the most obvious agrochemical dangers to bees resides in insecticides, many of which have broad efficacy across insect genera, including the Apocrita suborder that comprises many common insect pollinators [5]. Fortunately, an increasing emphasis is being placed on refining insecticides to improve safety for primary pollinators such as bees and wasps [6].

Less carefully scrutinized have been toxicological risks arising from herbicides, including the increasingly ubiquitously applied glyphosate (GPH). For more than a decade, GPH has been consistently been one of the three most extensively utilized herbicides in the United States, Canada, Brazil and Argentina, in both commercial agriculture and household applications [7]. Usage in Europe remains high, though it has been banned in some countries for personal use, due to an ongoing debate regarding possible human carcinogenicity [8], although the direct health implications to humans, mammals and birds remains a contentious issue [8,9].

Possible carcinogenicity is unlikely to significantly impact pollinator viability, as the short lifespan of most bees and wasps precludes cancer mortality in most situations of moderate carcinogenic exposure. Furthermore, although GPH structurally resembles several common cholinergic insecticides (i.e. those acting against the common neurotoxicological target acetylcholinesterase) in possessing an organophosphorus moiety, there is only weak evidence to suggest environmentally significant neurotoxicity along the standard cholinergic pathway [10].

These caveats, however, do not guarantee that GPH is fully safe for exposed pollinators. Given recent drastic insect population declines [11] that seem to parallel accelerated herbicide use [12], there is merit to investigating whether GPH may affect pollinator health via indirect mechanisms that diverge from commonly monitored pathways.

One theory attracting significant attention suggests that GPH exposure may significantly impinge on the gut microbiome, killing bacteria necessary for healthy host metabolism and otherwise responsible for pathogen resistance [13]. Such a mechanism might explain population decline, even in the absence of a mechanism for host-specific lethal toxicity.

Specifically, given the spatial cognitive sophistication that has co-evolved with complexities of nectar foraging and colony coordination [14], it is theoretically possible that disorientation arising from gut dysbiosis may sufficiently degrade forager function to the point where colony demands are no longer sustainable, but current characterization of gut dysbiotic cognitive effects is inadequate to fully address this issue.

A second consideration may be the prospect of non-cholinergic GPH neuro-intoxication, as exemplified by prior findings of a statistically significant, dose-dependent impairment in honey bee navigation performance [15]. To this end, it is hypothesized that GPH may be directly responsible for the observed intoxicative disorientation, via some form of nonfatal neuromodulation by GPH, which may be inferred from documented physiological effects arising from exposure to GPH or its close chemical analogs.

In preliminary pursuit of this hypothesis, a two-pronged research approach was developed. First, in order to achieve more specific neurobehavioral characterization of GPH-induced *A. mellifera* disorientation, behavioral studies were conducted to complement and elaborate upon the bee navigation observations made in the Balbuena study [15], adding controlled and quantifiable problem-solving metrics, as well as qualitative physical observations.

Secondly, neurological observations were combined with cheminformatic and structural biological arguments to identify and rationalize *A. mellifera* neurotransmission receptors with biochemical relevance to the behavior, and with quantifiable risk of toxicological susceptibility to GPH.

Methods

Behavioral analysis

Prior analysis of *A. mellifera* navigation determined, via harmonic radar tracking, that insects subjected to sublethal GPH-dose required greater time to return to their hive post-foraging and took less direct routes [15]. While these observations indicate, with strong likelihood of significance, that GPH administration poses a tangible impact on bee efficiency, the analysis does not necessarily pinpoint the cause. For example, both the slow return and the indirect trajectory could be indicative of degraded cognition (failure to interpolate efficient routes, given sensory inputs and prospective obstacles), memory (flaws in the imprinting of learned route), dulled sensory perception (in particular, the use of olfactory stimuli to locate the hive), motor control impairment (difficulties in translating from effort to objective) or even elevated fatigue (frequent detours to surfaces available for incremental recuperation).

To provide a more clear delineation of which of the above factors (alone or in combination) were most likely to account for degraded efficiency, a detailed *A. mellifera* behavioral study was performed according to the following protocol.

Bee cohorts: Behavioral analysis was performed on a total of 120 western honey bees, all chosen at random from the same indoor colony of *A. mellifera* which had been established in a controlled setting roughly six months prior to experiment. 40 of these bees were employed as non-intoxicated cohort evaluation and were returned to the hive after completion of the entire experiment. An additional 40 bees were employed within the half sublethal intoxication cohort. The final 40 were employed within the full sublethal dose group.

Sample preparation: A GPH/sucrose solution was prepared by combining 3g of glyphosate (RoundUp™ granules, measured via Digiweigh scale) with 100 mL of distilled H_2O to create a stock solution of 100 mg GPH/L. Using a 100 mL graduated cylinder, 5 mL of the GPH stock solution was added to 95 mL of supersaturated sucrose to obtain a full sublethal concentration (ED_{50}). Using a 100 mL graduated cylinder, 2.5 mL of the GPH stock solution was added to 97.5 mL of supersaturated sucrose to obtain a half sublethal concentration (ED_{25}).

GPH exposure: Over the course of a two hour period immediately prior to behavioral testing, each cohort of bees was exposed to cohort-specific sucrose-based feed solution in order to convey an appropriate sublethal GPH dose (or non dose). Specifically, the feed solutions for control, half-sublethal and full-sublethal cohorts were as follows:

- Control = 0 mg L^{-1} (supersaturated sucrose solution only; no GPH),
- ED_{25} = 5 mg L^{-1} (weight of GPH/volume sucrose solution), and
- ED_{50} = 10 mg L^{-1} (weight of GPH/volume sucrose solution).

Navigation test courses: As with all aspects of the experimental protocol, extensive details are provided regarding the construction of test courses within the Supporting Information, section 1. In brief, the two courses entailed:

- A straight bee-line path with a 2.54 x 2.54 cm cross-section and 50.8 cm length, leading directly into hive (hereafter referred to as 'simple maze'), and
- A convoluted path (spanning five right-angle turns) with a 2.54 x 2.54 cm cross-section and 93.98 cm total path length (hereafter referred to as 'complex maze').

In all tests, a quantity of ~5 mL raw liquid honey was placed, open air accessible, at the hive entrance to serve as a chemoattractant.

Test regimens: All bees in the study were challenged with the navigation course immediately after their two-hour feeding or dosing period, during which process their course performance was monitored and timed. Additionally, 20 bees from the ED_{25} cohort and 20 bees from the ED_{50} cohort were subjected to a repeat course challenge, commencing 24 hours after the end of their original dosing period.

Qualification and quantitation: Course timings were performed manually, one honey bee at a time, using an iPhone timer. Behavior observations were accrued visually, with the support of iPhone video recordings.

Error quantification: Intrinsically variable quantities (including variations in the precise amount of GPH-dosed food consumed by bees, variations in individual susceptibility to putative toxic effects, and variations in flight and behavior irrespective of dosing) have been treated according to rigorous sampling and statistical assessment. Prospective systematic errors in GPH dosing and flight measurements were minimized through experimental protocol. Specifically, in GPH dose preparation, weighing errors were diminished through careful scale calibration, and grain-by-grain transfer into preparative cylinders. Solvent volume was standardized through careful adherence to CMC meniscus reading protocol. Errors in transit-time recording were minimized by rigorous visual monitoring of bee progress, with any resulting inconsistencies assumed to be random, and thus addressed within statistical assessment.

Chemical informatics

In order to substantiate a hypothesis that GPH effects on *A. mellifera* navigation may arise from neuromodulation, molecular informatics and modeling analyses were applied in order to identify possible neurological targets that could rationalize behavioral observations, and are at an elevated risk of being specifically and selectively modulated by GPH. The procedure by which such targets were identified and evaluated is documented as follows.

Search for known GPH neuromodulatory activity: GPH (rendered in figure 1) and its closest available structural analogs (nitrosoglyphosate, N-amino-glyphosate and N-hydroxy-glyphosate) were mined via the PubChem Compound and PubChem Bioactivity utilities [16] in order to identify all biomolecular targets for which tangible reports of modulation were evident.

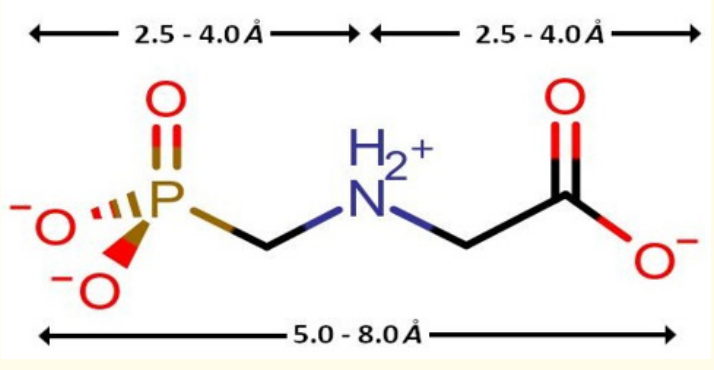

Figure 1: *Chemical structure of glyphosate (GPH) in a zwitterionic state predicted to be dominant (nearly 70% propensity in pH 7.4 plasma, according to Marvin pKa calculator [17]). Dimensions labels convey the relative positioning of charged atoms, as a basis for identifying structural analog*

Specifically, the text search 'glyphosate' in PubChem Compound returned a set of 62 compounds including GPH itself and various analogs. This set was culled down to those with evidence of measurable biological activity. The filtered list comprised GPH in two different preparation forms, plus the three close superstructural analogs listed in the above paragraph. For each of these compounds, the corresponding known active targets were parsed directly from bioactivity information listed within the compound record and filtered according to relevance to neurologically-relevant pathways.

Broader analog search: Realistically surveying for evidence of neuromodulative properties of GPH analogs should consider not only compounds with the high substructural conservation described in GPH neuromodulatory activity but should also reflect physicochemical analogy in toxicologically relevant properties such as size, shape, charge distribution, and the relative position of lipophilic groups (if present).

To pursue this broader search strategy, all PubChem compounds with at least 60% fingerprint similarity to GPH were identified using standard search filters, and again restricted to the subset of those having prior evidence of bioactivity. The resulting set of 132 compounds was then narrowed using the OpenBabel OB Conformer Search utility [18,19] by a mining for the three dimensional (3D) pharmacophore (or toxicophore) features identified in figure 1, including the presence of two ionizable nucleophile atoms situated between 5.0 - 8.0 Å apart, and one ionizable electrophile atom located between 2.5 - 4.0 Å away from each of the two nucleophiles. Compounds were further restricted to a maximum of 15 heavy atoms so as to remove from consideration larger superstructural entities with incidental fragmentary similarity to GPH. These criteria resulted in identification of the four neuroactive analogs shown in figure 2.

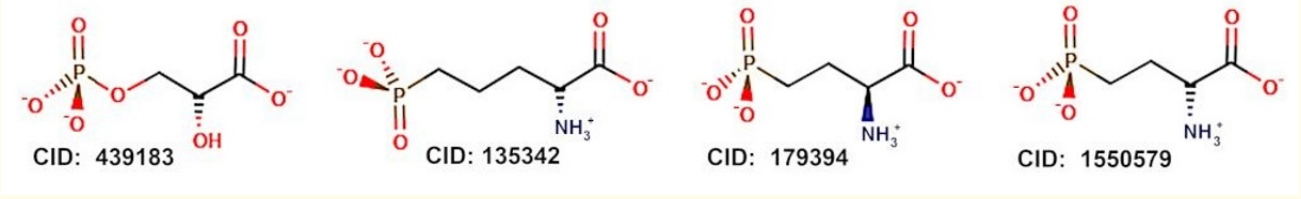

Figure 2: *Four neuroactive structural analogs of Glyphosate, identified by PubChem compound (CID) numbers.*

Target elaboration

Target class identification: Although glyphosate (CID: 3496) itself was not listed as being conclusively bioactive against any *H. sapiens* or *A. mellifera* neurological targets, the four GPH analogs listed in figure 2 have the following potentially relevant activities as follows:

- CID = 439183: 3-phosphoglycerate (3PG) is substrate for phosphoglycerate mutase (PGAM; uniprot: P18669), which is expressed in red blood cells. PGAM dysregulation is associated with anemic response [20] and potentially also age-related dementia [21].
- CID = 179394: Modulates human mGlu4 (uniprot: Q14833) and mGlu8 (uniprot: O00222) metabotropic glutamate receptors. Expressed in the cerebellum, hippocampus, hypothalamus and thalamus, Glu4 governs synaptic transmission and regulates neuron apoptosis [20]. Responsible for sensory processing, mGlu8 is expressed in the anterior cingulate cortex [20]. Dysfunction of mGlu4 has neuromuscular implications [22], while mGlu8 dysregulation is implicated in various psychiatric disorders [23].
- CID = 1550579: is another modulator for human mGlu8 metabotropic glutamate receptor whose specifics are detailed above.
- CID = 135342: Binds glutamate ionotropic NMDA receptors GRIN1 (uniprot: Q05586), GRIN2A (uniprot: Q12879), GRIN2B (uniprot: Q13224), GRIN2C (uniprot: Q14957), and GRIN2D (uniprot: O15399). These proteins control brain calcium flux, as modulated by glutamate and NMDA binding. GRIN1 is expressed mostly in the anterior cingulate cortex; GRIN2A in the primary visual cortex; GRIN2B in the fronto-parieto-temporal cortex, hippocampus and basal ganglia; GRIN2C in the hippocampus, amygdala, caudate nucleus, corpus callosum, subthalamic nuclei and thalamus; GRIN2D in the hypothalamus [20]. NMDA receptor excitotoxicity is implicated in a broad range of acute and chronic neurological disorders and in neurodegeneration [24].

Homolog search: Although the PubChem Bioactivity database is the world's largest compendium of measured interactions between chemical entities and physiologically relevant biomolecules, it reports only a tiny fraction of all ligand-receptor interactions and focuses heavily on human (non-insect) targets. To infer relevant neurological pathways that may be modulated by GPH-like substances, the set of targeting prospects listed in target class identification was extrapolated through sequence-based protein homolog searching via PBLAST search [25]. Sequences of preliminary *H. sapiens* proteins of interest from target class identification were searched across the known *H. sapiens* and *A. mellifera* proteomes for all homologs with at least 30% sequence identity. For a comprehensive survey of possible bee neurotoxicity, all *A. mellifera* protein variants were retained for further examination. Because the human equivalent proteins are only intended in this study to provide reference for assessing effects in bees, only a smaller set corresponding to dominant alleles of each distinct human gene type from the homolog search were considered. Specifically, splice variants,

non-physiological cleaved forms, and infrequent mutations were disregarded.

Preliminary target validation

Empirically, it may be assumed that the long-established absence of literature reports of GPH human neurotoxicity is evidence that GPH does not strongly interfere with neurocognitively important biochemical targets in humans; however, this does not directly inform the chances that GPH has unintended neuromodulatory consequences for *A. mellifera*. Preliminary molecular modeling studies provide a means for objectively quantifying the risk that GPH may induce neuromodulation in bees, but confidence in such predictions is contingent upon adequate counter-validation, as may be addressed as follows:

1. Modeling predictions must demonstrate, with reasonable significance, that GPH modulates relevant *A. mellifera* targets with greater effect than would be comparably predicted for normal physiological binders such as target substrate molecules, and
2. If GPH effects in *A. mellifera* exceed physiological binders for a given target, model predictions should further demonstrate that the extent of GPH effect is more pronounced for the *A. mellifera* target than for homologous human targets.

Target elaboration for *H. sapiens*: The PBLAST [19] protocol described in section 2.3 was repeated in a precisely analogous manner with the exception that, this time, the homolog search was performed over the Homo sapiens proteome, rather than *A. mellifera*.

3D target characterization: The three-dimensional molecular structures of all potentially relevant targets identified in searches 2.3 and 2.4.1 were produced in a semi-automated fashion via the Phyre2 protein threading server [26], with all program search and refinement settings set to default, except for specifying the 'Intense' mode to achieve more highly refined molecular structures. Resulting models were examined according to the norms of standard structural biology via the PROSESS tool [27]. Those structures adhering to reasonable PROSESS criteria, and containing a fully resolved small molecule binding site, were retained for further analysis. The list of all satisfactorily resolved *A. mellifera* and *H. sapiens* target structures is provided in Supporting Information, section 2.

Ligand structure representation: In addition to the chemicals shown in figure 2, static 3D structures of relevant reference ligands (i.e. physiological ligands or other documented modulators; see figure 3) were solved in Vega-ZZ [28] using the Tripos Molecular Force Field [29] and Gasteiger-Marsili charges [30] as a basis for standard steepest descent molecular mechanics optimization. These structures were saved as Tripos mol2 files.

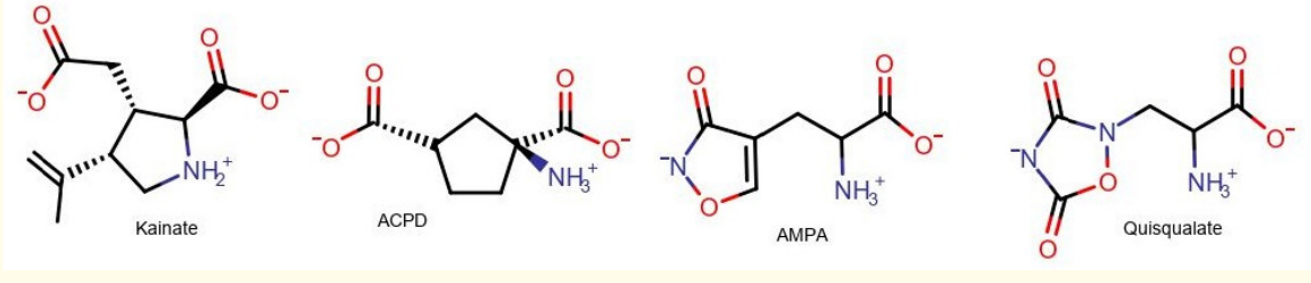

Figure 3: *Comparison of the structure of GPH with compounds of interest (physiological ligands and other documented modulators) known to bind to possible GPH modulation targets.*

Binding affinity prediction: The relative binding efficacies of each ligand from figure 3 were assessed against all biomolecular target models (from 3D target characterization) via the LeDock molecular docking software [31]. Twenty solved poses were requested for each ligand/receptor pair to an accuracy of 1.0 Å for pose cluster assignment, and by starting the pose search within a cuboid region encompassing a crystallographically characterized GPH-analog ligand bound to each resolved receptor template (see Supporting Information, section 2 for structure identifiers). These templates were used to validate the docking methodology. Specifically, the docking

poses were compared to each crystallographically characterized template to ensure reasonable reproduction of experimentally obtained ligand position and orientation within the receptor. In quantitative terms, a simulation was deemed successful if one (or more) of the three highest scoring poses exhibited an atomic root-mean-squared deviation (RMSD) of less than 1.5 Å relative to crystallographic reference.

Results

Behavioral studies

Simple maze experiment: This first test, gauging basic aspects of bee neuromuscular and sensory performance, displayed statistically significant ($p < 0.01$ in all cases) dose-varying GPH effects. From the transit times reported in figure 4A, the half sublethal GPH dose resulted in bees taking more than ~6.5 times as long to navigate the straight channel, whereas the full sublethal dose incurred transit times roughly 10.5 times more than control. Part B reports the number of detectable course corrections (regressions) made by bees during their maze transit. Since bees in the control group made zero course corrections, the GPH effect is strongly significant ($p < 0.01$ in all cases).

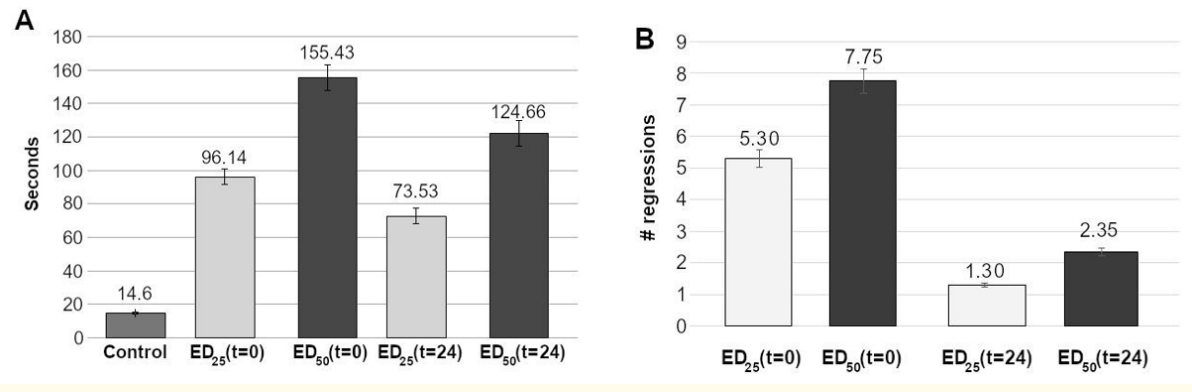

Figure 4: *Simple maze navigation performance for bees subjected to GPH at half sublethal (ED_{25}) and full sublethal (ED_{50}) concentrations immediately after dosing and 24 hours as measured by A) transit times, and B) transit path regressions. Error bars reflect 95% confidence intervals derived from T-test statistics.*

Complex maze experiment: This test sought to assess more complex problem solving abilities that have been documented for *A. mellifera* [14,32]. In this portion of the study, the mean transit times of bees completing the complex maze were all fairly close to triple the average duration required for each cohort to complete the simple maze. Independent of time of test (t = 0 vs. t = 24 hours) the general trends resemble the simple maze case, with the half sublethal cohort taking more than eight times as long as the undosed control to navigate the route, and the full sublethal group taking nearly 14 times as long (Figure 5A).

Statistics for transit regressions for the complex maze exhibit qualitative differences (Figure 5B) from the path precision observed for the earlier straight-line task (Figure 4B). First, the complex task proved somewhat challenging even to un-dosed insects who, on average, required two or more path recalibrations in order to learn the route. Interestingly, however, the complex maze did not produce a statistically significant difference in the number of regressions observed among the ED50 and ED25 cohorts for bee performance immediately following GPH exposure. Disregarding the fact that this intriguing anomaly is not consistent with transit times, or with later (24 hour post-dose) path regression performance, the question of plausible explanations for a short-duration intoxication phenomenon will be examined in the discussion section.

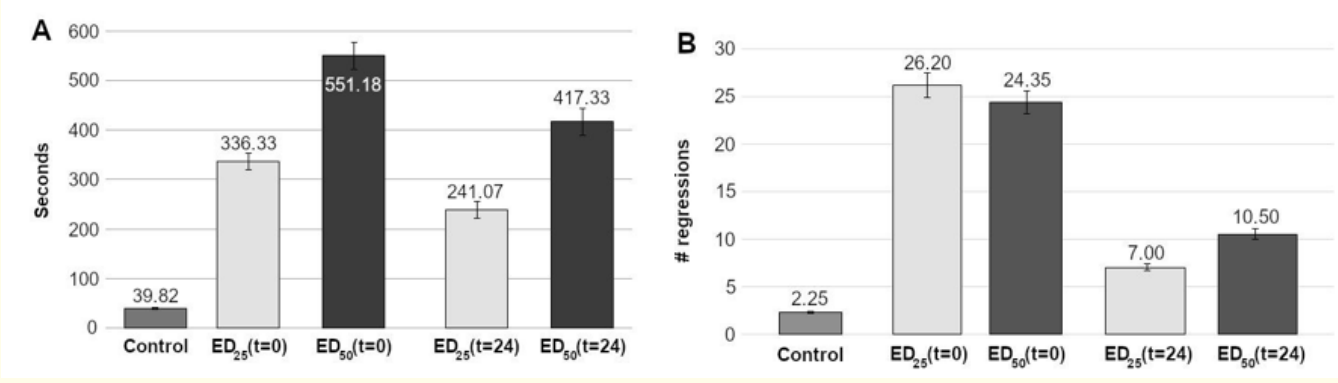

Figure 5: *Complex maze navigation performance for bees subjected to GPH at half sublethal (ED25) and full sublethal (ED_{50}) concentrations immediately after dosing and 24 hours as measured by A) transit times, and B) transit path regressions. Error bars reflect 95% confidence intervals derived from T-test statistics.*

Molecular docking simulations comparing GPH versus known receptor ligands

Phosphoglycerate mutase: By far the simplest receptor class to evaluate potential GPH modulation for was phosphoglycerate mutase, for which the homolog search only uncovered a single *A. mellifera* protein (uniprot: P15259). The molecular docking comparisons of GPH binding affinity versus the known PGAM substrate (3PG) thus produced only the following simple table 1.

Species	GPH	3PG
H. sapiens	-3.90	-4.10
A. mellifera	-4.69	-5.06

Table 1: *Comparison of LeDock predicted binding affinities for GPH and 3PG docked to human and bee forms of the PGAM enzyme. Affinities are values of the LeDock scoring function [31], which can be loosely interpreted as correlating with binding free energies, thus larger negative numbers reflect greater affinity.*

Although LeDock is generally regarded as one of the most accurate modern docking simulation tools for pose prediction and affinity estimation [33], the small difference between the docking scores of GPH and 3PG for human PGAM is insignificant, thus one may not clearly establish whether GPH is predicted to have a measurable modulation effect, as would be evinced by GPH binding sufficiently more potently to PGAM than the substrate.

The difference in docking scores for binding to *A. mellifera* PGAM is somewhat larger, and may be approaching a level that would be considered a significant prediction [34], however the ratio of scores between GPH and 3PG (0.93) point away from an assertion that GPH is likely to achieve appreciable PGAM modulation by outcompeting the substrate.

Without reliable quantitative guidance from docking simulations, instead, in section molecular dynamics simulations, the binding performance for GPH versus 3PG will be contrasted in more rigorous molecular dynamics (MD) simulations for receptors of interest.

Metabotropic glutamate receptors: From the metabotropic glutamate receptor class identified in section target class identification for possible GPH effect, a number of potentially relevant targets were identified, including a list of 11 *A. mellifera* homologs, which were contrasted against the six most closely related *H. sapiens* alleles, mGlu1, mGlu2, mGlu3, mGlu5, mGlu7 and mGlu8 (see Supporting Information, section 2 for all uniprot identifiers). In order to intuitively estimate the effect of GPH relative to glutamate for all of these receptors, figure 6 plots each receptor according to the predicted GPH docking score (ordinate) versus glutamate docking score (abscissa).

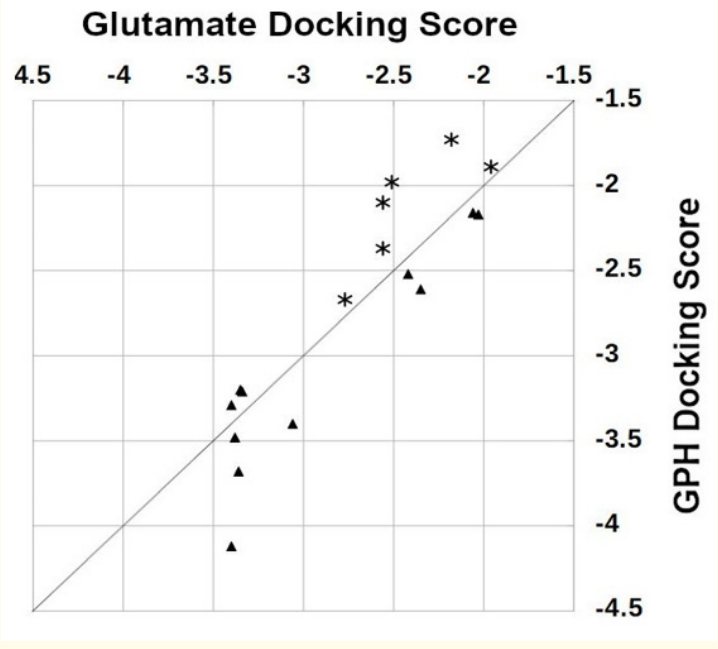

Figure 6: *Metabotropic glutamate receptors (∗ represents H. sapiens; ▲ represents A. mellifera) plotted according to LeDock pose scores for bound GPH and glutamate. The upper left portion of the plot shows receptors for which glutamate is predicted to bind more strongly than GPH, while the lower right shows receptors for which GPH is predicted to bind more strongly than glutamate.*

The first thing to note is that docking predicts an approximately linear relationship between binding affinity of GPH, as compared to glutamate. This is not surprising, given how similar the size, shape, flexibility and charge distributions are for the two ligands. This general linearity notwithstanding, one subtle yet interesting nonlinear trend does emerge. Across all of the *H. sapiens* receptors, glutamate is predicted in all cases (100%) to bind with slightly greater affinity than GPH, which would be consistent with a scenario where GPH is not considered to modulate the target. However, among the *A. mellifera* receptors, eight of 11 are predicted to bind GPH more potently than glutamate. Although the small glutamate-GPH affinity differences for most of the individual *A. mellifera* receptors are of questionable significance, the broader *A. mellifera* sampling trend is stronger. From binomial statistics, one finds that the affinity trend corresponds to only an 11.3% chance that glutamate binds with equal or greater strength than GPH.

From the perspective of the hypothesis regarding GPH effect on neurological receptors, the most interesting data point in figure 6 is the bottom-most *A. mellifera* point (at the following coordinates: glutamate = 3.40; GPH = 4.12), which corresponds to the metabotropic glutamate receptor 1, present jointly in insect brains and neuromuscular junctions. MD rationalization for the unusually high strength of GPH binding to this receptor will be explored in section molecular dynamics simulations.

Non-metabotropic glutamate receptors: The NMDA receptors identified in section target class identification are examples of a populous set of fairly homologous glutamate receptors that are functionally and structurally unrelated to the metabotropic glutamate receptors from section molecular docking simulations comparing GPH versus known receptor ligands. Specific proteins within this target class are generally named according to specific receptor agonists that are known to mimic glutamate action, including NMDA, kainate and AMPA (see figure 3 for agonist structures). From within the set of five NMDA receptors listed from the initial bioactivity search in section target class identification, 59 *A. mellifera* homologs and alleles were identified, which were contrasted against the predominant physiological allele for six closely related *H. sapiens* proteins: AMPA receptors 2 and 4, kainate receptor types 4 and 5 and NMDA receptor isoforms 2a and 3a. Docking of glutamate and GPH into the aggregate set of 65 receptors provided the distribution depicted in figure 7.

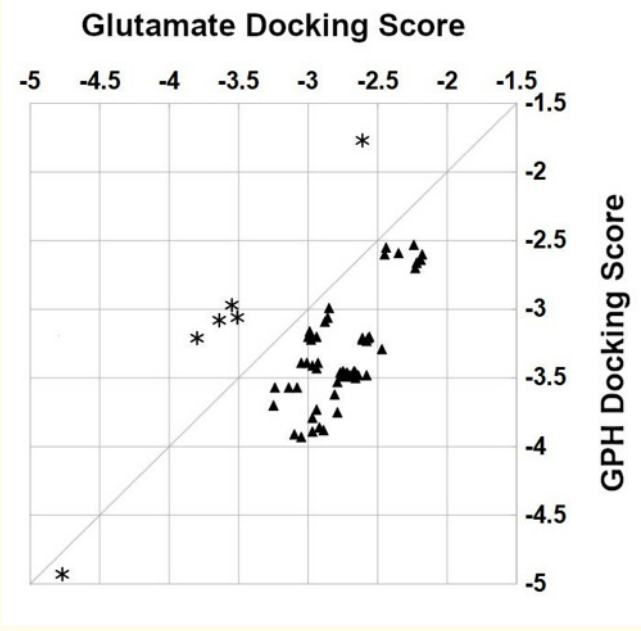

Figure 7: *Non-metabotropic glutamate receptors (✱ represents H. sapiens; ▲ represents A. mellifera) plotted according to LeDock pose scores for bound GPH and glutamate. The upper left portion of the plot shows receptors for which glutamate is predicted to bind more strongly than GPH, while the lower right shows receptors for which GPH is predicted to bind more strongly than glutamate..*

In figure 7, there is a strong trend among the docking results to suggest that many or most members of the *H. sapiens* family of non-metabotropic glutamate receptors bind glutamate with greater potency than GPH, with the possible exception of the single point near the lower left corner of the graph which corresponds to *H. sapiens* kainate receptor 2. Among human receptors, this latter system was unique in implying GPH out-competition of glutamate, thus it was prioritized for subsequent MD verification.

For *A. mellifera*, an opposite trend predominates, with the computed docking affinity of GPH being greater than glutamate for all 59 studied members of the honey bee non-metabotropic glutamate receptor family. While numerous receptors exhibited ligand affinity differences of potentially significant magnitude, molecular dynamics methodology was chosen to validate the point (corresponding to *A. mellifera* AMPA receptor 3, isoform X2) exhibiting the greatest difference in predicted docking score (-3.88 for GPH; -2.89 for glutamate).

Molecular dynamics simulations

The predictions derived in section phosphoglycerate mutase from LeDock simulations of GPH and 3PG binding to human and honey bee PGAM enzyme are effectively validated in the results of MD tracking of ligand binding energies, as reported in figure 8. For the human structure, GPH binding is initially substantially disfavored relative to the 3PG substrate but, after a period of roughly 400 μs, during which receptor induced-fit conformational relaxation is taking place, the difference between the mean GPH (-621 ± 22 kcal/mol) and 3PG (-655 ± 14 kcal/mol) binding enthalpies is much smaller. For the analogous honey bee receptor, however, there is a more significant difference in mean binding enthalpies over the final 600 μs of the simulation: -670 ± 14 kcal/mol for 3PG, versus -538 ± 19 kcal/mol for GPH.

Insight of a different nature is afforded by the MD simulations on GPH and glutamate binding to *A. mellifera* metabotropic glutamate receptor 1, as shown in figure 9. In this case, LeDock simulations implied that GPH might interact with the receptor with substantially greater affinity than glutamate, in contrast to other *A. mellifera* and *H. sapiens* metabotropic glutamate receptors where GPH and glutamate affinities were predicted to be rather similar. However, dynamic simulations do not fully bear this result.

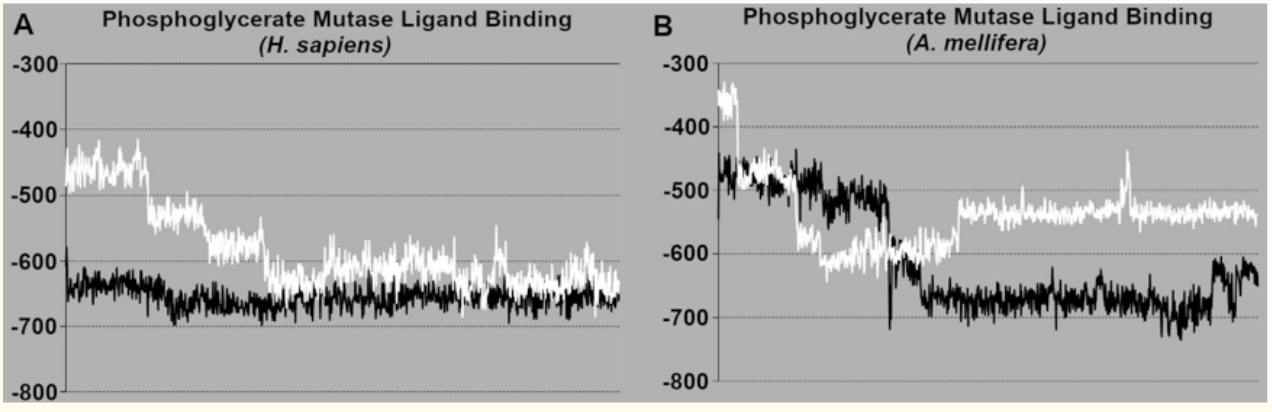

Figure 8: *Binding enthalpy traces for GPH (white line) and 3-phosphoglycerate (dark line) interacting with the phosphoglycerate mutase enzymatic active site for A) H. sapiens, and B) A. mellifera. Enthalpies (in kcal/mol) encompassing intermolecular energy terms (hydrogen bonds, van der Waals and electrostatics) are derived from NAMD molecular dynamics simulations. The length of each simulation is 1.0 ns.*

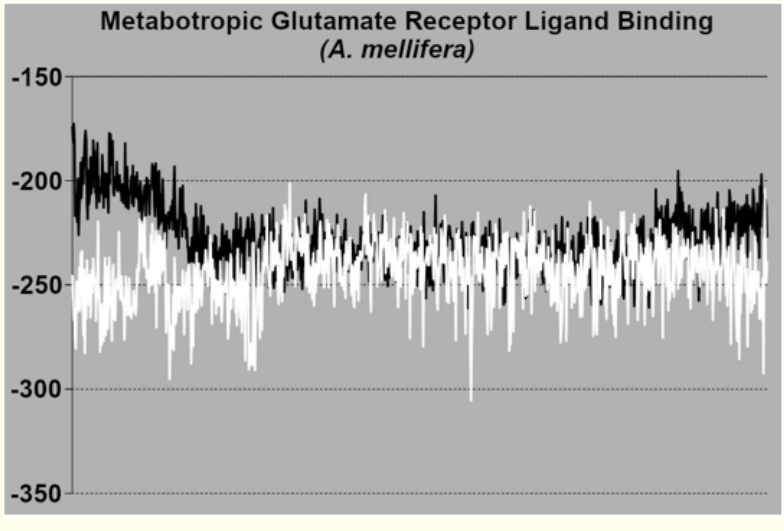

Figure 9: *Binding enthalpy traces for GPH (white line) and glutamate (dark line) interacting with the A. mellifera metabotropic glutamate receptor 1 active site. Enthalpies (in kcal/mol) encompassing intermolecular energy terms (hydrogen bonds, van der Waals and electrostatics) are derived from NAMD molecular dynamics simulations. The length of simulation is 1.0 ns.*

From figure 9, one sees that while glutamate binding to the initial receptor conformation is less favorable than GPH binding, this distinction diminishes after the initial simulation phase enables receptor relaxation. Over the last 600 μs, the difference in mean interaction enthalpies between the glutamate (-232 ± 11 kcal/mol) and GPH (-242 ± 14 kcal/mol) is insignificant, and the simulation does not strongly support an assertion that GPH is a potent competitor.

MD simulation of the *H. sapiens* kainate 2 glutamate receptor sought to verify if GPH might bind more potently than glutamate, and the results in figure 10 indicate that the more rigorous treatment essentially replicated the molecular docking prediction. Although binding

enthalpies for both ligands tended to fluctuate greatly during the initial phase of MD simulations, a general level of stability was achieved well in advance of the 400 μs point used as a cut-off between equilibration and analysis. Over the latter portion of the simulation, GPH was predicted to have a slightly greater (-400 ± 17 kcal/mol) mean binding affinity than glutamate (-380 ± 17 kcal/mol).

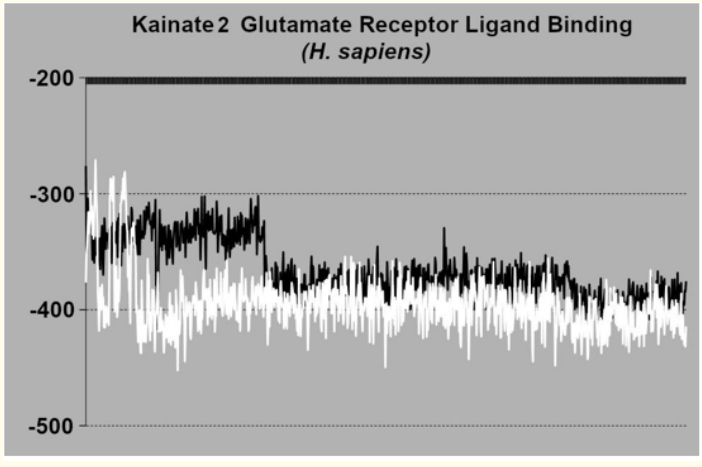

Figure 10: *Binding enthalpy traces for GPH (white line) and glutamate (dark line) interacting with the H. sapiens kainate 2 glutamate receptor active site. Enthalpies (kcal/mol) encompassing intermolecular energy terms (hydrogen bonds, van der Waals and electrostatics) are derived from NAMD molecular dynamics simulations. The length of simulation is 1.0 ns.*

The final MD validation examined the docking prediction that GPH had substantially greater affinity than glutamate for the *A. mellifera* AMPA 1 receptor isoform X2. While fluctuations in simulated ligand binding were substantial, the absolute difference in ligand performance is far more stark. Specifically, from figure 11, the predicted mean binding enthalpy of GPH over the final of the 600 μs of simulation time is predicted to be much greater (-1106 ± 39 kcal/mol) than that of glutamate (-831 ± 31 kcal/mol).

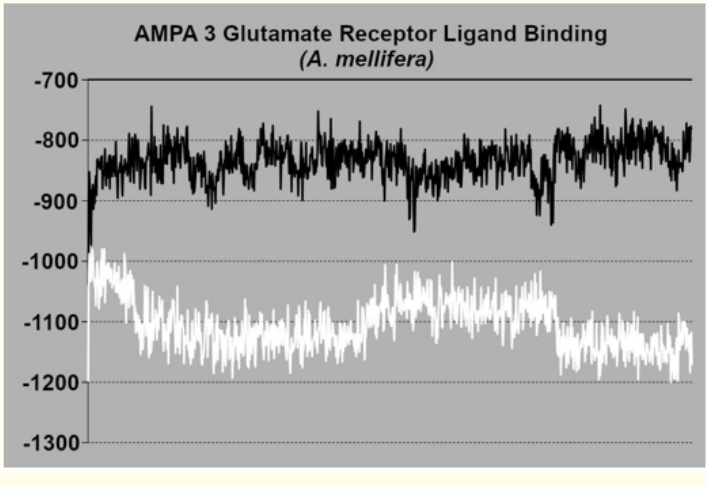

Figure 11: *Binding enthalpy traces for GPH (white line) and glutamate (dark line) interacting with the A. mellifera AMPA glutamate receptor 3 isoform X2 active site. Enthalpies (in kcal/mol) encompassing intermolecular energy terms (hydrogen bonds, van der Waals and electrostatics) are derived from NAMD molecular dynamics simulations. The length of simulation is 1.0 ns.*

Molecular docking comparison with other known glutamate receptor modulators

While the results thus far tend to point, consistently, to GPH being a weaker modulator to the PGM enzyme for both *H. sapiens* and *A. mellifera*, the debatable question of which ligand interacts most strongly with glutamate receptors prompts recognition that, for many of the glutamate receptors examined in sections molecular docking simulations comparing GPH versus known receptor ligands and molecular dynamics simulations, glutamate is not the only ligand of known physiological or pharmacological relevance. In particular, the molecules reported in figure 3 are all either standard agonists (e.g. AMPA, NMDA and kainate) or known modulators (ACPD, LAP4 and quisqualate) of various glutamate receptors. Rather than simply comparing GPH effect versus glutamate, it was illustrative to explore whether GPH docking predicts an effect comparable to, or greater than, a broader slate of positive control ligands.

In table 2, the results were consistent from results reported in sections molecular docking simulations comparing GPH versus known receptor ligands and molecular dynamics simulations that, in a preponderance of cases, GPH tends to show only modest evidence for outcompeting known physiological ligands and modulators of human glutamate receptors, but GPH is generally predicted to be significantly more competitive within the manifold of ligand interactions with *A. mellifera* glutamate receptors.

Receptor Class	Glutamate	AMPA	Kainate	NMDA	ACPD	LAP4	Quisqualate
H. sapiens							
Metabotropic	-0.30	-1.94	-0.58	**+0.15**	-0.46	-2.46	-2.18
AMPA	-0.58	-2.39	-0.07	**+0.62**	-0.47	-3.71	-2.47
Kainate	-0.15	-2.03	-0.70	**+0.46**	-0.04	-3.07	-1.67
NMDA	-0.71	-0.46	**+0.70**	**+0.16**	-0.46	-2.95	-2.51
A. mellifera							
Metabotropic	**+0.15**	-1.12	-0.38	**+0.34**	**+0.28**	-1.63	-1.17
AMPA	**+0.79**	**+0.17**	**+0.76**	**+0.66**	**+1.33**	-0.45	**+0.26**
Kainate	**+0.58**	-0.93	-0.24	**+0.61**	**+0.56**	-1.75	-1.14
NMDA	**+0.51**	-0.67	**+0.32**	**+0.41**	**+0.61**	-1.23	-0.79

Table 2: *Glyphosate competition statistics, indicating the mean extent to which various known glutamate receptor modulators outcompete GPH for various receptor classes. Values reflect the quantity E(ligand) - E(GPH), where 'E' refers to LeDock scoring function [31]. Negative values indicate that a specific ligand is predicted to outcompete GPH. Positive values suggesting greater GPH potency are shown in bold.*

Discussion

Prior to this paper, a diverse range of mechanistic hypotheses have been proposed for plausible GPH effects on insect health. One proposition stated that the herbicide may negatively affect the hypopharyngeal glands of the honey bee's endocrine system. The hypopharyngeal glands are a pair of coiled glands in the honey bee's head which are only active in workers and secrete proteins and hydrolytic enzymes [35] whose chemical modulation might interfere with release of developmental hormones in a manner comparable to GPH's herbicidal mode in blocking the shikimic acid pathway of plants.

Another potential clue emerges from a public health study that has identified a correlation between environmental GPH exposure in humans and elevated incidence of Parkinson's disease [36]. This potential link is noteworthy given the fact that behavioral observations of GPH-intoxicated bees (wing tremors, slowed crawling and decreased motor skills) qualitatively resemble Parkinson's symptoms in higher organisms.

Such hypotheses notwithstanding, our informatics and modeling studies suggest the possibility of a different, or complementary, physiological mechanism for GPH impact on *A. mellifera*. While likely discounting a toxicological targeting of PGAM1, where the prospect of GPH outcompeting the native substrate (3PG) was assessed at less than 50%, docking and dynamics simulations identify glutamate receptors as plausible toxicological targets. Results suggest that the chance that GPH binds to *A. mellifera* glutamate receptors more strongly than glutamate is better than 50% for metabotropic receptors, greater than 95% for NMDA and kainate receptors, and exceeds 98% for AMPA receptors (see data in Supporting Information, section 2).

AMPA receptors may represent the most interesting targets for GPH activity within *A. mellifera* physiology, in that there is better than 50% chance of GPH outcompeting nearly all of the common GluR modulators reported in table 2, including AMPA itself.

Ultimately, there are fairly clear structural biological arguments for why GPH should bind with substantially greater potency to *A. mellifera* AMPA receptors than to the corresponding *H. sapiens* form. These factors are apparent in figure 12.

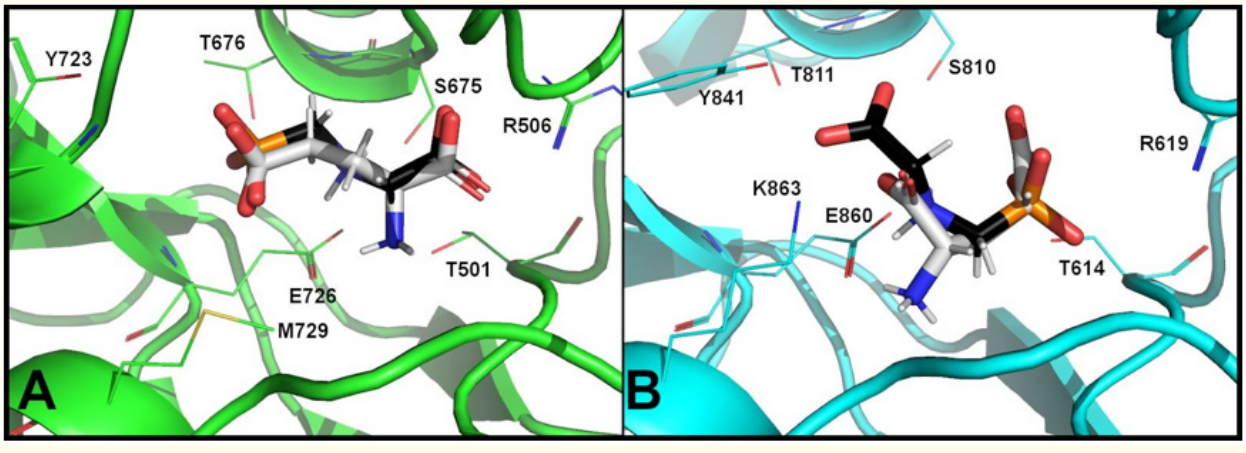

Figure 12: *Bound molecular structures of glutamate (grey C atoms; CPK-colored heteroatoms) and glyphosate (black C atoms; CPK-colored heteroatoms) interacting with A) H. sapiens AMPA glutamate receptor isoform 2, and B) A. mellifera AMPA glutamate receptor 1 isoform X2.*

Crucial differences between *H. sapiens* and *A. mellifera* AMPA receptors include both steric and electrostatic factors. In the first case, the homology model structure of *A. mellifera* AMPA receptor 1 predicts a more spacious receptor site, courtesy of the *A. mellifera* sequence having greater flexibility conferred by smaller rigid helices and longer low-structure coils. This permits receptor accommodation of the phosphonate tail on GPH, which is bulkier than the glutamate carboxyl or amino acid moieties, and wider than the flat oxazolone ring on AMPA. Secondly, while most amino acids in the immediate vicinity of the neurotransmitter active site are conserved between species, the *A. mellifera* AMPA receptor is a better electrostatic match for GPH binding courtesy of a Met → Lys mutation relative to the human protein (i.e. Met 729 for *H. sapiens*; Lys 863 for *A. mellifera*). This mutation is favorable, not only for providing an additional positive charge with which to stabilize a highly anion ligand (GPH has a net charge of -2 at physiological pH, whereas all other ligands subjected to simulation and binding assessment herein have -1 charges), but also to give the bee AMPA receptor a tripartite electrostatic match to GPH, such that two peripheral cationic residues (Arg 619 and Lys 863) attract the ligand's two anionic centers (the monoanionic carboxyl and the dianionic phosphonyl), while a central anion (Glu 860) is well situated to bind the cationic central secondary amine on GPH.

Statistically observed decline in bee navigation performance metric (Section behavioral studies) under the influence of non-lethal GPH dose, coupled with structural modeling trends, enable preliminary conjecture regarding possible GPH effects on *Apis mellifera* biochemistry. The mere observation of slower, less direct, insect flights in returning to the hive, with partial (but not complete) restoration

of physical function after 24 hours is consistent with excitotoxicity, such as glutamate receptor agonists would manifest, but is inadequate to narrow the phenomenon down to one receptor class, let alone a single receptor isoform.

Although the simulations have identified AMPA receptors as a plausible target for GPH-based intoxication, the modeling statistics are not fully definite. For example, there is significant evidence to suggest that GPH may out-compete NMDA binding to NMDA receptors ($z = 2.61$; 12 df; $P > 0.98$), which is further augmented by structural biology, vis a vis the NMDA receptor also possessing the cation-anion-cation tripartite receptor motif (*A. mellifera* NMDA receptors have an arginine in the position occupied by the *A. mellifera* AMPA receptor Lys 863 and the *H. sapiens* Met 729). Prospects for physiologically important GPH modulation of kainate and metabotropic receptors appear to be diminished relative to AMPA and NMDA receptors, but neither modeling statistics nor structural arguments conclusively rule out an effect.

To shed further light on the question of GPH toxico-targeting prospects, it may be useful to note several empirical, qualitative observations made during the course of bee flight monitoring. Although these observations were not rigorously quantified in this preliminary study, and can thus not yet be statistically argued, the following comparison of undosed control insect behavior versus GPH-dosed bees may prove useful for further assessment:

- **Control:** In the control groups, honey bees crawled various ways including upside down, facing up, and a side crawl technique. All honey bees in the simple maze formed a straight beeline to the maze and only two bees appeared mildly stressed, as indicated by a slight buzzing noise. In the complex maze the control honey bees successfully navigated the corners and turns with the different types of crawling techniques. The honey bees experienced minimal stress and crawled quickly in straight paths.
- **GPH-dosed:** In the glyphosate trials the honey bees continued to be directionally challenged. The insects experienced difficulty in entering the maze and buzzed vigorously. Glyphosate honey bees moved their abdomens in circular motions and would stop to rest. Bees frequently extended their proboscis, likely to recruit other senses to aid in navigation. GPH-dosed honey bees were observed to experience tremors with vibrating wings, slowed crawling, decreased motor skills, and abnormal movement. These insects struggled to complete tasks that were simple for the control honey bees, such as an insect righting itself after falling on its back.

Behavioral differences between control and GPH cohorts tend to suggest motor control malfunction, for which AMPA and NMDA receptor dysregulation is commonly associated, especially in view of extensive research on glutamate receptor agonism in mammalian epilepsy [37]. Observations regarding proboscis extension and evidence of the bee abdominal rotation likely both reflect sensory confusion (*A. mellifera* abdomens contain magnetic iron granules used in navigation [38]), which would tend to implicate metabotropic [39] or AMPA receptor [40] modulation.

Interestingly, one prior behavioral trend from Section molecular docking simulations comparing GPH versus known receptor ligands that best exemplifies possible GPH impact on higher order cognitive performance or memory may be the unusual observation that bee path regressions (deviations from standard bee lines) may have been lower in the most GPH-intoxicated bee cohort (24.35 ± 1.10 regressions at ED_{50}, immediately after exposure) than in the analogous ED_{25} dose cohort (26.20 ± 1.20 regressions). As opposed to the most visually obvious issues of garbled autonomic and sensory impulses, which exhibit standard dose response, it is reasonable to infer that the observed regression statistics reflect not only autonomic and sensory disability, but also a different dose-curve that reflects cognitive and/or memory challenges. Specifically, it is possible that the ED_{50} (but not the ED_{25}) exposure substantially disrupts higher order, acquired, behaviors governing the full 'forage and return' task program, and that this disruption impedes the impetus to make instinctive course corrections, as compared to lower dose bees with better adherence of their task regimen. This twofold intoxication paradigm seems to be consistent with the modeling results that predict AMPA receptors (key in both autonomic and sensory function) to be most vulnerable to GPH modulation, while NMDA receptors (more important for learned behaviors and memory [41,42]) are assessed as GPH-susceptible to a lesser (though still appreciable) extent that may thus be most apparent for higher GPH doses. This possible NMDA modulating effect is interesting, in light of tentative findings of depressive behavior of rats exposed to GPH [43].

The mechanistic complexity of AMPA and NMDA receptors being differentially modulated at different dose ranges may suggest the value of further studies that assess whether periodic or chronic GPH exposure may alter the effect on honeybee foraging behavior and colony health in field-realistic environments. Longer observational monitoring (48+ hours after initial dose) and repeated dosing may help to assess the relative risks of short-term intoxication versus irreversible neurodegenerative damage. Varied test criteria, including other maze constructs and different performance metrics, should help to control for intoxication effects versus chemically altered stress responses. This distinction could permit more clear characterization of the precise neurotoxic consequences of GPH exposure, which would prove helpful in identifying biochemical mechanisms through which these toxicological mechanisms manifest.

Mechanistic complexity inferred from studying honey bees may also warrant careful re-evaluation of human safety standards for GPH usage, in that there is a fair degree of homology between mammalian and insect glutamate receptors. Fortunately, structural studies have identified specific cross-species Met/Lys and Met/Arg mutations that lead to AMPA and NMDA receptor structures in *H. sapiens* that are substantially more resistant to GPH agonistic binding than would be expected for the honey bee homologs. This difference may explain why extensive human safety trials have typically not detected measurable neurotoxicity [44]. Nonetheless, docking predictions that GPH may bind competitively to human NMDA receptors are justification for careful further scrutiny that may detect subtle toxic effects.

Conclusions

Results of the observational study of glyphosate-intoxicated *A. mellifera* reveal a clear dose-dependent impact on navigation, as reflected in the ability of honey bees to complete simple and complex mazes. The null hypothesis of non-toxicity is rejected, in light of strong statistical evidence of effect. The two-pronged experimental results suggests that a sublethal GPH dose compromises performance as evinced by slower maze navigation and a more convoluted path (greater number of regressions) observed in homeward beelines.

This supports a hypothesis, inspired from prior research of Balbuena., *et al.* [15] reporting measurable navigation effects of non-lethal GPH doses, while extending the earlier work to show partial, incomplete, recovery, as shown in a 65% decrease in flight path regressions after 24 hours without herbicide exposure.

The analyses reported herein neither prove nor disprove prior conjectures regarding GPH toxicology, but do provide significant evidence of alternative neurological rationalization of *A. mellifera* behavioral effects arising from GPH exposure. Specifically, the fundamental chemical informatics principle of structurally similar chemicals demonstrating similar toxicology, led to identifying putative toxic glutamate receptor modulation. Exceptional steric and electrostatic similarity between glyphosate and known glutamate receptor agonists, including known excitotoxins kainic acid and NMDA, suggests that GPH may be an *A. mellifera* neurotoxin, acting on one, or multiple, post-synaptic glutamate receptors, likely among AMPA and NMDA subclasses.

While living pollinators and safe and effective herbicides are both crucial resources for feeding a growing 21st century population, their synergy must be safeguarded. Hopefully, an improved understanding of the possible molecular basis for observed GPH effects on *A. mellifera* colonies may help to guide continued refinement of benign agricultural solutions.

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